Neisseria gonorrhoeae 23S rRNA A2059G mutation is the only determinant necessary for high-level azithromycin resistance and improves in vivo biological fitness

Jianglin Zhang¹ and Stijn van der Veen¹–³*

¹Department of Microbiology and Parasitology, School of Medicine, Zhejiang University, Hangzhou, China; ²Department of Dermatology, Sir Run Run Shaw Hospital, School of Medicine, Zhejiang University, Hangzhou, China; ³State Key Laboratory for Diagnosis and Treatment of Infectious Diseases, Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, The First Affiliated Hospital, School of Medicine, Zhejiang University, Hangzhou, China

*Corresponding author. Tel: +86-571-88206684; Fax: +86-571-88208022; E-mail: stijnvanderveen@zju.edu.cn

Received 29 August 2018; returned 26 September 2018; revised 28 September 2018; accepted 28 September 2018

Objectives: The global emergence of Neisseria gonorrhoeae isolates displaying high-level azithromycin resistance is a major concern for the currently recommended azithromycin/ceftriaxone dual therapy. N. gonorrhoeae high-level azithromycin resistance has been associated with an A2059G mutation in 23S rRNA. Here we investigated the specific contribution of this 23S rRNA A2059G mutation to high-level azithromycin resistance and its impact on biological fitness.

Methods: A2059G/G2059A alleles were specifically cloned into all four genomic copies of 23S rDNA of an azithromycin-susceptible isolate and a high-level azithromycin-resistant isolate. WT and mutant strains were subsequently investigated for azithromycin susceptibility using the agar dilution method. In addition, their biological fitness was studied by comparative liquid growth in the presence of hydrophobic and amphipathic compounds, by competition assays in a mouse vaginal tract infection model and by competition assays for invasion and intracellular survival.

Results: Azithromycin susceptibility analyses showed that the 23S rRNA A2059G mutation is the only genetic determinant required for N. gonorrhoeae to display the high-level azithromycin resistance phenotype. Further analysis of biological fitness showed that strains containing 2059G outcompeted isogenic strains containing 2059A for colonization in the mouse vaginal tract infection model and for invasion of HeLa cervical epithelial cells. Furthermore, the A2059G mutation enhanced growth in the presence of lithocholic acid or Triton X-100.

Conclusions: Our findings that the 23S rRNA A2059G mutation is sufficient for high-level azithromycin resistance and that this mutation generally enhanced the biological fitness of N. gonorrhoeae have important implications for the currently recommended treatment policies and antimicrobial stewardship programmes.

Introduction

Neisseria gonorrhoeae is a global health concern because it has developed resistance to most of the commonly used antimicrobials.¹ ² Furthermore, resistance to extended-spectrum cephalosporins is increasing globally and confirmed treatment failures using ceftriaxone, the last available first-line empirical monotherapy, have already been reported in several countries.³ Therefore, dual therapies containing ceftriaxone and azithromycin are now the standard recommendation in Europe and the USA.⁴ However, azithromycin resistance is common in many countries, particularly in settings where this antimicrobial is frequently used and widely available.⁵ ⁶ In recent years, high-level azithromycin-resistant N. gonorrhoeae, defined by an MIC ≥256 mg/L, have been isolated in many countries, including Ireland, Scotland, England, Argentina, Italy, the USA, Sweden, Australia and China.⁸ ¹⁹ These high-level azithromycin-resistant isolates pose a serious threat for the currently used ceftriaxone/azithromycin dual therapy.

Azithromycin targets the 23S rRNA component of the 50S ribosomal subunit and blocks the peptide exit channel, thereby preventing the translocation of the peptidyl-tRNA. This results in the release of incomplete polypeptides. Resistance to azithromycin can arise from specific mutations in the target loop V of 23S rRNA. N. gonorrhoeae contains four copies of the 23S rRNA in its chromosome and the degree of resistance is dependent on the specific mutation and the number of copies of the 23S rDNA that are mutated.⁸ ²⁰ High-level azithromycin resistance has been
associated with the A2059G mutation, while low-level resistance has been shown for strains containing the C2611T mutation. In addition, the MtrCDE multidrug efflux pump is able to export azithromycin and up-regulation of the pump due to mutations in MtrR and the mtrCDE promoter region result in increased resistance to azithromycin and other macrolides.

Given the strong association between specific 23S rRNA mutations and resistance to azithromycin, it might only be a matter of time before they will be consolidated as a dominant trait. However, antibiotic resistance frequently comes with a biological cost for maintaining the mutation and therefore it is often assumed that antibiotic-resistant bacteria are outcompeted by susceptible bacteria when antibiotic pressure is alleviated.

For instance, Salmonella enterica serovar Typhimurium containing mutations conferring high-level resistance to ciprofloxacin showed a defect in their ability to colonize the gut of chickens. In addition, a recent modelling study on spreading of cefixime resistance in an English MSM population showed that cefixime-resistant strains were outcompeted by susceptible strains in the absence of cefixime and were unable to persist, which is important for antimicrobial stewardship programmes and recycling of antimicrobials.

Given the global emergence of high-level azithromycin-resistant N. gonorrhoeae isolates and their threat to ceftriaxone/azithromycin dual therapy, it is important to investigate the impact of the high-level azithromycin-resistance-associated 23S rRNA A2059G mutation on azithromycin resistance and biological fitness. Therefore, in this study, the A2059G mutation was specifically cloned in all four copies of 23S rDNA of an azithromycin-susceptible strain and similarly the G2059A mutation was cloned in all four copies of 23S rDNA of a high-level azithromycin-resistant strain. These strains were subsequently investigated for azithromycin resistance and biological fitness.

### Materials and methods

#### N. gonorrhoeae strains and mutants

For all experiments, the N. gonorrhoeae high-level azithromycin-resistant strains ZJXSH89 (ST1866) and ZJXSH73 (ST3102) and azithromycin-susceptible strain ZJXSH86 (ST774) and their derivatives (Table S1, available as Supplementary data at JAC Online) were revived from glycerol stocks on GC agar base (Oxoid) containing 1% (v/v) Vitox (Oxoid) and grown overnight at 37°C in the presence of 5% CO₂. The streptomycin-resistant derivatives ZJXSH89-4G and ZJXSH86-4A were selected on GC agar containing streptomycin (BBI) and used for further mutant construction. To generate markerless A/G mutations in the four copies of 23S rDNA (r02, r05, r08 and r11) present in the genomes of ZJXSH89-4G and ZJXSH86-4A, a strategy was used that employs a dominant streptomycin-susceptible rpsL gene for positive selection. The streptomycin-susceptible rpsL gene and 23S rDNA fragments were amplified from N. gonorrhoeae using rpsL- and 23S rDNA-specific primers (Table S2) and cloned into vector pUC57-kanR, thereby generating vectors pUC57-23S(2059G)::kanR-rpsL-23S and pUC57-23S(2059A)::kanR-rpsL-23S (Table S3). These vectors were subsequently linearized and used for transformation of strains ZJXSH89-4G and ZJXSH86-4A. Successful mutations were finally verified by PCR and sequencing. This procedure was repeated to replace 2059A/G in all four genomic copies of 23S rDNA. The ΔmtrCDE deletion mutants of strains ZJXSH89 and ZJXSH73 were generated by amplification of flanking regions to generate the vector pUC57-ΔmtrCDE::kanR. This vector was subsequently used to generate ZJXSH89-ΔmtrCDE and ZJXSH73-ΔmtrCDE. The derivatives ZJXSH89-4A-catA2 and ZJXSH86-4A-catA2, which contain the chloramphenicol resistance gene catA2 in the unrelated convergent locus between lctP and aspC, were generated by amplification of lctP and aspC to generate the pUC57-catA2 derivative pUC57-lctP-catA2-aspC. This vector was subsequently linearized and transformed into strains ZJXSH89-4A and ZJXSH86-4A.

#### Comparative WGS

WGS of N. gonorrhoeae strains ZJXSH89-4G, ZJXSH89-4A, ZJXSH86-4A and ZJXSH86-4G was performed using the HiSeq Illumina platform (Genewiz) following the 2×150 paired-end configuration. Sequence data have been deposited to the Sequence Read Archive under accession number SRP158823 (https://www.ncbi.nlm.nih.gov/sra/SRP158823). The reference genome of N. gonorrhoeae strain FA1090 was used for comparative analysis and calling of mutations between strains ZJXSH89-4G and ZJXSH89-4A and between strains ZJXSH86-4A and ZJXSH86-4G using GATK software (V3.4.6).

### Azithromycin susceptibility assays

Overnight grown bacteria were suspended into GC broth containing 1% Vitox and 10 μL of the suspension containing 10⁴ cfu was plated on GC agar plates containing 1% Vitox and a 2-fold dilution series of azithromycin. Plates were incubated for 24–48 h at 37°C in the presence of 5% CO₂ and the MIC was determined as the lowest concentration at which no growth was observed.
incubated with phorbol myristate acetate (Sigma; 10 μg/mL) for 24 h to obtain differentiated cells. Overnight grown N. gonorrhoeae strains were suspended in pre-warmed RPMI-1640 medium containing 1% Vitox and 4G/4A mixed bacterial suspensions were added at an moi of 100 (HeLa cells) or 20 (THP-1 cells). After 1 h of incubation, cells were washed and fresh RPMI-1640 medium supplemented with 200 mg/L gentamicin (Songon Biotech) was added. Samples were taken for up to 4 h and cells were lysed with 1% (v/v) Saponin (Sigma). Samples were serial diluted and plated on GC agar containing 1% Vitox and azithromycin (128 mg/mL) or chloramphenicol (5 mg/mL).

**Competition assays in murine vaginal tract infection model**

Murine vaginal tract competition assays were performed as described previously.16,37 Six-to-eight-week-old female BALB/c mice (Shanghai SLAC Laboratory Animal Company) at their dioestrus stage were subcutaneously administered with 0.1 mg of β-oestradiol (Aladdin) dissolved in sesame oil (Sigma) on days –2, 0 and 2. During this period, mice were also administered 0.6 mg of vancomycin (Meilunbio) and 1.2 mg of streptomycin twice daily. Furthermore, during the whole course of the experiment drinking water was supplemented with 0.4 g/L trimethoprim (Meilunbio). On day 0, mice were administered intravaginally with 4G/4A mixed bacterial suspensions in PBS containing 0.5 mM CaCl₂ (Sigma), 1 mM MgCl₂ (Sigma) and 1% (v/v) gelatin (Aladdin) at a total dose of 2 x 10⁷ cfu. The vaginal tracts of the mice were swabbed daily for up to 4 days and both the input mixed bacterial suspension and samples obtained from the mice were plated on GC agar supplemented with 1% Vitox, vancomycin (3 mg/L), colistin (Meilunbio, 7.5 mg/L), nystatin (Meilunbio, 2.8 mg/L), trimethoprim (5 mg/L), streptomycin (100 mg/L) and azithromycin (128 mg/mL) or chloramphenicol (5 mg/L). The competition index was calculated based on the 4G/4A ratio in the daily vaginal swabs (output) and the 4G/4A ratio of the inoculum (input) according to the formula (4G/4A)output/(4G/4A)input.

**Table 2. Selection for additional 23S rDNA A2059G mutations in colonies grown on agar plates with different azithromycin concentrations**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Azithromycin concentration in agar (mg/L)</th>
<th>Number</th>
<th>23S rDNA 2059A/G</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>r02a</td>
</tr>
<tr>
<td>ZJXSH89-3G</td>
<td>0.016</td>
<td>5</td>
<td>5G</td>
</tr>
<tr>
<td>ZJXSH89-2G</td>
<td>0.016</td>
<td>5</td>
<td>5A</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>5</td>
<td>5G</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5</td>
<td>5G</td>
</tr>
<tr>
<td></td>
<td>1024</td>
<td>5</td>
<td>5G</td>
</tr>
<tr>
<td>ZJXSH86-3G</td>
<td>0.016</td>
<td>5</td>
<td>5G</td>
</tr>
<tr>
<td>ZJXSH86-2G</td>
<td>0.016</td>
<td>5</td>
<td>5G</td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>5</td>
<td>5G</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5</td>
<td>5G</td>
</tr>
<tr>
<td></td>
<td>1024</td>
<td>2³</td>
<td>2G</td>
</tr>
</tbody>
</table>

³Only two colonies emerged on plates with this azithromycin concentration.

**Gonococcal high-level azithromycin resistance and biological fitness**

### N. gonorrhoeae liquid growth

N. gonorrhoeae strains were grown overnight and suspended in 12 mL of GC broth containing 1% Vitox at an OD₆₀₀ of 0.025. For competition assays, overnight grown strains were mixed to equal numbers and added to the culture medium. The competition index was calculated as (4G/4A)output/(4G/4A)input. For growth in the presence of hydrophobic or amphipathic compounds, sodium cholate (Aladdin; 70 μg/mL), lithocholic acid (Aladdin; 70 μg/mL), Tween 20 (Biosharp; 0.02% v/v) or Triton X-100 (VETEC; 0.00325% v/v) was added. Cultures were incubated at 37°C with shaking at 200 rpm until samples were taken in a time-series for OD₆₀₀ measurements or cfu determination. Serial diluted samples were plated on GC agar containing 1% Vitox and azithromycin (128 mg/mL) or chloramphenicol (5 mg/mL).

### 23S rRNA stability assays by quantitative real-time PCR (qPCR)

Overnight grown N. gonorrhoeae strains were suspended in 12 mL of GC broth containing 1% Vitox and grown at 37°C with shaking at 200 rpm until an OD₆₀₀ of 0.5 was reached. Rifampicin (BBI) was added at a concentration of 50 mg/mL to stop expression of new RNA. Samples were collected in a time-course up to 8 h after addition of rifampicin. Total RNA was extracted from the samples using the RNA isolator kit (Vazyme) according to the manufacturer’s protocols. A total of 0.5 μg of RNA from each sample was used for cDNA synthesis using Hiscript Q Reverse Transcriptase Super Mix (Vazyme), 2 μL of diluted cDNA and 200 nM 23S rRNA or 16S rRNA primers (Table S2). Reactions were run on a 7500 Real-Time PCR system (Applied Biosystems) with an initial step of 30 s at 95°C for 15 s. The relative 23S rRNA 2059 4G/4A stability was determined after normalization with the 16S rRNA ratios.

### Competition assays in HeLa and THP-1 cell lines

HeLa cells (ATCC CCL-2) and THP-1 cells (ATCC TIB-202) were seeded at a concentration of 2 x 10⁵ cells/well in 12-well plates in RPMI-1640 cell culture medium (Biological Industries) supplemented with 10% FBS (Bovagen) and grown overnight at 37°C in the presence of 5% CO₂. THP-1 cells were further

### Ethics

The animal experiments were approved by the Zhejiang University Animal Care and Use Committee under project license number ZJU2015-032-01. All animal procedures were performed according to the guidelines of the Administration of Affairs Concerning Experimental Animals of the People’s
Figure 1. Effects of the 23S rRNA A2059G mutation on growth of *N. gonorrhoeae* in the presence of bile and hydrophobic compounds. Strains ZJXSH89-4G (open circles), ZJXSH89-4A (open triangles), ZJXSH86-4G (filled circles) and ZJXSH86-4A (filled triangles) were grown in the absence of additional compounds (a and b) or in the presence of lithocholic acid (c and d), Triton X-100 (e and f), sodium cholate (g and h) or Tween 20 (i and j). Graphs represent the mean and SD of at least three independent biological repeats performed on separate occasions. Significant differences between strains at corresponding timepoints were identified by Student’s two-tailed unpaired t-test (GraphPad Prism). *P* < 0.05.
Gonococcal high-level azithromycin resistance and biological fitness

Results

23S rRNA A2059G is the only mutation required for high-level azithromycin resistance

Previous studies have shown a strong association between N. gonorrhoae high-level azithromycin resistance and the 23S rRNA A2059G mutation. In addition, all our high-level azithromycin-resistant clinical isolates contained the 23S rRNA A2059G mutation and a derepressed MtrCDE multidrug efflux pump due to an adenine deletion in the 13 bp inverted repeat in the promoter region of mtrR. To verify whether MtrCDE is actually involved in high-level azithromycin resistance, mtrCDE deletion mutants were generated in the two high-level azithromycin-resistant strains ZJXSH89 and ZJXSH73, which in our previous studies belonged to the two most commonly observed STs among high-level azithromycin-resistant strains. However, no difference in azithromycin susceptibility was observed between WT and ΔmtrCDE strains (Table 1), indicating that MtrCDE is not important for high-level azithromycin resistance. Subsequently, the high-level azithromycin-resistant strain ZJXSH89 and the azithromycin-susceptible strain ZJXSH86 were used to specifically mutate the A/G at position 2059 of 23S rDNA in all four copies in the genome, without using selection procedures involving azithromycin or other macrolides, which might introduce unwanted secondary mutations. WGS was performed on ZJXSH89-4G, ZJXSH89-4A, ZJXSH86-4A and ZJXSH86-4G, and confirmed the absence of any other unwanted secondary mutations. Subsequently, azithromycin susceptibility analysis showed that both ZJXSH89-4G and ZJXSH86-4G, containing 2059G in all four copies of 23S rDNA (4G), were high-level azithromycin-resistant, while ZJXSH89-4A and ZJXSH86-4A, containing four copies of 2059A (4A), were completely susceptible to azithromycin (Table 1). Interestingly, while strains containing three copies of 2059G (3G) still showed high-level azithromycin resistance and strains containing one copy (1G) were fully susceptible, strains containing two copies (2G) showed variable outcomes between technical and biological repeats (Table 1). Variable azithromycin susceptibility outcomes might be explained by additional recombination events within the experiment that result in changes in 23S rRNA copies containing 2059G. Therefore, ZJXSH89 and ZJXSH86 containing two (2G) and three (3G) copies of 2059G were plated on agar containing various azithromycin concentrations. After incubation of the plates, for each strain and azithromycin concentration, five colonies were randomly selected for location-specific sequencing of the 2059A/G polymorphism of 23S rRNA to identify whether additional recombination events had occurred (Table 2). While at the lowest azithromycin concentration (0.016 mg/L), all colonies displayed the expected location-specific 2059G/A, at elevated azithromycin concentrations additional A2059G mutations were readily selected for.

23S rRNA A2059G mutation impacts growth under stress conditions

To investigate whether the 23S rRNA A2059G mutation comes with a biological cost that affects gonococcal fitness, growth of the strains containing 4G and 4A was monitored in the presence and absence of hydrophobic and amphipathic compounds (Figure 1). In optimal growth medium, no significant differences were observed in the growth of ZJXSH89-4G and ZJXSH89-4A (Figure 1a) or ZJXSH86-4G and ZJXSH86-4A (Figure 1b). However, in the presence of lithocholic acid (Figure 1c and d) or Triton X-100 (Figure 1e and f), for both strains 4G showed significant better growth compared with 4A. In contrast, in the presence of sodium cholate, ZJXSH89-4G showed significantly better growth than ZJXSH89-4A (Figure 1g) while the opposite was observed for ZJXSH86-4G and ZJXSH86-4A (Figure 1h). Similarly, in the presence of Tween 20, ZJXSH89-4A grew significantly better than ZJXSH89-4G (Figure 1i) while again the opposite was observed for ZJXSH86-4G and ZJXSH86-4A (Figure 1j). Differences in growth phenotypes between 4G and 4A were not the result of rpsl mutations introduced to generate the 4G and 4A mutants (Figure S1). Overall, these data indicate that the 23S rRNA A2059G mutation has an impact on biological fitness under certain stress conditions.

23S rRNA A2059G mutation has no impact on 23S rRNA degradation

To investigate whether the 23S rRNA A2059G mutation of N. gonorrhoae affects its degradation, qPCR experiments were performed on ZJXSH89-4G, ZJXSH89-4A, ZJXSH86-4A and ZJXSH86-4G, after incubation of the plates, for each strain and azithromycin concentration, five colonies were randomly selected for location-specific sequencing of the 2059A/G polymorphism of 23S rRNA to identify whether additional recombination events had occurred (Table 2). While at the lowest azithromycin concentration (0.016 mg/L), all colonies displayed the expected location-specific 2059G/A, at elevated azithromycin concentrations additional A2059G mutations were readily selected for.

Figure 2. A2059G mutation has no impact on N. gonorrhoeae 23S rRNA stability or degradation. Relative 23S rRNA levels were quantified by qPCR in a time-series after addition of rifampicin to inhibit transcription. Relative 23S rRNA levels were divided by relative levels of 16S rRNA to correct for sample variations. (a) Relative 23S rRNA levels of strains ZJXSH89-4G (white) and ZJXSH89-4A (black). (b) Relative 23S rRNA levels of strains ZJXSH86-4G (white) and ZJXSH86-4A (black). Graphs represent the mean and SD of three biologically independent repeats performed on separate occasions. No significant differences were observed.
performed to investigate the relative reduction of 23S rRNA levels compared with 16S rRNA levels in a time-series after halting transcription in mid-log cells. However, no significant differences in relative 23S rRNA levels were observed in the time-series between ZJXSH89-4G and ZJXSH89-4A (Figure 2a) or between ZJXSH86-4G and ZJXSH86-4A (Figure 2b). Therefore, these results show that the *N. gonorrhoeae* 23S rRNA A2059G mutation has no impact on its stability or degradation.

### 23S rRNA A2059G mutation contributes to colonization in a mouse vaginal tract infection model

To investigate whether the 23S rRNA A2059G mutation contributes to host colonization, competition assays were performed in liquid culture and in a mouse vaginal tract infection model. Bacterial suspensions containing equal numbers of ZJXSH89-4G and ZJXSH89-4A (Figure 2a) or between ZJXSH86-4G and ZJXSH86-4A (Figure 2b). Therefore, these results show that the *N. gonorrhoeae* 23S rRNA A2059G mutation has no impact on its stability or degradation.

#### Figure 3. 23S rRNA A2059G mutation contributes to in vivo biological fitness of *N. gonorrhoeae* in a mouse vaginal tract infection model.

Bacterial mixtures of strains ZJXSH89-4G and ZJXSH89-4A and of strains ZJXSH86-4G and ZJXSH86-4A were used for competition assays in liquid growth medium and a mouse vaginal tract infection model. (a) Recovery of strains ZJXSH89-4G (circles) and ZJXSH89-4A (triangles) during competitive growth in liquid growth medium. (b) Recovery of strains ZJXSH86-4G (circles) and ZJXSH86-4A (triangles) during competitive growth in liquid growth medium. Liquid growth curves represent the mean and SD of three biologically independent experiments performed on separate occasions. (c) Competition indices between strains ZJXSH89-4G and ZJXSH89-4A during competitive colonization and survival in a mouse vaginal tract infection model. (d) Competition indices between strains ZJXSH86-4G and ZJXSH86-4A during competitive colonization and survival in a mouse vaginal tract infection model. Filled symbols indicate the mice in which cfu were recovered for one strain only. Mice for which no bacteria were recovered for either strain are not shown. Competition indices were calculated as (4G/4A)$_{output}$/ (4G/4A)$_{input}$. Significant differences in competition indices between liquid growth medium and the mouse vaginal tract infection model were identified by Student’s two-tailed unpaired t-test (GraphPad Prism). *$P < 0.05$.

#### 23S rRNA A2059G mutation contributes to invasion of epithelial cells

As the 23S rRNA A2059G mutation contributes to *in vivo* biological fitness, next the contribution of this mutation to bacterial invasion
and intracellular survival in epithelial cells and macrophages was investigated. Bacterial suspensions containing equal numbers of ZJXSH89-4G and ZJXSH89-4A and of strains ZJXSH86-4G and ZJXSH86-4A were used to perform competition assays for invasion and intracellular survival in gentamicin protection assays using HeLa cells and differentiated THP-1 human monocytic cells. (a) Recovery of strains ZJXSH89-4G (white) and ZJXSH89-4A (black) during competitive survival inside HeLa cells. (b) Recovery of strains ZJXSH86-4G (white) and ZJXSH86-4A (black) during competitive survival inside HeLa cells. (c) Recovery of strains ZJXSH89-4G (white) and ZJXSH89-4A (black) during competitive survival inside differentiated THP-1 cells. (d) Recovery of strains ZJXSH86-4G (white) and ZJXSH86-4A (black) during competitive survival inside differentiated THP-1 cells. All graphs represent the mean and SD of three biologically independent repeats performed on separate occasions. The broken line indicates the detection limit of 25 cfu. Significant differences in recovery of intracellular cfu between 4G and 4A were identified by Student’s two-tailed paired t-test (GraphPad Prism). *P < 0.05.

**Discussion**

The emergence of *N. gonorrhoeae* high-level azithromycin-resistant isolates over the past decade poses a major risk for the security and continuity of the currently used ceftriaxone/azithromycin dual therapy. The 23S rRNA A2059G mutation has been strongly associated with *N. gonorrhoeae* high-level azithromycin resistance. Our current study has now confirmed that the 23S rRNA A2059G mutation is the only mutation required to change azithromycin-susceptible strains into high-level azithromycin-resistant strains. Further analysis of copy number of the 23S rDNA mutation and azithromycin resistance showed that three copies of A2059G was sufficient for displaying the high-level azithromycin resistance phenotype, which was similar to previous observations. However, investigation of single colonies growing on agar plates containing different concentrations of azithromycin indicated that internal 23S rDNA recombination events happen frequently and affect copy number analyses.

Given that ceftriaxone monotherapy and ceftriaxone/azithromycin dual therapy are currently the last available empirical antimicrobial treatments for *N. gonorrhoeae*, antimicrobial stewardship programmes aimed at prolonging the effectiveness of these therapies appear to be essential. Emergence and spreading of antimicrobial resistance is dependent on the pattern of antibiotic use and the formation and biological fitness of resistant
strains. However, antimicrobial resistance often comes at a biological fitness cost for maintaining the mutation, particularly when these mutations are chromosomally located. For instance, 23S rRNA mutations giving rise to macrolide-resistant Campylobacter jejuni showed a reduced growth rate and were outcompeted by their parent strains in mixed cultures. Similarly, a 23S rRNA G2576T mutation in Streptococcus pneumoniae, which provides resistance to linezolid, imposes a growth defect. In contrast, our results showed that the N. gonorrhoeae 23S rRNA A2059G mutation did not affect in vitro growth under optimal conditions, while in the presence of hydrophobic or amphipathic compounds that mimic stress conditions encountered in the host, a growth benefit was observed for some strains. However, variable strain-dependent growth benefits were observed for one or the other allele in the presence of other stresses, thereby also indicating a possible influence of the genetic background. Interestingly, for Escherichia coli it has been shown that mutations providing resistance to rifampicin and streptomycin come with a biological cost and reduce growth rate; however, survival of these mutants inside macrophages was enhanced. Therefore, it appears that even though some antibiotic resistance mutations incur a fitness cost, they could still contribute to survival in the host. In our experiments, no significant differences in intracellular survival of macrophages were observed. However, the 23S rRNA A2059G mutants showed enhanced biological fitness during the colonization of a mouse vaginal tract infection model and enhanced invasion of epithelial cells. Increased biological fitness in the mouse vaginal tract infection model has previously also been shown for N. gonorrhoeae mutations providing fluoroquinolone resistance, while similar mutations in E. coli required additional growth defect compensatory mutations before increased in vivo biological fitness was observed. In contrast, no compensatory mutations were required for the N. gonorrhoeae 23S rRNA A2059G mutants to display increased in vivo biological fitness. However, it is important to note that in vitro cell culture and mouse vaginal tract infection models are very different from human infections. In addition, the bacterial genetic background may also play an important role, as variable results were obtained for the two strains during in vitro growth experiments in the presence of hydrophobic and amphipathic compounds. Therefore, we should be careful with translating the obtained results to the situation of high-level azithromycin-resistant strains in the human population.

In conclusion, this study confirmed the previous observation that N. gonorrhoeae high-level azithromycin resistance is the result of a single point mutation in 23S rRNA and that no other mutations are required. Furthermore, 23S rRNA A2059G mutants showed enhanced in vivo biological fitness in a mouse infection model, indicating that these strains might have a selective advantage under certain conditions in the absence of antimicrobial selective pressure. Therefore, these results could have major implications for antimicrobial stewardship programs aimed at maintaining the efficacy of the current antimicrobial treatment regimens.

Acknowledgements
We thank the company Genewiz (Shanghai) for performing WGS and comparative genome analyses.

Funding
This work was supported by the Zhejiang Provincial Natural Science Foundation of China (grant number LR16H190001).

Transparency declarations
None to declare.

Supplementary data
Tables S1–S3 and Figures S1 and S2 are available as Supplementary data at JAC Online.

References
Gonococcal high-level azithromycin resistance and biological fitness

29 Warner DM, Shafer WM, Jerse AE. Clinically relevant mutations that cause derepression of the Neisseria gonorrhoeae MtrC-MtrD-MtrE Efflux pump system confer different levels of antimicrobial resistance and in vivo fitness. Mol Microbiol 2008; 70: 462–78.