Influence of Iron-limited and Replete Continuous Culture on the Physiology and Virulence of *Neisseria gonorrhoeae*

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Neisseria gonorrhoeae strains P9-2 (Pen^S) and KW2 (Pen^R) were grown in chemostats of non-ferrous design at constant growth rate, pH and dissolved oxygen tension. Iron limitation ($\mu_{\max}$ 0.1 h⁻¹) was imposed by omitting iron salts from the defined medium and titrating increasing concentrations of the non-metabolizable iron chelators ovotransferrin and Desferal, to progressively decrease the growth yield. Metabolic activity during iron limitation was very high, with a q_{Glc} which was 2- or 11-fold greater than during cystine- or glucose-limited growth, respectively. More aspartate and isoleucine were metabolized during cystine-limited growth, while more glutamate, proline and serine were metabolized during glucose- or iron-limited growth. Significant concentrations of alanine or valine were excreted during cystine- or glucose-limited growth, respectively. Iron-limited growth of an initial inoculum of non-piliated, transparent colony-forming (P⁻O⁻) gonococci resulted in the selection of 100% piliated bacteria. Initial inocula of P⁺O⁻ gonococci retained this phenotype for over 100 generations. Iron-limited gonococci were extremely virulent in the guinea-pig subcutaneous chamber model and inocula of even 12 bacteria grew rapidly and persisted. By contrast, cystine-limited (iron-replete) gonococci retained piliation but did not survive in the chambers. Transition from iron-limited to glucose-limited growth resulted in marked loss of piliation but the bacteria remained virulent. Loss of virulence did not correlate with susceptibility to killing by normal human serum, nor with changes in the content or composition of lipooligosaccharide, which contained 2.9, 3.7, 4.3 and 4.8 kDa moieties. Additional proteins were detectable in Sarkosyl-purified outer membranes of iron-limited gonococci but several proteins with molecular masses similar to those described in the literature for iron-restricted gonococci were detectable in cystine- or glucose-limited bacteria.

INTRODUCTION

A versatile physiology (Keevil *et al.*, 1986) or rapid antigenic variation in pili (P⁺), proteins II (O⁺), or other outer-membrane proteins (OMPs) (Heckels, 1984) might explain the ability of *Neisseria gonorrhoeae* to colonize and persist at anatomically distinct sites despite the host immune response. One important environmental factor which pathogens frequently encounter in Man is iron deprivation. Iron is an essential nutrient for the majority of bacteria, particularly aerobes. Potential pathogens must therefore acquire this nutrient in competition with the host, since many body fluids contain iron-binding proteins (Weinberg, 1978; Griffiths, 1983; Brown & Williams, 1985). For example, the mucosal surfaces invaded by *N. gonorrhoeae* secrete

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Abbreviations: KDO, 2-keto-3-deoxyoctonate (3-deoxy-D-manno-2-octulosonate); MIRP, major iron-regulated protein; OMP, outer-membrane protein.

lactoferrin whilst plasma contains transferrin. These chelators have an extremely high association constant for iron and are normally only partially saturated. Consequently, the amount of free iron available to the invading pathogen is of the order of 10^{-18} M, far less than the minimum requirement of 10^{-7} M which is considered necessary for normal growth. Many pathogens are able to secrete high-affinity, iron-chelating siderophores of low molecular mass to abstract the iron from the transferrin and lactoferrin. However, gonococci and meningococci are unable to synthesize their own siderophores. They must therefore be able to adapt to iron-restricted environments by either developing mechanisms for acquiring and transporting the protein-bound iron (Mickelsen & Sparling, 1981; Mickelsen *et al.*, 1982) or assimilating it from other sources such as the haem available during menstruation (Yancey & Finkelstein, 1981; Dyer *et al.*, 1987).

Several OMPs of *Neisseria* spp. which have been reported to be introduced during conditions of iron restriction are believed to be concerned with uptake of sequestered iron. However, with the notable exception of continuous culture studies of *Neisseria meningitidis* (Archibald & DeVoe, 1978; DeVoe, 1982), these experiments were performed in batch culture with no control over the available iron, oxygen and carbon concentrations nor, indeed, the growth rate, which was greatly reduced (Norqvist *et al.*, 1978; Mietzner *et al.*, 1984; West & Sparling, 1985). We have therefore conducted an *in vitro* study of the influence of a fluctuating iron supply on the physiology and the regulation of the OMPs of *N. gonorrhoeae* using the unique advantages of continuous culture (see Keevil *et al.*, 1986). Virulence was assessed by resistance to human serum killing and ability to survive in the guinea-pig subcutaneous chamber model. Iron-replete conditions were established using other nutrient limitations which might occur *in vivo*, including glucose and cystine.

METHODS

Strains and maintenance. Penicillin-resistant *Neisseria gonorrhoeae* KW2 contained the so-called Asian R plasmid and has been described elsewhere (Keevil *et al.*, 1987). Penicillin-sensitive *N. gonorrhoeae* P9-2 was kindly provided by Dr J. E. Heckels (Southampton University). Both strains were stored in liquid nitrogen and colonies expressing the P⁺O⁻ or P⁻O⁻ phenotypes after growth on gonococcal clear typing agar (GCCT; Swanson, 1978) at 37 °C in an atmosphere of 5% (v/v) CO₂ in air were selected for investigation.

Media and growth. Gonococci were grown in continuous culture in defined medium, as described by Keevil *et al.* (1986), at dilution rates of up to 0.1 h⁻¹. The fermenter was a modified version of the Series 500 chemostat (LH Fermentation) with the fermenter head and all other ferrous materials replaced with autoclavable nylon and glass. The culture was maintained automatically at pH 7.0, 37 °C and a dissolved oxygen tension of 20% air saturation. Iron limitation was achieved by omitting iron salts from the medium, using the highest purity reagents available and aseptically titrating into the medium reservoir increasing concentrations of the non-assimilable iron chelators conalbumin (ovotransferrin; Sigma) or Desferal (desferrioxamine mesylate; Ciba Laboratories) (Mickelsen *et al.*, 1982) to decrease the growth yield. Conalbumin complexes 2 mol Fe³⁺ per mol whilst Desferal complexes 1 mol Fe³⁺ per mol; their final concentrations in the growth media were 7.5 and 15 µM, respectively. When conalbumin was present the medium also contained 10 g sodium bicarbonate l⁻¹ since this chelator requires bicarbonate ion to aid iron binding. All glassware was washed with 1% (v/v) HCl followed by deionized water to remove adsorbed, contaminating iron. Iron-replete growth was obtained by omitting the chelators, adding 12.5 µM-ferric nitrate and limiting the concentrations of glucose (83 mM reduced to 5.6 mM) or cystine (370 µM reduced to 20 µM).

Analytical procedures. Monitoring of the cultures for optical density and biomass concentration was as described previously (Keevil *et al.*, 1986). Glucose, ammonia and amino acids in the uninoculated media and the clarified culture supernatants were analysed by the methods of Ellwood *et al.* (1974). Iron was measured by the ferrozine method of Stookey (1970), which allowed concentrations to be determined at the sub-micromolar level.

Lipopolysaccharide analysis. LPS was extracted by the phenol/water procedure (Westphal & Jann, 1965). For each sample, pooled aqueous phases and the phenol phase were dialysed separately for 3 d against running water and then against phosphate-buffered saline. The 2-keto-3-deoxyoctonate (KDO) content of the LPS was assayed by the method of Karkhanis *et al.* (1978). The LPS was resolved on slab gels using the Laemmli (1970) buffer system. The separating gel was 15% (w/v) acrylamide, 0.5% (w/v) bis-acrylamide containing 4 M-urea. Samples containing up to 0.4 mg LPS ml⁻¹ were diluted 1:1 with double-strength sample buffer and 50 µl applied to each track of the gel. Gels were run at 125 V until the bromophenol blue tracking dye had migrated to within 1 cm of the end of the gel. The gel was stained for LPS using a silver stain (Tsai & Frasch, 1982). The molecular masses of the resolved components were estimated by comparison with purified *Salmonella minnesota* LPS (Sigma; Schneider *et al.*, 1984).

Determination of virulence characteristics. Cultures were monitored at regular intervals for changes in colonial morphology on agar, such as highlighting of colonies by transmitted light (indicating piliation) and opacity (due to proteins II; Keevil *et al.*, 1986). The presence of proteins II or other OMPs was verified by analysing membrane preparations by SDS-PAGE (Keevil *et al.*, 1986). Partially purified outer membranes were prepared by the method of Heckels (1977) and contaminating inner membrane material was subsequently removed with Sarkosyl (Nordqvist *et al.*, 1978). In addition to staining the gels with Coomassie brilliant blue, any proteins which did not stain well with this dye (Mietzner *et al.*, 1984; West & Sparling, 1985) were resolved by subsequently overstaining with the more sensitive silver stain procedure of Wray *et al.* (1981). Other characteristics to be assessed included ability of harvested bacteria to survive either in polypropylene chambers implanted subcutaneously into the flanks of guinea-pigs (Veale *et al.*, 1975), exactly as described by Keevil *et al.* (1986), or to exposure to human serum pooled from five volunteers and diluted 1:4 with phosphate-buffered saline (Rittenberg *et al.*, 1977). For the latter procedure chemostat samples were diluted 1:10 in the dilute serum and incubated at 37 °C for 30 min. Controls consisted of replacing the normal serum with serum inactivated by heat at 56 °C for 30 min before exposure to gonococci. The gonococcal samples recovered after serum treatment were serially diluted with prewarmed GCCT broth and incubated in GCCT agar at 37 °C in a candle jar to determine the number of survivors.

Error determination. All errors were in the ranges described previously by Keevil *et al.* (1986) whilst the LPS analyses did not vary by more than 15%.

RESULTS

Nutrient limitation

N. gonorrhoeae strain KW2 grew well in glucose-limited continuous culture at pH 7.0 and a dissolved oxygen tension of 20% of air saturation. Steady-state growth, defined by a constant culture turbidity (OD_{540}) and cell yield (g dry wt l⁻¹), was achieved within 10 generations, as found previously for strains KW1 and P9-2 (Keevil *et al.*, 1986). Supplementing the air supply with 5% carbon dioxide or the medium with 10 g sodium bicarbonate l⁻¹ (see later) had no effect on growth. Analysis of the nitrogen constituents of the clarified culture supernatants confirmed that this strain also prefers to metabolize cystine rather than cysteine in the chemostat (data not shown). Cysteine was therefore omitted from further growth media. The cystine requirement of strain KW2 was similar to the of strain P9-2: only 0.02 mM was required to give cystine limitation when the glucose concentration was increased to repletion (Table 1). Ammonium salts and all other amino acids included in the growth medium remained in excess during growth with the various nutrient limitations. However, more aspartate and isoleucine were metabolized by strain KW2 during cystine-limited growth, while more glutamate, proline and serine were metabolized during glucose- or iron-limited growth. Iron-limited strain P9-2 metabolized much less aspartate, cystine, methionine and serine than strain KW2. A significant concentration of alanine was excreted during cystine-limited growth of strain KW2 (Table 1) whilst valine was the major product of both strains during glucose limitation.

Growth yield

A high molar growth yield on glucose (Y_{Glc}) was obtained for strain KW2 (Table 2) with apparently 73% of the glucose carbon metabolized being converted to biomass. The Y_{Glc} decreased during cystine-limited growth but the lowest Y_{Glc} and glucose carbon conversion to biomass were observed during iron-limited growth of both strains KW2 and P9-2, even though the yields and culture turbidities were comparable to those obtained during other nutrient limitations. This was probably due to the high rates of glucose conversion: the q_{Glc} was over 10-fold greater than during glucose-limited growth and reflects the metabolic versatility of these strains. The maximum specific growth rate (μ_{max}) was over 0.7 h⁻¹ during glucose limitation but never exceeded 0.1 h⁻¹ during iron limitation, when washout of the cultures occurred.

Phenotypic variation

Previous experiments established that *N. gonorrhoeae* strains KW1 and P9-2 maintained the P⁺O⁻ phenotype during prolonged cystine- or oxygen-limited continuous culture but not during glucose limitation (Keevil *et al.*, 1986). This change of phenotype was also observed for strain

Table 1. Concentrations of nutrients (mM) in unused growth medium and in supernatants obtained from continuous cultures of *N. gonorrhoeae* strains KW2 and P9-2

Nutrient limitation . . .	Growth medium	Strain KW2			Strain P9-2
		Glucose	Cystine	Iron	Iron
Glucose*	5.6/83	0	43.6	28.6	25
Iron*	0/0.013†	0.011	0.014	0	0
Ammonia	5.6	4.4	3.6	2.0	3.2
Arginine	0.6	0.34	0.48	0.22	0.3
Aspartate	1.8	1.3	0.09	0.17	1.3
Cystine*	0.02/0.37	0.05	0	0.05	0.2
Glutamate	1.4	0.03	0.32	0.05	0.1
Isoleucine	1.0	1.6	0.07	0.03	0.1
Methionine	1.3	0.94	0.44	0.12	0.7
Proline	2.2	0.1	0.65	0.1	0.2
Serine	2.4	0.06	0.14	0.04	0.9
Alanine		0.01	0.55	0.05	0.01
Glycine		0.02	0.01	0.02	0.01
Histidine		0	0	0	0.01
Leucine		0	0	0	0.01
Lysine		0	0	0.07	0.02
Ornithine		0.01	0	0.01	0.01
Phenylalanine		0.03	0	0.01	0
Threonine		0.03	0.08	0.01	0.01
Valine		0.38	0.18	0.01	0.01

* The concentrations in the unused growth medium describe those providing subsequent limited or replete growth, respectively.

† These concentrations of iron do not include the approximately 10 μ M contamination present in the other nutrients and water.

Table 2. Concentrations of bacteria, yield and glucose consumption in continuous cultures of *N. gonorrhoeae* strains KW2 and P9-2

Nutrient limitation . . .	Strain KW2			Strain P9-2
	Glucose	Cystine	Iron	Iron
OD ₅₄₀	2.8	2.7	2.1	2.5
Yield (g dry wt l ⁻¹)	0.59	0.7	0.51	0.52
10 ⁻⁹ × Cells ml ⁻¹	1.5	1.7	1.6	2.0
Y _{Glc} (g mol ⁻¹)	105	17.8	9.4	8.8
Biomass (% C metabolized)	73.1	12.3	6.7	6.3
q _{Glc} (mmol g ⁻¹ h ⁻¹)	0.95	5.6	10.7	11.1

KW2 when grown in glucose-limited continuous culture (Fig. 1). After approximately 30 generations of growth the majority of recovered bacteria expressed the P⁻O⁻ phenotype when cultured on GCCT agar. This phenotype was confirmed by SDS-PAGE of whole cell preparations extracted at 37 or 100 °C (Keevil *et al.*, 1986) since no bands corresponding to the presence of pili or heat-modifiable proteins II were apparent (data not shown). Only 2% of strain KW2 grown glucose-limited remained pilated, with the P⁻O⁻ phenotype predominating.

The P⁺O⁻ phenotype was also stable for both strains P9-2 and KW2 when grown in iron-limited continuous culture with either conalbumin or Desferal as the iron chelator (Fig. 2). However, when the nutrient limitation for strain P9-2 was changed to glucose there occurred a progressive loss of the P⁺O⁻ phenotype and non-piliated gonococci predominated. In support of this finding, a P⁻O⁻ isolate of strain KW2 maintained this particular phenotype when grown initially in glucose-limited continuous culture (Fig. 3). Nevertheless, when the supply of iron was restricted after 16 generations of growth by increasing the glucose concentration to 43 mM and progressively increasing the concentration of Desferal in the nutrient feed (up to 7.5 mg l⁻¹),

Fig. 1. Changes in the colonial composition of *N. gonorrhoeae* KW2 during prolonged glucose-limited growth. Gonococci were harvested from continuous culture and serial dilutions were plated onto GCCT agar and incubated for 24 h at 37 °C to identify the P⁺O⁻ (○), P⁻O⁻ (□) and P⁻O⁺ (●) phenotypes, as described in Methods.

Fig. 2. Changes in the colonial composition of *N. gonorrhoeae* P9-2 during the transition between iron-limited and glucose-limited growth. Glucose-limited growth was obtained by removing Desferal, adding ferric nitrate and decreasing the glucose concentration (➡), as described in Methods. The gonococcal phenotypes were identified as described for Fig. 1. ○, P⁺O⁻; □, P⁻O⁻.

approximately 50% of the recovered gonococci expressed the P⁺O⁻ phenotype, even though no glucose was yet detectable in spent culture supernatants. Further addition of glucose and Desferal (83 mM and 10 mg l⁻¹ final concentration, respectively) after 43 generations of growth resulted in a decreased growth yield, and excess glucose was now detectable in the steady-state culture, suggesting true iron limitation. As this change occurred, the phenotype of the culture became exclusively P⁺O⁻ after a further 20 generations of growth. This pilated phenotype was then stably maintained up to 120 generations of growth, when the experiments were terminated.

Virulence studies

The influence of the various continuous culture growth environments on the virulence of *N. gonorrhoeae* strains P9-2 and KW2 was assessed by the ability of recovered gonococci to survive in the guinea-pig subcutaneous chamber model (Veale *et al.*, 1975). Samples were taken directly from independent continuous cultures and inoculated into the chambers at various doses. For example, glucose-limited strain KW2 was harvested at 150 generations and inoculated into

Fig. 3. Conversion of the P^-O^- phenotype of *N. gonorrhoeae* KW2 to P^+O^- during the transition between glucose-limited and iron-limited growth. Iron-restricted (glucose-limited) growth was obtained by increasing the glucose concentration to 43 mM and adding 7.5 mg Desferal l^{-1} ($\blacksquare$). Iron-limited (glucose-excess) growth was obtained by increasing the glucose concentration to 83 mM and adding 10 mg Desferal l^{-1} ($\Rightarrow$). The gonococcal phenotypes were identified as described for Fig. 1. $\circ$, P^+O^- ; $\square$, P^-O^- .

Table 3. Persistence of infection in guinea-pig subcutaneous chambers of *N. gonorrhoeae* grown in iron, cystine- or glucose-limited continuous culture

Strain	Nutrient limitation	Dose (c.f.u.)	Viable organisms* recovered at day:				
			1	4	7	14	21
P9-2	Iron†: (conalbumin)	200	ND	ND	0.32 ± 0.11	2.9 ± 0.54	1.8 ± 0.37
	(Desferal)	12	ND	0.93 ± 0.21	1.4 ± 0.25	0.4 ± 0.15	1.9 ± 0.44
KW2	Iron†: (Desferal)	200	0.40 ± 0.15	0.76 ± 0.19	0.82 ± 0.22	0.33 ± 0.12	0.74 ± 0.23
	Glucose	200	0.32 ± 0.11	0.88 ± 0.28	0.96 ± 0.31	2.3 ± 0.63	0.86 ± 0.19
	Cystine	2×10^5	0.0002 ± 0.0001	0	0	0	0

ND, Not determined.

* Results are expressed as $10^{-6} \times$ c.f.u. ml^{-1} , $\pm$ SEM.

† Iron limitation was obtained by adding conalbumin or Desferal to the growth medium, as described in Methods.

separate chambers at 200 cells per chamber. Viable counts on GCCT agar of samples recovered from the chambers indicated that the gonococci grew rapidly *in vivo* and persisted at approximately 10^6 cells ml^{-1} (Table 3). Strain P9-2, cultivated iron-limited with 7.5 μ M-conalbumin as chelator, was also virulent in the animal model and gave similar results to glucose-limited strain KW2 over a 21 d incubation period. To eliminate any possibility that the conalbumin might be interfering with the *in vivo* survival of gonococci these experiments were repeated on iron-limited gonococci with 15 μ M-Desferal as chelator. For strain P9-2, even a dose of 12 gonococci per chamber proved virulent in the model and the gonococci again persisted at approximately 10^6 cells ml^{-1} . Similar results were also obtained for strain KW2 with Desferal as chelator. By contrast, cystine-limited gonococci were not virulent in the chamber model at any dose up to 10^6 cells per chamber and no viable bacteria were detected after 4 d.

McBride *et al.* (1981) and Keevil *et al.* (1986) have shown previously that when gonococci of different PO phenotypes are inoculated into the chamber model the predominant phenotype quickly becomes P^+O^+ . This was also found in the present study within 4 d when viable strains P9-2 or KW2 were recovered from the chambers and cultured on GCCT agar.

Fig. 4. Resolution by SDS-PAGE of purified lipooligosaccharide of *N. gonorrhoeae* KW2 grown in prolonged iron- or cystine-limited continuous culture. Iron- (lanes 1 and 3) or cystine-limited (lanes 2 and 4) gonococci were extracted, separated on the gels (1 μ g, lanes 1 and 2; 2 μ g, lanes 3 and 4) and stained with silver, as described in Methods. *Salmonella minnesota* wild-type LPS (10 μ g) is shown in lane 5.

Table 4. Resistance to killing by diluted normal human serum of *N. gonorrhoeae* KW2 grown in continuous culture

Nutrient limitation	C.f.u. recovered after incubation with*:		Percentage of resistant gonococci
	Inactivated serum†	Normal serum	
Iron	75 $\pm$ 6	47 $\pm$ 5	63
Glucose	28 $\pm$ 3.4	17.4 $\pm$ 1.8	62
Cystine	25 $\pm$ 1.9	20 $\pm$ 1.4	80

* Samples were diluted 1:10 in the diluted serum and incubated at 37 °C for 30 min before serial dilution and culture on GCCT agar, as described in Methods. Results are expressed as $10^{-6} \times$ c.f.u. ml $^{-1}$, $\pm$ SEM.

† The serum was heated at 56 °C for 30 min prior to incubation with gonococci.

Sensitivity to serum killing

Survival of *N. gonorrhoeae* in the subcutaneous chamber model did not correlate with the presence or absence of pili or OMPs II. In an attempt to elucidate why only cystine limitation prevents virulence, gonococci were harvested from the various growth-limiting environments and incubated with normal human serum. The data (Table 4) indicated that all of the gonococci were relatively resistant to killing by the serum since there is usually greater than 95% killing of sensitive bacteria. Moreover, although cystine-limited gonococci were avirulent they were actually more resistant to serum killing than were glucose- or iron-limited organisms.

There was also no apparent correlation between virulence and LPS content of *N. gonorrhoeae*. The extraction procedure from iron- or cystine-limited gonococci indicated a KDO content of 4.7 or 3.6 mg g (dry wt cells) $^{-1}$, respectively. Polyacrylamide gels stained with silver showed only a rough lipooligosaccharide structure with no apparent differences between cystine-limited or replete gonococci (Fig. 4). This observation was also confirmed by a NMR study (C. W. Jones, personal communication). Comparison with *S. minnesota* LPS on resolved polyacrylamide gels (Schneider *et al.*, 1984) indicated multiple components of 2.9, 3.7, 4.3 and 4.8 kDa.

Variation in membrane protein composition

The OMP composition of *N. gonorrhoeae* strain KW2 recovered from the various continuous cultures was analysed by SDS-PAGE of Sarkosyl-insoluble extracts. The major OMP (PI) of this strain ran on the gels with a molecular mass of 35 kDa under all continuous cultivation

Fig. 5. Resolution by SDS-PAGE of purified OMPs of *N. gonorrhoeae* KW2 grown in continuous culture or as colonies on agar. Gonococci were grown in prolonged cystine- or iron-limited continuous culture or as 24-h-old colonies on agar, as described in Methods. Samples were derivatized at 37 °C (lanes 2, 4 and 6) or 100 °C (lanes 3, 5 and 7) and after separation on the gels the proteins were stained with Coomassie brilliant blue. Molecular mass standards are shown in lanes 1 and 8.

conditions. As reported previously for strain P9-2, the growth environment modulated the expression of certain other OMPs of strain KW2. Thus, a comparison of the OMP profile of gonococci grown on agar with those grown in the chemostat revealed that cystine limitation resulted in the appearance of proteins of 20.5, 21, 22, 27, 28, 31, 32, 80, 88, 97, 110 and 114 kDa (Fig. 5). Nevertheless, some of these proteins were not unique to cystine limitation since iron-limited gonococci contained 22, 23, 25, 29, 44 (when extracted at 37 °C), 74, 80, 88, and 97 kDa OMPs. However, the higher molecular mass material appeared in low concentration on the gels when extracted at 37 °C whilst lower molecular mass material predominated. The presence of a protease causing degradation was confirmed in iron-limited gonococci by including 10 µM of the protease inhibitor phenylmethylsulphonyl fluoride (PMSF; Meitzner *et al.*, 1984) at the start of the extraction procedure. There was then no discernible difference between material

Fig. 6. Resolution by SDS-PAGE of a 37 kDa OMP of *N. gonorrhoeae* KW2 grown in iron-limited or replete continuous culture. Gonococci were grown in prolonged cystine-, glucose- or iron-limited continuous culture, as described in Methods. Samples were derivatized at 37 °C (lanes 1, 3 and 5) or 100 °C (lanes 2, 4 and 6). After separation on the gels, proteins were stained initially with Coomassie brilliant blue and then overstained with silver, as described in Methods.

extracted at 37 or 100 °C, other than the presence in material extracted at 37 °C of the apparently heat-modifiable 110 kDa OMP described by Leith & Morse (1980) and Keevil *et al.* (1986). No evidence of significant protease activity was found for any of the other growth environments.

Interestingly, the 35 kDa PI of strain KW2 grown on agar was not the major protein but rather a 37 kDa protein. A protein of similar molecular mass has also been described to be induced in high concentration in batch cultures of strains F62 and FA19, but only during iron deprivation and when visualized on gels by silver staining (Mietzner *et al.*, 1984; West & Sparling, 1985). However, a monoclonal antibody raised against the so-called major iron-regulated protein (MIRP) has allowed detection of this protein in the strains during apparently iron-replete agar or batch culture (P. Norrod, personal communication). Although the 36 kDa PI of strain P9-2 was too close to the 37 kDa protein to allow clear resolution, a partially developed silver stain on top of the Coomassie-blue-stained gel of gonococci grown under cystine or iron limitation highlighted a discrete black band against the more diffuse blue background of the PI (data not shown). The 35 kDa PI of strain KW2 ran sufficiently distant from the 37 kDa protein on the gels to allow their partial resolution: the latter was present in both glucose- or cystine-limited (iron-replete) and iron-limited gonococci after silver staining (Fig. 6). The 37 kDa OMP was particularly susceptible to the protease degradation when iron-limited gonococci were extracted at 37 °C in the absence of PMSF (Fig. 6, track 5). Strain KW2 also contained a 33 kDa OMP during glucose-limited growth but not during cystine- or iron-limited growth (Fig. 6).

DISCUSSION

The present study has shown that gonococci grow well in iron-limited continuous culture, although the $\mu_{\max}$ does not exceed 0.1 h⁻¹. This is equivalent to a mean generation time (MGT) of at least 6.9 h and is in accord with the even longer MGTs reported during batch culture experiments where Desferal was used to deprive gonococci of iron (Mietzner *et al.*, 1984; West & Sparling, 1985). This highlights an important, and often ignored, constraint on investigating nutrient deprivation in batch culture: not only is the growth environment in constant change, unlike continuous culture, but also the growth rate may be significantly reduced. Thus, it is difficult to establish whether changes in physiology, cell morphology or virulence are due to deprivation of essential nutrients or to changes in the growth rate. Some experimenters have attempted to differentiate between these phenomena by growing micro-organisms in standard, nutrient-excess media and deliberately slowing the growth rate of the culture by, for example, decreasing the incubation temperature. However, any effects then noted could equally be due to the effects of temperature rather than the growth rate *per se*. The unique advantages of continuous culture have therefore been utilized in the present work to maintain a constant growth rate and constant chemical environment during the prolonged nutrient limitations

investigated. By this procedure it is possible to make meaningful comparisons and while, for example, it is clear that any nutrient limitation induces the inclusion of a range of proteins into the outer membrane of gonococci, some of these do not appear to be unique to the particular limitation investigated. These might perhaps be more usefully described as 'stress' proteins, a term which could encompass changes in nutrient availability, temperature, pH, growth rate etc. Thus, OMPs of molecular mass 32, 32.5, 34, 42, 48, 50, 58, 98 and 110 kDa were apparently induced in strain P9-2 grown under glucose-, cystine- or oxygen-limitation (Keevil *et al.*, 1986), whilst 22, 37, 80, 88 and 97 kDa OMPs were detectable in strain KW2 grown under either iron- or cystine-limitation. Of course, these might be different proteins of coincident molecular masses but this apparent correlation does serve to illustrate the caution necessary when ascribing induction phenomena and possible function of proteins in particular growth environments.

In particular, the 37 kDa MIRP protein has been so-called due to its apparent induction during iron-deprived batch culture (Mietzner *et al.*, 1984). However, a similar protein appears to be present in gonococci grown under other nutrient limitations described here. Induction of the MIRP may be affected by the availability of sulphur compounds and it is also found in the cytoplasm and periplasm of gonococci (P. Norrod, personal communication). Perhaps significantly, the 37 kDa protein described here was present in good yield during cystine-limited growth. Nevertheless, the protein was also present during the glucose-limited growth experiments described here and elsewhere (Rock *et al.*, 1988). Several possibilities arise from these findings. Firstly, that there are several proteins of 37 kDa, inducible under different growth limitations. The studies of P. Norrod and S. A. Morse and colleagues indicate otherwise, but future studies will attempt to obtain a monoclonal antibody reacting with the MIRP and confirm whether the 37 kDa protein reported here is the same protein. Secondly, that MIRP is not regulated by iron availability but influenced by other environmental parameters, such as 'stress'. Thirdly, that the different nutrient limitations described impose physiological constraints upon gonococci; for example depleting the energy generation necessary for the active transport of iron during iron-replete (glucose- or cystine-limited) growth, which would cause a low intracellular iron concentration and induce proteins normally associated with iron transport during growth in iron-depleted media. The finding that MIRP is present in high concentration in the soluble cell fractions suggests that its presence in the outer membrane might either be transitory (possibly due to its high basic amino acid content interacting with membrane phospholipids) or that some of it continues to exist as a pro-protein with its intact leader sequence enabling insertion in the outer membrane. If so then it is analogous to the FecB or FepB periplasmic iron transport proteins described in *Escherichia coli* (molecular mass 30–37 kDa; Pierce & Earhart, 1986; Pressler *et al.*, 1988) and might serve to interact with the high-molecular-mass transferrin- and lactoferrin-binding proteins on the outer membrane, abstracting their bound iron and providing a shuttle mechanism to convey the iron across the periplasm to the inner membrane for active transport into the cytoplasm. The TonB protein of *E. coli* is essential for the uptake of iron via all the transport systems so far discovered. It is located in the inner membrane but is partially exposed to the periplasm (C. J. Higgins, personal communication), suggesting that it might be the essential receptor on the cytoplasmic membrane to which periplasmic iron transport proteins bind. To confirm this hypothesis of iron transport in gonococci it will be essential to verify the location of the 37 kDa protein and determine the concentration of bound and free iron in intracellular pools during different nutrient limitations.

Restriction in the availability of free iron *in vivo* plays a key role in the host's defence strategy against potential pathogens. It is noteworthy, therefore, that iron-limited continuous culture actually maintained the expression of pili in P⁺ gonococci and promoted this phenotype in P⁻ strains. The appearance and maintenance of pilus expression was probably due to selection of that particular genotype in response to environmental pressure, since the transition kinetics for populations of P⁺ gonococci to become almost exclusively non-piliated or *vice versa* were too slow for phenotype induction in continuous culture (Lancaster *et al.*, 1988). Thus, P⁻ gonococci appear spontaneously at a low rate in a P⁺ population and might outcompete the latter during

glucose limitation, possibly because pilus formation places more energetic and nutrient demands on the cell. Glucose-excess growth is presumably not energy limited and P^+ revertants can then compete successfully with P^- gonococci. Why P^+ gonococci actually take over the whole of the population during iron limitation with few or no P^- survivors is unclear but points to an essential role for pili during growth in particular environments. Furthermore, some O^+ variants of strain KW2 expressing PII were selected during glucose-limited growth, as found previously for strains KW1 and P9-2 (Keevil *et al.*, 1986). Pili might be important in enhancing attachment of gonococci to epithelial surfaces (Virji & Heckels, 1984) whilst PII are involved in resistance to host defences (Virji & Heckels, 1985). Thus, the versatility of gonococci in being able to change their cell surface characteristics in response to environmental pressures is of obvious advantage when attempting to colonize a host and overcome the local defences. This versatility is also apparent in the greatly increased metabolic activity during iron limitation. The ability to increase the q_{Glc} over 10-fold might be presumed to confer some competitive advantage, such as outcompeting other organisms for glucose when invading sites occupied by an indigenous flora (McBride *et al.*, 1978). The increased metabolism might also explain the significantly enhanced, KCN- or antimycin-A-sensitive, respiration of gonococci when exposed to the iron-restricted environments of serum, neutrophils, myeloid cells or McCoy cells (Britigan *et al.*, 1988). Increased glucose metabolism would necessitate an increased regeneration or reduced cofactors such as NADH and this is classically accomplished in aerobes by increased respiration. Furthermore, although gonococci are aerobes they contain little or no superoxide dismutase activity; increased respiration might deplete the local oxygen concentration and thereby decrease the likelihood of potentially lethal superoxide formation, particularly by neutrophils (Cohen & Cooney, 1984).

The failure of cystine-limited strain KW2 to establish infections in the subcutaneous chamber model is reminiscent of earlier observations with strains KW1 and P9-2 (Keevil *et al.*, 1986). Furthermore, strain KW2 also prefers to metabolize the dimer rather than cysteine. The cystine requirement for strain KW2 was similar to that of strain P9-2 but fivefold less than that of strain KW1 (Keevil *et al.*, 1986). These observations, together with those of Manchee *et al.* (1980), suggest that a preference for cystine over cysteine might be widespread among gonococci and might be clinically important in that aerobic sites of infection could be considered to have a higher ratio of the dimer to the monomer (Keevil *et al.*, 1986). It is still not clear why cystine limitation prevents infection because the subcutaneous chambers apparently contain excess of this essential nutrient (Keevil *et al.*, 1986). However, our work suggests that this avirulence is not due to increased susceptibility to serum killing by the host. On the contrary, cystine-limited gonococci were actually more resistant than those grown under other nutrient limitations. In addition, increased resistance to serum killing has been associated with changes in LPS structure and content in glucose-limited gonococci (Morse *et al.*, 1983). Our data on serum resistance with glucose-limited gonococci are in agreement with those obtained with the glucose-limited strain of Morse *et al.* (1983) grown at a similar dilution rate. Furthermore, Schneider *et al.* (1984) have described the diverse heterogeneity of lipooligosaccharide molecular size among different strains of gonococci whilst Apicella *et al.* (1988) have suggested that phenotypic variation of lipooligosaccharide may occur which is under environmental and/or genetic control. However, no significant changes in the rough structure of the LPS or the KDO content of strain KW2 were detected in the cystine-limited or excess environments described in the present study when serum resistance was maintained. The multicomponent composition of the lipooligosaccharide was similar to that of other serum-resistant strains, such as strain 220, described previously (Schneider *et al.*, 1984) and contained the 3.6–3.7 kDa component found in all serum-resistant strains (Schneider *et al.*, 1985). These observations do not exclude the possibility that subtle changes in epitope expression might have occurred. Future studies should address the possibly enhanced sensitivity of cystine-limited gonococci to other host defences such as phagocytic ingestion and killing.

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