Mutations in *Neisseria gonorrhoeae* grown in sub-lethal concentrations of monocaprin do not confer resistance

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Abstract

*Neisseria gonorrhoeae*, due to its short lipooligosaccharide structure, is generally more sensitive to the antimicrobial effects of some fatty acids than most other Gram negative bacteria. This supports recent development of a fatty acid-based potential treatment for gonococcal infections, particularly ophthalmia neonatorum. The *N. gonorrhoeae* genome contains genes for fatty acid resistance. In this study, the potential for genomic mutations that could lead to resistance to this potential new treatment were investigated. *N. gonorrhoeae* strain NCCP11945 was repeatedly passaged on growth media containing a sub-lethal concentration of fatty acid myristic acid and monoglyceride monocaprin. Cultures were re-sequenced and assessed for changes in minimum inhibitory concentration. Of note, monocaprin grown cultures developed a mutation in transcription factor gene *dksA*, which suppresses molecular chaperone DnaK and may be involved in the stress response. The minimum inhibitory concentration after exposure to monocaprin showed a modest two-fold change. The results of this study suggest that *N. gonorrhoeae* cannot readily evolve resistance that will impact treatment of ophthalmia neonatorum with monocaprin.

Introduction

Monocaprin is a powerful fast-acting bactericidal agent against *Neisseria gonorrhoeae* [1,2]. It has recently been proposed as a candidate for treatment of gonococcal eye infections, such as ophthalmia neonatorum, where topical treatment would rapidly kill the bacteria without irritating the eye [2]. As with any antimicrobial, attention must be given to possible resistance mechanisms. There are many ways that bacteria can acquire resistance to antimicrobials and some of these can be induced in the laboratory experimentally. Growth of the bacteria on media containing a sub-lethal concentration of the antimicrobial will select for genomic mutations that confer an addition level of fitness in this environment to out-compete non-mutated cells. Previously, *N. gonorrhoeae* has been passaged on media containing increasing amounts of the fluoroquinolone ciprofloxacin, resulting in an isolate that had 10,000 times greater resistance than the parental isolate [3]. Mutations in the *gyrA* and *parC* genes were identified;
mutations in the same genes also developed naturally in clinical isolates [4,5]. By 2006, the US Centers for Disease Control and Prevention no longer recommended fluoroquinolones for treatment of gonococcal infections [6]. Trying to predict how an organism will change genetically and / or phenotypically given a set of conditions or given a certain stimuli in the laboratory is known as experimental evolution. Bacteria are commonly studied in this way as they have short generation times which make them ideal for this method of study. The now low cost of next generation sequencing means that resequencing whole genomes following experimental evolution is feasible. This “evolve and re-sequence” strategy [7,8] can identify mutations in unexpected regions of the genome.

Although the lipopolysaccharide (LPS) of most Gram negative bacteria provides some intrinsic resistance to fatty acids, N. gonorrhoeae have short lipoooligosaccharide (LOS) structures and are generally more sensitive to fatty acids [1]. N. gonorrhoeae possesses other mechanisms to protect itself from the antimicrobial action of fatty acids. It has previously been shown that isolates from men that have sex with men have reduced permeability of hydrophobic agents [9]. The FarA-FarB-MtrE efflux pump has been demonstrated to confer decreased sensitivity to certain fatty acids [10]. Transcription of farAB is controlled by the FarR protein and integration host factor [11,12]. Therefore, mutations in farA, farB, mtrE, their promoters, or any of their regulators could have an effect on the resistance profile of the bacteria.

The purpose of this study was to identify genomic mutations that resulted from passage of N. gonorrhoeae on media containing sub-lethal concentrations of the monoglyceride monocaprin and to determine any changes in the minimum inhibitory concentration (MIC). Parallel cultures grown in a sub-lethal concentration of the saturated fatty acid myristic acid (C14:0). Myristic acid was chosen as a positive control for selection of genomic mutations and increase of MIC because it has very good bacteriostatic properties against N. gonorrhoeae [2] but also has a known mechanism of resistance in the farAB-mtrE encoded efflux pump system [10–12]. An ocular formulation of monocaprin is a promising candidate for the treatment of ophthalmia neonatorum [2], particularly in cases of antibiotic resistant gonococcal infections, therefore understanding the potential to develop resistance and the mechanisms involved are important.

Results and discussion

The MIC values before experimental evolution were 125 μM for myristic acid and 250 μM for monocaprin. Therefore, sub-lethal concentrations of 62.5 and 125 μM were used in the experimental evolution for myristic acid and monocaprin, respectively. The non-selective, myristic acid-, and monocaprin-containing cultures were successfully grown for a total of twenty passages each. Growth on plates containing myristic acid and monocaprin was observed to be slower than those on non-selective plates, especially the first few passages on the myristic acid-containing plates. The MICs were unchanged between the starting culture and the non-selective cultures after 20 passages (Table 1).

It is known that N. gonorrhoeae have an efflux pump-based mechanism of resistance against myristic acid [10–12]. Loss of the FarR regulator of expression of the farAB efflux pump genes results in an increase in the MIC for those fatty acids that are substrates of the efflux pump, including myristic acid [11]. After 20 passages, the bacteria grown on media with sub-lethal myristic acid had a sixteen-fold increase in MIC compared to the starting inoculum (Table 1). One myristic acid grown replicate (14:0–1) appeared to have a growth rate advantage over the other replicate, although the MICs were the same. The MICs of the bacteria grown on sub-lethal monocaprin increased eight-fold for myristic acid in both duplicate samples. It appears that growth in the presence of sub-lethal monocaprin has conditioned the N. gonorrhoeae for
growth on myristic acid resulting in an increase in the myristic acid MIC. From these results, it is evident that resistance to myristic acid can readily develop, which is not unexpected considering previous studies [10–12].

After experimental evolution, the monocaprin MIC for the samples passaged on plates containing myristic acid or monocaprin were both 500 μM (Table 1). Unlike the eight and sixteen fold change for the myristic acid MIC, the MIC for monocaprin has simply doubled that of the starting inoculum (Table 1). This suggests that passage on sub-lethal concentrations of both myristic acid and monocaprin prepares the bacterial cells for growth on a modest increase in monocaprin. Mechanisms contributing to such an increase may be related to a general stress adaptation.

The paired sequencing reads from MicrobesNG were successfully mapped to the GenBank N. gonorrhoeae strain NCCP11945 reference genome [13]. Twelve mutations within coding sequences (CDSs) were present in all the sequenced samples in comparison to the reference sequence (Table 2). These differences are either the result of sequencing errors from the original sequencing, sequencing errors in our data, or mutations that have occurred in the N. gonorrhoeae strain NCCP11945 isolate during the few passages between it being sequenced [13] and reaching our laboratory. The original sequencing was done by Sanger sequencing with an eight-fold coverage and predicted error rate of 0.15 per 10,000 bases [13], which would equate to 33 errors in the 2,232,025 bp genome. These sequencing differences identified here were either in pseudogenes or are predicted by SNAP2 [14] not to cause a functional effect in the encoded protein.

| Locus ID   | Gene product                           | Position     | Codons       | Change                           |
|------------|----------------------------------------|--------------|--------------|----------------------------------|
| NGK_RS00010 | DNA polymerase III subunit beta, pseudogene | 2,329        | GAC → GCC    | D179L, nonsynonymous             |
| NGK_RS00195 | Glutamate-1-semialdehyde 2,1-aminomutase | 37,377       | GCC → GGC    | A290G, nonsynonymous             |
| NGK_RS07005 | Glycine dehydrogenase (decarboxylating) | 1,287,872    | ACG → GCG    | T336A, nonsynonymous             |
| NGK_RS07575 | Ubiquinone biosynthesis regulatory protein kinase UbiB | 1,395,910    | AAC → AGC    | N382S, Nonsynonymous             |
| NGK_RS09065 | Hypothetical protein, pseudogene       | 1,670,097    | GGT → G-T    | Frameshift                       |
| NGK_RS09355 | Autotransporter, pseudogene             | 1,718,262    | GGT → G-T    | Frameshift                       |
| NGK_RS10485 | DNA mismatch repair protein MutS        | 1,926,831    | ATC → AGC    | I681S, nonsynonymous             |
| NGK_RS11075 | 3OS ribosomal protein S10               | 2,031,372    | TTT → TCT    | F21S, nonsynonymous              |
| NGK_RS11400 | Lipid-A-disaccharide synthase           | 2,086,880    | GAT → GAA    | D114E, nonsynonymous             |
| NGK_RS11740 | Diaminopimelate decarboxylase           | 2,155,020    | GAC → CTG    | D355L, nonsynonymous             |

Table 2. Mutations in CDSs present in all sequenced samples, but not in the reference sequence.

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Ten unique mutations were found in CDSs of the non-selectively passage cells (Table 3), although none of these were present in both replicates (Table 3). These changes are indicative of the general rate at which mutations can occur and become part of the bacterial population. Four of the mutations are present in all of the sequencing reads from the isolate, whilst the remainder are present in the majority of reads. Each of these CDS mutations would result in a nonsynonymous change, including two that generate premature termination codons, albeit late in the gene for NGK_RS05395 at 351/400, and one that alters the initiation codon to the less favoured TTG (Table 3). These cultures have been continuously maintained on standard gonococcal media and passaged to fresh media every two to three days. This is often standard practice in research laboratories where cultures can be kept growing in incubators for extended periods of time. However as evidenced here, mutations can arise via repeated passage that may impact downstream experimental use of the bacterial culture. Care must be taken to use minimally passaged isolates from well-maintained freezer stocks particularly when comparing parent and experimentally generated mutants. When comparing results to the reference genome, the potential for mutations to have arisen in the course of experiments must be considered.

Six sequence differences were found in CDSs from the samples passaged on sub-lethal myristic acid (Table 4). A SNP mutation was identified in the 24th codon of farR that changed it from a glutamine (Q) codon to an ochre stop codon in one isolate (Fig 1). This mutation is present in all sequence reads from this sample, however it is not present in any of the sequence reads of the duplicate myristic acid sample. This mutation would prevent expression of FarR, resulting in overexpression of the farAB encoded fatty acid efflux pump system [10–12]. This sample was observed to have a growth advantage over its other replicate but did not show a greater MIC compared to the other replicate (Table 1). The FarR transcriptional regulator also activates glnA responsible for glutamine biosynthesis [15] in N. gonorrhoeae and nadA in N. meningitidis [16]. This mutation is not surprising as mutations in the promoter region and open reading frame of another transcription regulator of an efflux pump system, mtrR, has

Table 3. CDS mutations found only in non-selectively passaged N. gonorrhoeae experimental evolution sequencing data.

| Locus ID   | Gene product                                      | Position   | Codons      | Change            | Isolate* | Reads^ |
|------------|---------------------------------------------------|------------|-------------|-------------------|----------|--------|
| NGK_RS00080| Preprotein translocase subunit SecG               | 14,279     | CCG → CTG   | A51V, nonsynonymous| NS-2     | 15/18  |
| NGK_RS00215| Ribosomal protein L11 methyltransferase           | 39,992     | CGG → CTG   | G191V, nonsynonymous| NS-2     | 13/13  |
| NGK_RS00290| Hypothetical protein                              | 57,720     | AAG → GAG   | K86G, nonsynonymous| NS-1     | 15/29  |
| NGK_RS00605| Two component sensor kinase                       | 124,256    | ATG → TGT   | Start to less favourable start| NS-2 | 2/3    |
| NGK_RS03680| Membrane protein                                  | 668,244    | GAC → TAC   | D84Y, nonsynonymous| NS-1     | 18/18  |
| NGK_RS05395| Type I restriction endonuclease subunit S         | 978,753    | CAA → TAA   | Q351STOP, premature stop| NS-2 | 2/2    |
| NGK_RS06935| Adenine phosphoribosyltransferase                 | 1,273,919  | GGC → GAC   | G135D, nonsynonymous| NS-2     | 15/16  |
| NGK_RS09755| Fimbrial protein                                  | 1,795,528  | AAA → AAG   | Q90T, nonsynonymous| NS-2     | 4/7    |
|            |                                                   | 1,795,532  | AAG → ACG   | K91T, nonsynonymous| NS-2     |        |
| NGK_RS11565| Bifunctional glutamine synthetase adenyltransferase / deadenyltransferase | 2,118,245 | CAA → TAA   | Q364STOP, Premature stop| NS-1 | 34/34  |

* mutation identified in either the non-selective isolate 1 (NS-1) or the non-selective isolate 2 (NS-2) culture.

^ number of sequencing reads containing the mutation identified.

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been demonstrated to confer decreased susceptibility to some common antimicrobials [17, 18]. It is interesting to note that the mutation does not confer a higher level resistance to myristic acid compared to the parallel culture.

The monocaprin passaged isolates had two non-synonymous SNP mutations (Table 5), both of which were predicted by SNAP2 to have a functional effect. The mutation in the phosphate permease protein was only present in one isolate. This is a membrane protein which is responsible for passively allowing entry of phosphate ions into the cell. It is not clear what effect this mutation would have on the cells ability to protect it from monocaprin. The mutation in dksA (NGK_RS106025) is present in all of the sequencing reads from both monocaprin grown cultures. The main role of DksA, a zinc-containing multi-functional protein, is as a transcription factor that binds directly to RNA polymerase and negatively regulates rRNA expression by destabilizing rRNA::promoter complexes [19], positively regulates several amino acid biosynthesis genes [19], and regulates fis expression [20]. An important gene regulator, DksA also inhibits transcript elongation, exonucleolytic RNA cleavage, pyrophosphorolysis, and increases intrinsic termination (http://www.uniprot.org/uniprot/P0ABS1), as well as being involved with RecN in repair of DNA double-strand breaks [21] and suppression of dnaK [22]. The mutation causes a substitution of the 75th amino acid from a threonine to an isoleucine (Fig 2), predicted by SNAP2 to have a functional effect with a change in the predicted secondary structure. The D71, D74, or A76 residues of the E.coli homolog form part of the coiled-coil tip that is responsible for the DksA-specific effects on open complex formation [23, 24]. These amino acids are conserved in N. gonorrhoeae and the T75I change is 12 amino acids away from these conserved residues (Fig 2). The protein appears to be highly conserved within N. gonorrhoeae; at the time of writing there were 322 DksA protein sequences in the GenPept database and of these 319 (99%) have an amino acid sequence

Table 4. CDS mutations found only in sub-lethal myristic acid passaged N. gonorrhoeae experimental evolution sequencing data.

| Locus ID   | Gene product          | Position | Codons    | Change                        | Isolate^ | Reads^\* |
|------------|-----------------------|----------|-----------|-------------------------------|-----------|----------|
| NGK_RS00315| FarR regulatory protein | 61,543   | CAA → TAA | Q24STOP, premature stop       | 14:0–1    | 40/40    |
| NGK_RS03680| Membrane protein       | 669,035  | Deletion of 4 codons | Deletion                     | 14:0–1    | 13/15    |
| NGK_RS07715| Lysine-tRNA ligase     | 1,428,182| CGG → TCG | P151S, nonsynonymous           | 14:0–1    | 24/24    |
| NGK_RS09755| Fimbrial protein       | 1,795,512| TTC → TTA | TTA → TCA                     | 14:0–1 14:0–2 | 9/18 3/36 |
| NGK_RS11260| Valine-tRNA ligase     | 2,055,439| GTG → ATG |                                | 14:0–2    | 26/26    |

* mutation identified in either the myristic acid passaged isolate 1 (14:0–1) or the myristic acid passaged isolate 2 (14:0–2) culture.

^ number of sequencing reads containing the mutation identified.

non-consensus.

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FarR sequence in myristic acid grown culture 14:0-2 (identical to wild-type)

\[
\begin{align*}
\text{M} & \text{P} \text{T} \text{Q} \text{S} \text{K} \text{H} \text{A} \text{S} \text{I} \text{N} \text{I} \text{G} \text{L} \text{I} \text{Q} \text{A} \text{R} \text{E} \text{A} \text{L} \text{M} \text{T} \text{Q} \text{F} \text{R} \text{P} \text{I} \text{L} \text{N} \\
\text{A} \text{T} \text{G} \text{C} \text{T} \text{A} \text{C} \text{C} \text{A} \text{A} \text{T} \text{C} \text{A} \text{A} \text{A} \text{A} \text{C} \text{A} \text{T} \text{G} \text{C} \text{T} \text{A} \text{A} \text{T} \text{C} \text{G} \text{T} \text{C} \text{G} \text{T} \text{A} \text{C} \text{A} \text{G} \text{G} \text{A} \text{G} \text{C} \text{T} \text{G} \text{A} \text{T} \text{A} \text{C} \text{G} \text{C} \text{T} \text{A} \text{T} \text{T} \text{C} \text{G} \text{T} \text{A} \text{C} \text{A} \text{G} \text{G} \text{A} \text{G} \text{C} \text{T} \text{G} \text{A} \text{T} \\
\text{A} \text{T} \text{G} \text{C} \text{T} \text{A} \text{C} \text{C} \text{A} \text{A} \text{T} \text{C} \text{A} \text{A} \text{A} \text{C} \text{A} \text{T} \text{G} \text{C} \text{T} \text{A} \text{A} \text{T} \text{C} \text{G} \text{T} \text{C} \text{G} \text{T} \text{A} \text{C} \text{A} \text{G} \text{G} \text{A} \text{G} \text{C} \text{T} \text{G} \text{A} \text{T} \text{C} \text{T} \text{G} \text{A} \text{T} \\
\text{M} & \text{P} \text{T} \text{Q} \text{S} \text{K} \text{H} \text{A} \text{S} \text{I} \text{N} \text{I} \text{G} \text{L} \text{I} \text{Q} \text{A} \text{R} \text{E} \text{A} \text{L} \text{M} \text{T} * \\
\end{align*}
\]

FarR sequence in myristic acid grown culture 14:0-1 (premature termination codon)

\[
\begin{align*}
\text{M} & \text{P} \text{T} \text{Q} \text{S} \text{K} \text{H} \text{A} \text{S} \text{I} \text{N} \text{I} \text{G} \text{L} \text{I} \text{Q} \text{A} \text{R} \text{E} \text{A} \text{L} \text{M} \text{T} * \\
\end{align*}
\]

Fig 1. Mutations identified in N. gonorrhoeae grown with sub-lethal myristic acid. The SNP in myristic acid grown culture 14:0–1 is present at the 70th nucleotide into farR (black highlight) and results in the formation of a premature stop codon after 23 amino acids (*). The full length FarR is 146 amino acids.

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identical to *N. gonorrhoeae* strain NCCP11945. The remaining three have separate single amino acid substitutions, none of which were at residue 75. DksA is involved in the ‘stringent response’ which is the stress response that occurs when bacteria are exposed to heat shock, experience stress conditions, or are starved of essential cellular components such as amino acids, fatty acids, or iron [25].

All four samples passaged on media containing sub-lethal antimicrobials showed an increase in MIC for both antimicrobials, suggesting that mechanisms of survival in the presence of one confer cross-resistance to the other. However, no common mutations in CDSs across these four sets of sequencing data were present. The increases in MICs may therefore be a result of general adaptation to stressful growth conditions, rather than being due to specific resistance mechanisms against monocaprin or myristic acid. A modest increase in MIC such as those observed here for monocaprin (Table 1) could arise due to an adaptation of the bacterial cell to the stresses experienced by exposure to the antimicrobials, due to selection for a portion of the population expressing a different phase variable repertoire of outer membrane proteins or LOS with different permeability, or other general changes that are not specific to monocaprin resistance.

The promoters of *farR, farAB, mtrR,* and *mtrCDE* were examined for any signs of mutation in the genome sequence data. In *N. gonorrhoeae,* modified promoter regions can enhance the expression of efflux pumps; a mutation in the sequence upstream of *mtrC* acts as an alternative promoter region enabling transcription of *mtrCDE* without MtrR control [26]. No mutations in these regions were observed in the experimental evolution sequencing data. Of note, *N. gonorrhoeae* strain NCCP11945 used in this study has a single base deletion in the promoter located inverted repeat within the *mtrR mtrC* promoter region. This single base mutation results in a loss of expression of MtrR and increased expression of the MtrCDE efflux pump in *N. gonorrhoeae* strain FA19 [27], which suggests that overexpression of the MtrCDE efflux pump is insufficient to confer resistance to monocaprin. Further, the FarR mutant here would

| Table 5. CDS mutations found only in sub-lethal monocaprin passaged *N. gonorrhoeae* experimental evolution sequencing data. |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Locus ID | Gene product | Position | Codons | Change | Isolate* | Reads^ | |
|----------|-------------|----------|--------|--------|----------|--------| |
| NGK_RS03680 | Membrane protein | 668,977 | Deletion of 4 codons | Deletion and frameshift | MG10-2 | 26/31 | |
| NGK_RS07610 | Iron complex outer membrane protein | 1,402,968 | AGC → AGT | S21S, synonymous | MG10-2 | 40/40 | |
| NGK_RS08405 | Phosphate permease | 1,565,103 | GCC → GTC | A509V, nonsynonymous | MG10-1 | 16/31 | |
| NGK_RS08890 | Magnesium transporter | 1,635,913 | CCG → CCA | P48P, synonymous | MG10-1 | 31/59 | |
| NGK_RS10625 | RNA polymerase-binding protein DksA' | 1,953,691 | ACC → ATC | T75I, nonsynonymous | MG10-1 | 53/53 | |

* mutation identified in either the monocaprin passaged isolate 1 (MG10-1) or the monocaprin passaged isolate 2 (MG10-2) culture.

^ number of sequencing reads containing the mutation identified.

' identified via pBLAST analysis.

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DksA  *E. coli*                    ANFPDPVDRAAQEEFSLELRNDRERKLKKIETLK
DksA  *N. gonorrhoeae* C10         SA-PDPADRAQEEYALELRRDRERKLSSKIQTIR
Consensus                "---DP-DR-AQEE---LRLR-DRERKL---T---"
be overexpressing both MtrCDE and FarAB-MtrE, yet the MIC of monocaprin in this isolate (14:0–1, Table 4) remains 500 μM (Table 1).

Ophthalmia neonatorum, infection by *N. gonorrhoeae* of the eyes of newborns, is due to transmission of the bacteria from the infected birth canal of the mother to the eyes of the infant during birth. This is a ‘dead-end’ infection; the bacteria cannot transmit to a new host from the infant eye infection. As a result, any treatment applied to eliminate ophthalmia neonatorum that is not used to also treat reproductive tract infections is isolated to the patient, with no chance of resistant organisms evolving in the host and being transmitted to others. Monocaprin-based formulations have been shown to rapidly kill *N. gonorrhoeae* without causing irritation [2]. For this reason, we did not apply the strategy used previously by Belland *et al.* [3] of subjecting the bacteria to increasingly higher concentrations of monocaprin over time. For this particular clinical manifestation and this particular proposed application, given the rapid killing time of monocaprin and ‘dead-end’ nature of the infection, there is no scope for resistance to evolve within the host. Therefore, our experimental design here did not include a progressive increase in monocaprin concentration as had previous resistance studies [3]. Additional evidence presented here demonstrates that the opportunity for evolution of resistance to such a treatment is minimal within the patient and within the timeframe of treatment, therefore further supporting the development of these candidates as treatments for gonococcal eye infections.

Our results parallel those of previous researchers investigating the potential for resistance to emerge to monoglycerides. In *Staphylococcus aureus*, continuous passage on media containing sublethal concentrations of monolaurin for a year did not generate resistance [28]. It is hypothesized that resistance to monoglycerides is not seen and are less likely to develop than resistance to antibiotics because there are multiple modes of antibacterial action that would all need to be overcome [29].

In conclusion, *N. gonorrhoeae* strain NCCP11945 adapted to growth in media containing sub-lethal concentrations of the fatty acid myristic acid and the monoglyceride monocaprin. Passage on either of these two antimicrobials resulted in a doubling of the MIC for monocaprin, likely due to general stress adaptation. Passage on monocaprin or myristic acid resulted in eight- and sixteen-fold increases, respectively, in the myristic acid MIC. *N. gonorrhoeae* is known to have mechanisms of resistance to myristic acid, such as efflux pumps [10–12]. It appears that the bacteria were able to increase their ability to withstand the presence of myristic acid just by being in an environment surrounded by a similar hydrophobic agent. However, the MIC for monocaprin showed only a modest two-fold increase in MIC, suggesting that it would be a suitable candidate for treatment of gonococcal infections, such as ophthalmia neonatorum [2]. Genome sequencing revealed that the mutations from the monocaprin-containing culture are of a more general nature than those from the myristic acid cultures. It is likely that the mutations in *dksA*, present in both monocaprin cultures, have compensated for stress upon the cell due to the presence of monocaprin in the culture and that the small MIC increase observed here is the extent of what can be achieved by general adaptive mutations. The likelihood that monoglycerides such as monocaprin have multiple modes of antimicrobial action and that resistance have not emerged after a year of passage in other species [28, 29] is encouraging for their application as antimicrobials. Monocaprin is therefore a promising candidate for the treatment of gonococcal infections such as ophthalmia neonatorum.

**Methods**

**Bacterial growth and experimental evolution**

*N. gonorrhoeae* strain NCCP11945 was grown in three conditions: on standard GC agar (GC base (Oxoid) with Kellogg’s supplements [30]); on GC agar with sub-lethal monocaprin
(Sigma-Aldrich; C10:0MG; 125 μM); and on GC agar with sub-lethal myristic acid (Sigma-Aldrich; C14:0; 62.5 μM). Duplicate cultures were grown for each condition. Minimally passaged N. gonorrhoeae strain NCCP11945 received from the sequencing project [13] was incubated at 37˚C, 5% CO₂ for 48 hours. Bacterial cells were removed into 1 ml GC broth to a turbidity of 0.5 McFarland standard and used to create a continuous streak over a whole GC agar plate using a sterile cotton swab. For the first passage, six GC plates were inoculated: two GC agar; two GC agar with 125 μM monocaprin; and two GC agar with 62.5 μM myristic acid. Cultures were passaged every 48–72 hours for 20 passages.

Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) was determined by agar dilution. GC agar plates were made containing 500 mM, 250 mM, 125 mM, 62.5 mM, 32.25 mM, and 16.125 mM of either myristic acid or monocaprin. Bacterial cells were suspended in 1 ml GC broth to a turbidity of 0.5 McFarland standard and used to create a continuous streak over the whole plate with a sterile cotton swab. Plates were incubated at 37˚C, 5% CO₂ for 48 hours. The lowest concentration which prevented growth was deemed the MIC. The sub-lethal concentration was the highest concentration which did not prevent growth.

DNA extraction and genome sequencing

A Qiagen Gentra Puregene Yeast/Bac kit was used to extract DNA from 500 μl of a bacterial suspension in GC broth equivalent to a 0.5 McFarland standard. DNA from the 20th passage of the six experimental evolution cultures and DNA from the starting inoculum were sent to MicrobesNG (University of Birmingham, Birmingham, UK, supported by the BBSRC, grant number BB/L024209/1) for whole genome Illumina HiSeq 2x250 bp paired-end sequencing.

Genome sequence data quality control, assembly, and analysis

FastQ files of trimmed paired read data, were downloaded from the MicrobesNG server. Read files were checked using FastQC version 0.11.2 (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and aligned using UGENE version 1.20.0 [31, 32] Bowtie2 plug-in [33] against the published sequence of N. gonorrhoeae strain NCCP11945 (accession number NC_011035.1) [13]. Assemblies in UGENE were checked manually for identified variant positions. Genes containing sequence variants were identified and predicted consequences determined using SNAP2 [14]. The promoter regions of farR, farAB, mtrR, and mtrCDE were subject to in-depth analysis.

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Author Contributions

Conceptualization: Colin P. Churchward, Lori A. S. Snyder.

Formal analysis: Colin P. Churchward.

Funding acquisition: Lori A. S. Snyder.

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Methodology: Colin P. Churchward, Alan Calder.
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References
1. Bergsson G, Steingrimsson O, Thormarch H. In vitro susceptibilities of Neisseria gonorrhoeae to fatty acids and monoglycerides. Antimicrob Agents Chemother. 1999; 43(11): 2790–2792. PMID: 10543766
2. Churchward C.P, Alany R.G, Kirk R.S, Walker A.J, Snyder L.A.S. Prevention of ophthalmia neonatorum from Neisseria gonorrhoeae using a fatty acid-based formulation. mBio 2017; 8(4): e00534–17. https://doi.org/10.1128/mBio.00534-17 PMID: 28743809
3. Belland R.J, Morrison S.G, Ison C, Huang W.M. Neisseria gonorrhoeae acquires mutations in analogous regions of gyrA and parC in fluoroquinolone-resistant isolates. Mol Microbiol. 1994; 14(2): 371–380. PMID: 7830580
4. Deguchi T, Yasuda M, Nakano M, Ozeki S, Ezaki T, Saito I, et al. Quinolone-resistant Neisseria gonorrhoeae: correlation of alterations in the GyrA subunit of DNA gyrase and the ParC subunit of topoiso-erase IV with antimicrobial susceptibility profiles. Antimicrob Agents Chemother. 1996; 40(4) 1020–1023. PMID: 8849219
5. Su X, Lind I. Molecular basis of high-level ciprofloxacin resistance in Neisseria gonorrhoeae strains isolated in Denmark from 1995 to 1998. Antimicrob Agents Chemother. 2001; 45(1) 117–123. https://doi.org/10.1128/AAC.45.1.117-123.2001 PMID: 11120953
6. Centers for Disease Control and Prevention Workowski K.A, Berman S.M. Sexually transmitted diseases treatment guidelines. Morb. Mortal. Wkly. Rep. 2006; 55(RR-11) 1–94.
7. Long A, Liti G, Luptak A, Tenaillon O. Elucidating the molecular architecture of adaptation via evolve and resequencing experiments. Nat Rev Genet. 2015; 16(10) 567–582. https://doi.org/10.1038/nrg3937 PMID: 26347030
8. Schlotterer C, Koller R, Versace E, Tobler R, Franssen S.U. Combining experimental evolution with next-generation sequencing: a powerful tool to study adaptation from standing genetic variation. Heredity 2015; 114(5) 431–440. https://doi.org/10.1038/hdy.2014.86 PMID: 25269380
9. Lee E.H, Shafer W.M. The farAB-encoded efflux pump mediates resistance of gonococci to long-chained antibacterial fatty acids. Mol Microbiol. 1999; 33(4) 839–845. PMID: 10447892
10. Lee E.H, Rouquette-Loughlin C, Folster J.P, Shafer W.M. FarR regulates the farAB-Encoded Efflux Pump of Neisseria gonorrhoeae via an MtrR Regulatory Mechanism. J Bacteriol. 2003; 185(24) 7145–7152. https://doi.org/10.1128/JB.185.24.7145-7152.2003 PMID: 14649274
11. Lee E.H, Hill S.A, Napier-R, Shafer W.M. Integration Host Factor is required for FarR repression of the farAB-encoded efflux pump of Neisseria gonorrhoeae. Mol Microbiol. 2006; 60(6) 1381–1400. https://doi.org/10.1111/j.1365-2958.2006.05185.x PMID: 16796676
12. Chung G.T, Yoo J.S, Oh H.B, Lee Y.S, Cha S.H, Kim S.J, et al. Complete genome sequence of Neisseria gonorrhoeae NCCP11945. Journal of Bacteriol. 2008; 190(17) 6035–6.
13. Hecht M, Bromberg Y, Rost B. Better prediction of functional effects for sequence variants. BMC Genomics 2015; 16(Suppl 8) S1.
14. Johnson P.J, Stringer V.A, Shafer W.M. Off-target gene regulation mediated by transcriptional regulators of antimicrobial efflux pump genes in Neisseria gonorrhoeae. Antimicrob Agents Chemother 2011; 55(6): 2559–2565. https://doi.org/10.1128/AAC.0010-11 PMID: 21422177
15. Schielke S, Spatz C, Schwarz R.F, Joseph B, Schoen C, Schulz S.M, et al. Characterization of FarR as a highly specialized growth phase-dependent transcriptional regulator in Neisseria meningitidis. JIMM 2011; 301(4) 325–333. https://doi.org/10.1016/j.jimm.2010.11.007 PMID: 21292554
16. Chen P.L, Lee H.C, Yoo J.S, Oh H.B, Lee Y.S, Cha S.H, Kim S.J, et al. Complete genome sequence of Neisseria gonorrhoeae NCCP11945. Journal of Bacteriol. 2008; 190(17) 6035–6.
17. Allen V.G, Farrell D.J, Rebbapragada A, Tan J, Tijet N, Perussi S.J, et al. Molecular analysis of antimicrobial resistance mechanisms in Neisseria gonorrhoeae isolates from Ontario Canada. Antimicrob Agents Chemother. 2011; 55(2) 703–712. https://doi.org/10.1128/AAC.00768-10 PMID: 21098249
19. Paul B.J, Barker M.M, Ross W, Schneider D.A, Webb C, Foster J.W, et al. DksA: a critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. Cell 2004; 118(3) 311–322. https://doi.org/10.1016/j.cell.2004.07.009 PMID: 15294157

20. Mallik P, Paul B.J, Rutherford S.T, Gourse R.L, Osuna R. DksA is required for growth phase-dependent regulation growth rate-dependent control and stringent control of fis expression in Escherichia coli. J Bacteriol. 2006; 188(16) 5775–5782. https://doi.org/10.1128/JB.00276-06 PMID: 16885445

21. Meddows T.R, Savory A.P, Grove J.I, Moore T, Lloyd R.G. RecN protein and transcription factor DksA combine to promote faithful recombinational repair of DNA double-strand breaks. Mol Microbiol. 2005; 57(1) 97–110. https://doi.org/10.1111/j.1365-2958.2005.04677.x PMID: 15948952

22. Kang P.J, Craig E.A. Identification and characterization of a new Escherichia coli gene that is a dosage-dependent suppressor of a dnaK deletion mutation. J Bacteriol. 1990; 172(4) 2055–2064. PMID: 2180916

23. Furman R, Sevostyanova A, Artsimovitch I. Transcription initiation factor DksA has diverse effects on RNA chain elongation. Nucleic Acids Res. 2012; 40(8) 3392–3402. https://doi.org/10.1093/nar/gkr1273 PMID: 22210857

24. Lee J.H, Lennon C.W, Ross W, Gourse R.L. Role of the coiled-coil tip of Escherichia coli DksA in promoter control. J Mol Biol. 2012; 416(4) 503–517. https://doi.org/10.1016/j.jmb.2011.12.028 PMID: 22200485

25. Blaby-Haas C.E, Furman R, Rodionov D.A, Artsimovitch I, de Creycy-Lagard V. Role of a Zn-independent DksA in Zn homeostasis and stringent response. Mol Microbiol. 2011; 79(3) 700–715. https://doi.org/10.1111/j.1365-2958.2010.07475.x PMID: 21255113

26. Ohneck E.A, Zalucki Y.M, Johnson P.J, Dhulipala V, Golparian D, Unemo M, et al. A novel mechanism of high-level broad-spectrum antibiotic resistance caused by a single base pair change in Neisseria gonorrhoeae. mBio 2011; 2(5) e00187–11. https://doi.org/10.1128/mBio.00187-11 PMID: 21933917

27. Hagman K.E, Shafer W.M. Transcriptional control of the mtr efflux system of Neisseria gonorrhoeae. J Bacteriol. 1995; 177(14): 4162–4165. PMID: 7608095

28. Schlievert P.M, Peterson M.L. Glycerol monolaurate antibacterial activity in broth and biofilm cultures. PLoS One 2012; 7: e40350. https://doi.org/10.1371/journal.pone.0040350 PMID: 22808139

29. Mueller E.A, Schlievert P.M. Non-aqueous glycerol monolaurate gel exhibits antibacterial and anti-biofilm activity against Gram-positive and Gram-negative pathogens. PLoS One 2015; 10(3): e0120280. https://doi.org/10.1371/journal.pone.0120280 PMID: 25799455

30. Kellogg D.S Jr, Peacock W.L Jr, Deacon W.E, Brown L, Pirkle D.I. Neisseria gonorrhoeae. I. Virulence Genetically Linked to Clonal Variation. J Bacteriol. 1963; 85 1274–1279. PMID: 14047217

31. Okonechkov K, Golosova O, Fursov M, team U. Unipro UGENE: a unified bioinformatics toolkit. Bioinformatics 2012; 28(8) 1166–1167. https://doi.org/10.1093/bioinformatics/bts091 PMID: 22368248

32. Golosova O, Henderson R, Vaskin Y, Gabrielian A, Grekhov G, Nagarajan V, et al. Unipro UGENE NGS pipelines and components for variant calling RNA-seq and ChIP-seq data analyses. Peer J. 2014; 2 e644. https://doi.org/10.7717/peerj.644 PMID: 25392756

33. Langmead B, Salzberg S.L. Fast gapped-read alignment with Bowtie 2. Nature Methods 2012; 9(4) 357–359. https://doi.org/10.1038/nmeth.1923 PMID: 22888286