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ksgA* mutations confer resistance to kasugamycin in *Neisseria gonorrhoeae

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Abstract

The aminoglycoside antibiotic kasugamycin (KSG) inhibits translation initiation and thus the growth of many bacteria. In this study, we tested the susceptibilities to KSG of 22 low-passage clinical isolates and 2 laboratory strains of *Neisseria gonorrhoeae*. Although the range of KSG minimum inhibitory concentrations (MICs) was narrow (seven-fold), clinical isolates and laboratory strains fell into three distinct classes of KSG sensitivity, susceptible, somewhat sensitive and resistant, with MICs of 30, 60–100 and 200 µg/mL, respectively. Two genes have previously been shown to be involved in bacterial KSG resistance: *rpsI*, which encodes the 30S ribosomal subunit S9 protein; and *ksgA*, which encodes a predicted dimethyltransferase. Although sequencing of *rpsI* and *ksgA* from clinical isolates revealed polymorphisms, none correlated with the MICs of KSG. Ten spontaneous KSG-resistant (KSG^R) mutants were isolated from laboratory strain FA1090 at a frequency of $<4.4 \times 10^{-6}$ resistant colony-forming units (CFU)/total CFU. All isolated KSG^R variants had mutations in *ksgA*, whilst no mutations were observed in *rpsI*. *ksgA* mutations conferring KSG resistance included four point mutations, two in-frame and one out-of-frame deletions, one in-frame duplication and two frame-shift insertions. These data show a narrow range of susceptibilities for the clinical isolates and laboratory strains examined; moreover, the differences in MICs do not correlate with nucleotide polymorphisms in *rpsI* or *ksgA*. Additionally, spontaneous KSG^R mutants arise by a variety of *ksgA* mutations.

Keywords

Mutagenesis; Gonorrhoea; Antibiotic resistance

1. Introduction

The aminoglycoside antibiotic kasugamycin (KSG), first isolated from *Streptomyces kasugaensis*, has been used to prevent rice blast disease caused by the fungus *Pyricularia oryzae* [1–3]. KSG inhibits the growth of a wide variety of microorganisms, with reported low toxicity against plants, humans and other animals [2,4,5]. However, as an aminoglycoside, some degree of nephrotoxicity and ototoxicity is expected. Several Gram-negative bacteria, including *Pseudomonas* spp. and *Escherichia coli* strains, as well as the Gram-positive *Bacillus* spp. are sensitive to KSG [6]. Although clinical use of KSG has been explored as treatment for *Pseudomonas aeruginosa* infection in the bladder [4], it is currently only used agriculturally. KSG inhibits translation initiation by blocking transfer RNA (tRNA) binding

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to the 30S ribosomal subunit; it can be bacteriostatic or bactericidal depending on the concentration used [7]. Recent structural analysis of KSG translation inhibition has provided a detailed mechanism for KSG binding and inhibition [8,9]. KSG is thought to mimic messenger RNA (mRNA) codon nucleotides and to occupy the peptidyl (P) and exit (E) sites of the ribosome, causing distortion of the mRNA–tRNA codon–anticodon interaction and blocking translation initiation in susceptible organisms [8,9].

Several KSG resistance mutations have been identified and characterised in *E. coli* [10–13] and *Bacillus subtilis* [14]. The *E. coli* gene product of *ksgA*, an adenosine dimethyltransferase KsgA, is responsible for methylation of 16S ribosomal RNA (rRNA) adenosines at positions 1518 and 1519 [15–17]. Interestingly, this rRNA modification by KsgA appears to be conserved in all species of bacteria, archaea and eukarya studied to date [18,19]. The exact biological function of this rRNA modification is unknown, and in many bacteria loss of KsgA-dependent methylation is not lethal [20–22]. Mutations that disrupt KsgA-dependent rRNA methylation are the most common mechanism of KSG resistance in bacteria [11]. Other mutations known to confer KSG resistance include mutation of the target nucleotides A1518 and A1519 in the 16S rRNA [12] and amino acid substitutions in the 30S protein subunit S9, the gene product of *rpsI* [10]. These mutations may stabilise the mRNA–tRNA interaction perturbed by KSG leading to KSG resistance. Interestingly, *rpsI* mutations can result in both resistance to and dependence on KSG [10,23]. For example, one KSG-resistant (KSG^R) *rpsI* mutant, *E. coli* strain MV101, required KSG to depress the rate of protein synthesis, otherwise the enhanced ribosomal activity caused by the *rpsI* mutation was lethal [10]. Both 16S rRNA and *rpsI* mutations occur less frequently than mutations in *ksgA* [11,12].

Neisseria gonorrhoeae is an obligate human pathogen and is the only causative agent of the sexually transmitted infection gonorrhoea. In the USA, gonorrhoea is the second most frequently reported communicable disease, with 339 593 reported cases in 2005 and as many as 700 000 total cases yearly [24]. Many infected individuals may be asymptomatic [25]; however, serious symptomatic infections can occur both in men and women. Notably, *N. gonorrhoeae* can cause pelvic inflammatory disease (PID) in women [26], epididymitis in men and arthritis both in men and women [27]. Additionally, the pharynx, rectum and conjunctiva are sites often infected by *N. gonorrhoeae*, thus antibiotics must be effective at controlling the bacteria at multiple mucosal sites. Although antibiotic treatment has historically been effective in control of the disease, the increased prevalence of antibiotic-resistant *N. gonorrhoeae* has severely limited available treatment options [28]. Accordingly, the US Centers for Disease Control and Prevention (CDC) has classified *N. gonorrhoeae* as a ‘superbug’, and only cephalosporin drugs are recommended for treatment of gonorrhoea in the USA [29]. With ever increasing resistance to antibiotics and with disease incidence on the rise in many countries, novel therapies are needed to control the spread of disease. Here we examined the susceptibilities of 22 clinical isolates and 2 laboratory strains of *N. gonorrhoeae* to KSG and isolated 10 spontaneous KSG^R mutants.

2. Methods and materials

2.1. Bacterial strains and growth conditions

KSG susceptibility was examined for a previously assembled panel of characterised, low-passage clinical isolates of *N. gonorrhoeae* from three US laboratories (Table 1) [30]. These included seven isolates from PID patients collected by the CDC, seven isolates from disseminated gonococcal infections (DGIs) described by O’Brien et al. [27], four endometrial isolates provided by the laboratory of Peter A. Rice and four local infection (i.e. urethritis or cervicitis) isolates from the Bell Flower Clinic in Indianapolis, IN. The laboratory strains FA1090 (pilin variant 1-81-S2 [31] and pilin variant RM11.2 *recA6* [32]) and MS11 (pilin variant VD300) were used. The RM11.2 strain was only grown in the presence of isopropyl

β -D-1-thiogalactopyranoside (IPTG) when *recA* expression was required, after which the *pilE* locus was sequenced to confirm that the resulting bacteria maintained the parental pilin sequence.

Clinical isolates were grown on GC Medium Base (GCB) plates supplemented with 1% IsoVitalEx (BBL, Becton Dickinson, Cockeysville, MD) and laboratory strains were grown on GCB plates with Kellogg's supplements I and II [33]. All bacterial strains were incubated at 37 °C in a 5% CO₂ humidified atmosphere. KSG (BIOMOL International, Plymouth Meeting, PA) was suspended in H₂O at 100 µg/mL and added to sterilised GCB media to give final concentrations of 30–300 µg/mL as indicated in the results.

2.2. Minimum inhibitory concentration (MIC) determination

MICs of KSG were determined for all strains by the agar dilution method in accordance with the guidelines of the National Committee for Clinical Laboratory Standards [34,35]. Briefly, strains were grown for 16 h on GCB plates (with appropriate supplements) and were re-suspended in liquid GCB (with appropriate supplements) to an optical density at 550 nm (OD₅₅₀) between 0.2 and 0.4. Ten-fold serial dilutions were plated in duplicate both on non-selective GCB and GCB plates containing various concentrations of KSG. Dilutions plated on non-selective GCB were used to determine the colony-forming units (CFU)/mL of each strain. Spots of dilutions representing ca. 10⁴ CFU in a spot were used to determine the inhibition of bacterial growth after 24 h, with the endpoint defined as the lowest concentration of KSG that completely inhibits growth while disregarding single colonies or a slight haze [34]. Given the relatively narrow range of MIC values and the lack of approved sensitive/resistant breakpoints for KSG, fractional MIC concentrations were used as listed in the results. MIC determinations were performed at least three times for each strain.

2.3. Sequence analysis and polymerase chain reaction (PCR)

DNA sequencing was carried out commercially (SeqWright, Houston, TX). The primers used for sequencing the *ksgA* and *rpsI* genes were designed to anneal to DNA flanking these genes, so any mutations at the extreme 5' or 3' end could be detected. Primer ksgtop (5'-AAAGGGCGGGGTTTCAACC-3') anneals to DNA 33 bp upstream of the start of translation of *ksgA*, and primer ksgbot (5'-CGAATATTGTGCGTGCAGG-3') anneals to DNA 86 bp downstream of the stop codon of *ksgA*. Primer rpsIbot2 (5'-TATGCTGCCCAAAGGTCCG-3') anneals to DNA 123 bp upstream of the start of *rpsI*, and primer rpsIbot2 (5'-TTCGTCCGTGTAGTCGATAC-3') anneals to DNA 115 bp downstream of *rpsI*. Colony PCR was performed as previously described [36]. PCR conditions were 1× buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.5 pmol of each primer, 1.25 U of *Taq* polymerase (Promega, Madison, WI) and 5 µL of colony lysis solution (1% Triton X-100, 20 mM Tris–Cl (pH 8.3), 2 mM ethylene diamine tetra-acetic acid (EDTA)) as the template. Following initial denaturation at 95 °C for 2 min, PCR for *rpsI* amplification consisted of 30 cycles of denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min and extension at 72 °C for 1 min 25 s. PCR for amplification of *ksgA* was identical except 61 °C was used as the annealing temperature. DNA sequence analysis of *pilE* was performed as previously described [37].

2.4. Cloning of *ksgA* from the 431Δ99 KSG^R mutant

PCR amplification of *ksgA* from the 431Δ99 KSG^R mutant was performed as above except using primers ksgGUS+top1 (5'-TCGTATGCCGTCTGAAAACG-3'), ksgbot3 (5'-CCAGATAATTGCTCAACGCC-3'), annealing at 57 °C, and using genomic DNA from the 431Δ99 *ksgA* mutant as template. The resultant PCR product was blunted by adding 10 mM dNTPs and 0.625 U of *Pfu* DNA polymerase and incubating at 72 °C for 30 min. The blunted PCR products were cloned into the pCR-Blunt vector (Invitrogen, Carlsbad, CA) as per the

manufacturer's instructions and transformed into *E. coli* TOP10 cells (Invitrogen, Carlsbad, CA).

2.5. Determination of the frequency of spontaneous KSG^R mutants

The frequencies of spontaneous mutations conferring resistance to KSG were determined using *N. gonorrhoeae* strains RM11.2 [38], both in the presence and absence of IPTG, and the 1-81-S2 pilin variant of FA1090 [31]. Briefly, cells were grown for 18 h and were swabbed into 3 mL of liquid GCB to an OD₅₅₀ of ca. 0.3. A total of 500 µL of the re-suspended bacteria was grown for 48 h on five separate GCB plates (100 µL plated on each plate) containing 150 µg/mL KSG. The total CFU/mL was determined by plating serial dilutions (1:10) on GCB plates and counting colonies after 24 h. The mutation frequency was calculated by dividing the total number of resistant CFU/mL by the total CFU/mL. Since some experiments did not yield any spontaneous KSG^R mutants, the values from experiments where spontaneous KSG^R mutants were detected were used. There were ten experiments out of 15 total experiments where KSG^R variants were isolated and these values were used to calculate the mean frequency of spontaneous mutations that confer resistance to KSG. Since the mutation frequency in the other five experiments was less than the frequency in the ten experiments where mutants were detected, the actual mutation frequency may be less than the mean reported. The DNA sequence of the *rpsI* and *ksgA* genes from each KSG^R mutant was determined. To confirm that the mutant *ksgA* sequences identified were responsible for KSG resistance, the *ksgA* gene from each of the ten KSG^R mutants was PCR amplified as above and transformed into RM11.2 selecting for KSG resistance. The *ksgA* gene from the resultant KSG^R transformants was sequenced and compared with the input sequence.

2.6. Transformation assays

Transformation assays were performed as previously described [39] with the following modifications. Following bacterial re-suspension, 30 µL of the re-suspension was added to tubes containing the transforming DNA. Both genomic DNA isolated from *ksgA* 431Δ99 and cloned *ksgA* from this mutant (100 ng of DNA) were used as the transforming DNA, and GCB plates containing 150 µg/mL KSG were used for selection of transformants. Transformation efficiencies are reported as KSG^R CFU divided by total CFU. Transformation assays were performed in triplicate.

3. Results

3.1. *Neisseria gonorrhoeae* strains vary in susceptibility to the antibiotic KSG

Since KSG is active against various Gram-negative bacteria [6,7] and has never been examined in *N. gonorrhoeae*, we determined the MICs of KSG for 2 laboratory strains and 22 low-passage clinical isolates of *N. gonorrhoeae* (Table 1). These clinical isolates are from diverse disease courses including PID, DGI, endometrial involvement and local infection (urethritis or cervicitis) (Table 1). The MIC for the laboratory strain FA1090 (RM11.2) was 100 µg/mL, whilst the laboratory strain MS11 was slightly more susceptible with a MIC of 60 µg/mL. The panel of clinical isolates had MICs ranging from 30 µg/mL to 200 µg/mL (Table 1). A total of 12 isolates were susceptible to KSG concentrations of ≤60 µg/mL, 9 were inhibited by KSG of 60–90 µg/mL and only 1 isolate (PID 8) was found to be resistant to 200 µg/mL of KSG (Table 1). These results demonstrate a relatively narrow range of susceptibility of *N. gonorrhoeae* isolates to KSG and indicate that susceptibility to KSG does not correlate with sites of isolation or disease course.

3.2. Spontaneous mutation frequency and mutations in *ksgA* conferring resistance to KSG

Three mechanisms have been identified that confer resistance to KSG in bacteria: changing the target methylated nucleotides in 16S rRNA [12]; disruption of the KsgA methyltransferase [11,19]; or mutations in *rpsI* that stabilise the rRNA–tRNA interaction in the 30S ribosomal subunit S9 in the presence of KSG [10,13,40,41]. To determine the frequency of spontaneous mutations conferring resistance to KSG in *N. gonorrhoeae*, we isolated spontaneous KSG^R colonies from the laboratory strain FA1090. Given that different pilin variants have variable resistance to kanamycin and penicillin in *N. gonorrhoeae* [42], two different pilin variants of FA1090 were used, 1-81-S2 and RM11.2. Similarly, RecA expression is known to affect the spontaneous mutation frequency in *E. coli* [43], thus the inducible *recA* locus in RM11.2 was utilised to control RecA expression. KSG^R colonies were isolated after 18 h of growth on plates containing 150 µg/mL KSG. The mutation frequency without RecA induction was 2.1×10^{-6} KSG^R CFU/total CFU and the frequency with RecA induction was seven-fold higher (1.5×10^{-5} KSG^R CFU/total CFU), which is not statistically different by Student's *t*-test. Similarly, the 1-81-S2 pilin variant of FA1090, which was isolated from human volunteer studies, had a spontaneous mutation frequency of 4.4×10^{-6} KSG^R CFU/total CFU (not statistically different from RM11.2 with or without RecA induction by Student's *t*-test).

To determine the sequence changes responsible for KSG resistance, the *rpsI* and *ksgA* genes were sequenced from all KSG^R FA1090 variants recovered from the spontaneous mutants isolated above (Fig. 1). All KSG^R variants had mutations in *ksgA* and no changes in *rpsI* relative to the parental strain. A total of 10 different *ksgA* mutations were identified, comprising four point mutations, three deletions, one duplication and two insertions (Fig. 1B). To ensure that each of the ten *ksgA* mutations were the cause of the KSG^R phenotype, the *ksgA* gene from each of the mutants was moved back into the KSG-sensitive strain RM11.2 by transformation (back-crossed) and checked for resistance to KSG. All ten mutations produced KSG^R phenotypes after back-crossing. The four point mutations are all found in the first half of the gene (total gene length 777 bp). Both nucleotide substitutions G118A and G119A result in amino acid substitutions at glycine 40 (Gly40Ser and Gly40Asp, respectively) (Fig. 2). Interestingly, two of the three deletion mutations isolated, 202Δ33 and 431Δ99, are in-frame deletions (Fig. 1B(II)), whereas the most downstream deletion, 740Δ1, results in a frame shift at position 247 and a subsequent premature stop codon at amino acid 250 (Figs 1B(II) and 2). One mutant with a duplication within *ksgA*, 413dup18, was also isolated (Fig. 1B(III)). This mutant had duplications of nucleotides that result in direct repeats (Fig. 1B(III)) but did not result in a frame-shift (Fig. 2). Two frame-shift insertions were isolated at position 739 (Fig. 1B(III)). With the exception of the one C-terminal deletion, 740Δ1, all other changes in *ksgA* isolated would be predicted to produce a full-length gene product. The MIC for the *ksgA* 431Δ99 mutant was found to be three-fold higher than the parental strain (Table 1). These results demonstrate that spontaneous KSG^R mutants in *N. gonorrhoeae* arise through mutations in *ksgA*, which are likely to reduce KsgA activity and lead to undermethylated rRNA and thus resistance to KSG.

3.3. Transformation of *ksgA* mutant DNA into sensitive *Neisseria gonorrhoeae* confers resistance to KSG

We investigated the ability of *ksgA* 431Δ99 to confer resistance to KSG by transformation to a KSG-sensitive strain of *N. gonorrhoeae*. Genomic DNA from *ksgA* 431Δ99 was isolated and used to transform RM11.2. The transformation efficiency was 2.04×10^{-3} resistant CFU/total CFU for RM11.2. To confirm further that the *ksgA* 431Δ99 locus confers KSG resistance, the mutant *ksgA* gene was cloned and the cloned DNA was used to transform RM11.2. The transformation efficiency was 1.11×10^{-2} resistant CFU/total CFU. These results clearly demonstrate the capacity of *ksgA* mutations to serve as a selective transformation marker of

N. gonorrhoeae and confirm that the *ksgA* 431Δ99 locus is responsible for KSG resistance of this strain.

3.4. *rpsI* and *ksgA* sequences from clinical isolates

To determine whether the levels of KSG sensitivity in the *N. gonorrhoeae* clinical isolates correlated with sequence variation in *ksgA* and *rpsI*, these genes from each clinical and laboratory strain were sequenced and compared with the sequences of these genes from FA1090. Whilst no amino acid substitutions in *rpsI* were found relative to FA1090, several isolates contained one or two silent nucleotide polymorphisms (Table 2). These results demonstrate that *rpsI* is highly conserved in *N. gonorrhoeae*.

Sequencing of *ksgA* revealed that all strains contained one amino acid substitution (Asp198Glu) and two silent nucleotide polymorphisms, C654T and A663G, relative to FA1090 (Table 2). Additionally, several strains were found to contain other silent nucleotide polymorphisms (Table 2). Strikingly, PID 302 contained an additional 21 polymorphic nucleotides, 4 of which result in the amino acid substitutions (Table 2). These results demonstrate that KSG sensitivities of clinical isolates examined do not correlate with sequence differences of either *rpsI* or *ksgA* or with mutant *ksgA* sequences identified from spontaneous KSG^R mutants. Furthermore, these results show that both *rpsI* and *ksgA* are highly conserved in numerous clinical isolates of *N. gonorrhoeae*.

4. Discussion

Here we investigated the activity of the aminoglycoside antibiotic KSG against numerous clinical isolates and laboratory strains of *N. gonorrhoeae*. Although a previous report showed that pathogenic *E. coli*, *P. aeruginosa*, *Klebsiella pneumoniae* and *Serratia* spp. can have KSG resistance at levels between 100 µg/mL and 400 µg/mL [6], most clinical isolates of *N. gonorrhoeae* investigated in this study were sensitive to lower levels (30–100 µg/mL) of KSG. Although there are no established MIC resistant/sensitive breakpoints for KSG, the clinical isolates and laboratory strains fell into three distinct classes of KSG sensitivity, defined as susceptible (MIC = 30 µg/mL), somewhat sensitive (MIC = 60–100 µg/mL) or resistant (MIC = 200 µg/mL). Nineteen of the 22 clinical strains and all of the laboratory strains were sensitive to KSG with MICs of 60–100 µg/mL (Table 1). This group includes isolates from DGI, PID and localised infections (cervical or urethral) (Table 1). Only isolate PID 8 was resistant to KSG (MIC = 200 µg/mL). These results demonstrate that there is no correlation between disease course or site of isolation and KSG sensitivity.

In an effort to understand why some clinical isolates exhibited more or less sensitivity to KSG, the *rpsI* and *ksgA* genes from the clinical isolates were sequenced and compared with the laboratory strain FA1090. Two variable nucleotides were found in *rpsI* relative to FA1090 in several clinical isolates, although neither polymorphism resulted in an amino acid change (Table 2). Interestingly, mutations in 16S rRNA in *Neisseria* spp. have been shown to confer resistance to the antibiotic spectinomycin [44]. The absolute conservation of the amino acid sequence of RpsI in the clinical isolates together with the lack of isolated *rpsI* mutations demonstrate the highly conserved nature of RpsI, an essential component of the ribosome. The *ksgA* gene showed divergence from FA1090, with all clinical isolates containing an Asp198Glu substitution from FA1090 and two silent polymorphic nucleotide changes (Table 2). Since all clinical isolates contained this amino acid substitution, it is unlikely that it contributes to any change in KSG susceptibility. Additionally, several clinical strains contained other silent polymorphisms (Table 2). Strikingly, PID 302 contained 21 additional polymorphic nucleotides in *ksgA* (data not shown), 4 of which result in amino acid substitutions (Table 2), but none of these substitutions are likely to participate in KSG resistance because the MIC of this strain was 60 µg/mL. Moreover, none of the amino acid substitutions identified in the

clinical isolates correspond to substitutions isolated from spontaneous KSG^R RM11.2 mutants (Figs 1 and 2). It is clear that there is no correlation between the susceptibilities of the clinical isolates and the sequences of *ksgA* and *rpsL*. Although we do not know the cause of the different KSG susceptibilities in these clinical strains, we hypothesise that it is due to differences in cellular physiology that affect antibiotic resistance.

The enzymatic activity of KsgA is conserved in all organisms examined to date, and a recent report has shown that both archaeal and eukaryotic orthologues of KsgA can complement the enzymatic methyltransferase activity in bacteria in vitro and in vivo [45]. Sixteen residues are absolutely conserved in all KsgA enzymes examined, including *N. gonorrhoeae*, which further demonstrates the evolutionarily conserved post-transcriptional rRNA methylation activity of KsgA [45]. Whilst null mutations in *ksgA* conferring KSG resistance are not lethal for *E. coli*, the yeast orthologue of KsgA, Dim1, is essential for growth as a null mutation leads to the accumulation of misprocessed pre-rRNA, an effect independent of methyltransferase activity [46]. These observations have led to the notion that KsgA has additional and unidentified biological functions independent of methyltransferase activity [17]. Supporting this hypothesis, *E. coli* KsgA has been found to play a role in suppression of a cold-sensitive phenotype [17,47].

We identified ten distinct mutations in *ksgA* that lead to KSG resistance in *N. gonorrhoeae* (Figs 1 and 2); all were distinct from polymorphisms identified in the clinical isolates. In an effort to elucidate the possible consequences of these mutations on KsgA structure and function, we compared each *ksgA* mutation with the three-dimensional *E. coli* crystal structure of KsgA [18] (Fig. 2). *Neisseria gonorrhoeae* KsgA shares 46.9% identity and 61.9% similarity to the *E. coli* KsgA. Three of the four point mutations resulted in amino acid substitutions at absolutely conserved residues of KsgA; Gln12Pro and Gly40Asp/Ser (Fig. 2). The other point mutation identified to confer KSG resistance, Gly102Asp, is found adjacent to the conserved consensus sequence NLPY within Motif IV in the β_6 region, perhaps affecting the ability of the consensus region NLPY to fold correctly. The 202 Δ 33 mutation deletes amino acids 68–78 and affects the consensus α B and α B' helices. The 431 Δ 99 causes deletion of amino acids 143–175 (Fig. 2) and loss of the entire α F helix, β_6 strand and the turn leading into the β_7 strand. The C-terminal deletion, 740 Δ 1, results in a frame-shift in the α J helix and a stop codon 10 amino acids before the parental stop codon (Fig. 2). Similarly, the two C-terminal insertions (739insG and 739insA) result in frame shifts within the α J helix but do not cause premature stop codons (Fig. 2). Finally, the 413dup18 mutation results in addition of the sequence ERKEVV in the α E helix at amino acid 135 (Fig. 2). Although we cannot know the exact structural implications of these 10 mutations identified in *ksgA* and we did not measure methylase activity, these *ksgA* mutations may result in depressed enzymatic activity resulting in KSG resistance in *N. gonorrhoeae*.

There is an increasing need for novel therapies effective in treating *N. gonorrhoeae* infections as resistance to most antibiotics develops. We show that most clinical strains examined have relatively high KSG MICs compared with clinically used aminoglycoside antibiotics [48], that *ksgA* mutants with increased resistance to KSG are readily isolated and that some clinical isolates show intrinsically high levels of KSG resistance. Thus, it appears unlikely that KSG will provide a viable treatment option for gonorrhoea.

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Fig. 1.

(A) Map of *ksgA* and surrounding genes in *Neisseria gonorrhoeae*. Open boxes indicate intergenic regions. *NG0271* and *NG0270* (hypothetical proteins) are transcriptionally downstream of *ksgA*. *mug* and *trpB* are transcribed in the opposing direction. Drawn to scale.

(B) Location and type of spontaneous mutations in *ksgA* conferring kasugamycin (KSG) resistance in *N. gonorrhoeae*. Mutants were isolated from mutation frequency analysis and were transformed into a KSG-sensitive strain followed by sequencing of *ksgA*. Numbers indicate the location of the mutations. Drawn to scale. (I) Point mutations identified in *ksgA* that confer KSG resistance. (II) Deletion mutations identified in *ksgA* that confer KSG resistance. Note that the two most upstream deletions are in-frame and the most downstream deletion causes a frame shift and a premature stop codon. (III) Duplication (dup) and insertion (ins) mutations identified in *ksgA* that confer KSG resistance. The wavy lines and the far right numbers indicate the location and the length of the duplicated sequence, respectively.

Fig. 2. Sequence alignment of KsgA from *Escherichia coli* (Ec) and *Neisseria gonorrhoeae* (Ng) showing mutations that confer resistance to kasugamycin in *N. gonorrhoeae*. Identical residues are highlighted in dark grey and similar residues are highlighted in light grey. Vertical lines show the location of each mutation, and for single amino acid substitutions and deletions the text above the line indicates the identity of the mutations. Each of the two solid boxes designates residues removed from two unique deletion mutations. The triangle indicates the duplication mutation that results in the insertion of the indicated residues. The single dashed line indicates the location of three unique C-terminal frame-shift mutations. (A) Predicted amino acid sequence of the 739insG mutation; (B) predicted amino acid sequence of the 739insA mutation; (C) LWR.

and (C) predicted amino acid sequence of the 740 Δ 1 mutation that causes a stop codon at position 250.

Table 1

Kasugamycin minimum inhibitory concentrations (MICs) for laboratory strains and various clinical isolates

Strain	Patient gender	Site of isolation	MIC (µg/mL)	Source/reference
MS11	F	C	60	E.C. Gotschlich
FA1090 (RM11.2)	F	C	100	Long et al. (1998)
RM11.2 (<i>ksgA</i> 431Δ99)	N/A	N/A	300 ^a	This work (Fig. 1)
PID 1	F	C	60–75	O'Brien et al. [27]
PID 6	F	C	60	O'Brien et al. [27]
PID 8	F	C	200 ^a	O'Brien et al. [27]
PID 18	F	C	30	O'Brien et al. [27]
PID 20	F	C	60	O'Brien et al. [27]
PID 26	F	C	60	O'Brien et al. [27]
PID 302	F	C	60	O'Brien et al. [27]
DGI 4	F	B	60	O'Brien et al. [27]
DGI 5	M	B	60	O'Brien et al. [27]
DGI 8	F	B,C,P,R ^b	60–90	O'Brien et al. [27]
DGI 10	F	P	60	O'Brien et al. [27]
DGI 11	M	U	60	O'Brien et al. [27]
DGI 14	F	C	60–75	O'Brien et al. [27]
DGI 20	F	B,C ^b	30	O'Brien et al. [27]
EM 003-147	F	EM	75	P.A. Rice
EM 1069-12	F	EM	90	P.A. Rice
EM 2017-31	F	EM	90	P.A. Rice
EM 2291-124	F	EM	60–90	P.A. Rice
IN 229	F	C	90	Bell Flower Clinic
IN 400	M	U	60	Bell Flower Clinic
IN 578	M	U	60	Bell Flower Clinic
IN 644	F	C	60–90	Bell Flower Clinic

N/A, not applicable; C, cervix; B, blood; P, pharynx; R, rectum; U, urethra; EM, endometrium.

^a Classified as kasugamycin-resistant.^b Multiple sites of isolation are listed in the original report [27], therefore the actual site of isolation is not known.

Table 2
Nucleotide polymorphisms in *ksgA* and *rpsI* sequences relative to FA1090

Isolate	<i>ksgA</i>				<i>rpsI</i>			
	A135G	T444G	C447T	T471C	T594G ^a	C654T	A663G	T288C
PID 1		X	X		X	X	X	
PID 6					X	X	X	
PID 8 ^b					X	X	X	X
PID 18					X	X	X	X
PID 20					X	X	X	X
PID 26					X	X	X	X
PID 302 ^c		X		X	X	X	X	X
DGI 4					X	X	X	X
DGI 5					X	X	X	X
DGI 8					X	X	X	X
DGI 10					X	X	X	X
DGI 11					X	X	X	X
DGI 14					X	X	X	X
DGI 20					X	X	X	X
EM 003-147				X	X	X	X	
EM 1069-12					X	X	X	X
EM 2017-31					X	X	X	X
EM 2291-124				X	X	X	X	
IN 229	X				X	X	X	
IN 400		X	X		X	X	X	
IN 578					X	X	X	
IN 644		X	X		X	X	X	

^aT594G results in an Asp198Glu substitution, whereas all other polymorphisms are silent.

^bPID 8 is kasugamycin-resistant.

^cPID 302 contains 21 additional nucleotide polymorphisms in *ksgA*, 4 of which result in the amino acid substitutions Gly94Asn, Ser96Glu, Glu137Asp and Leu161Met.