Published in final edited form as:

Mol Microbiol. 2011 October ; 82(2): 489–501. doi:10.1111/j.1365-2958.2011.07826.x.

Identification of a conserved protein involved in anaerobic unsaturated fatty acid synthesis in *Neisseria gonorrhoeae*: implications for facultative and obligate anaerobes that lack FabA

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SUMMARY

Transcriptome analysis of the facultative anaerobe, *Neisseria gonorrhoeae*, revealed that many genes of unknown function were induced under anaerobic conditions. Mutation of one such gene, NGO1024, encoding a protein belonging to the 2-nitropropane dioxygenase-like superfamily of proteins, was found to result in an inability of gonococci to grow anaerobically. Anaerobic growth of an NGO1024 mutant was restored upon supplementation with unsaturated fatty acids (UFA), but not with the saturated fatty acid palmitate. Gonococcal fatty acid profiles confirmed that NGO1024 was involved in UFA synthesis anaerobically, but not aerobically, demonstrating that gonococci contain two distinct pathways for the production of UFAs, with a yet unidentified aerobic mechanism, and an anaerobic mechanism involving NGO1024. Expression of genes involved in classical anaerobic UFA synthesis, *fabA*, *fabM*, and *fabB*, was toxic in gonococci and unable to complement a NGO1024 mutation, suggesting that the chemistry involved in gonococcal anaerobic UFA synthesis is distinct from that of the classical pathway. NGO1024 homologs, which we suggest naming UfaA, form a distinct lineage within the 2-nitropropane dioxygenase-like superfamily, and are found in many facultative and obligate anaerobes that produce UFAs but lack *fabA*, suggesting that UfaA is part of a widespread pathway involved in UFA synthesis.

Keywords

UFA; *ufaA*; nitropropane dioxygenase; anaerobiosis; COG2070

INTRODUCTION

Neisseria gonorrhoeae is capable of anaerobic growth using nitrite or nitric oxide as a terminal electron acceptor (Knapp & Clark, 1984, Clark, 2009). The ability of this obligate human pathogen to utilize denitrification for anaerobic growth is believed to be of physiological significance, as gonococci are often isolated from infected individuals in co-culture with obligate anaerobes (Newkirk, 1996). Furthermore, antibodies directed against the anaerobically induced outer membrane nitrite reductase, AniA, can be found in the sera of infected women, demonstrating that this protein is expressed *in vivo* (Clark *et al.*, 1988). The gonococcal reduction of nitric oxide via the reductase, NorB, during denitrification has been reported to have immunomodulatory effects on the host, and in fact may be required to sustain cervical bacterial disease (Edwards, 2010, Clark, 2009).

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We have previously used deep sequencing to identify gonococcal genes differentially expressed in response to anaerobiosis (Isabella & Clark, 2011). In this study, approximately 10% of the gonococcal genome was reported to show altered expression anaerobically, and many anaerobically induced genes encoded proteins of unknown function. To define the role of such induced genes as they related to anaerobic growth, we attempted to construct mutant strains and screen for gross phenotypic differences both aerobically and anaerobically. These mutations fell into three categories: [1] genes that were apparently essential (no mutant could be constructed), [2] mutations that had no apparent difference in phenotype between aerobic and anaerobic growth conditions, and [3] mutations that had no apparent phenotype aerobically, but resulted in a deficiency in anaerobic growth. As we are most interested in anaerobiosis in gonococci, mutations that were found to result in only an anaerobic growth defect were selected for further investigation.

There are several functions known to be essential for anaerobic growth of bacteria. Unlike many facultative anaerobes, gonococci are unable to utilize sugars other than glucose, and substrate level phosphorylation, on its own, is incapable of supporting growth anaerobically. In the absence of oxygen, gonococci require an alternative electron acceptor in the form of either nitrite or nitric oxide (Knapp & Clark, 1984, Clark, 2009). The truncated denitrification system in gonococci, AniA and NorB, has been well characterized, though the route of electron transfer to AniA has yet to be completely resolved (Hopper *et al.*, 2009). Bacteria also require an alternative mechanism to synthesize deoxyribonucleotides anaerobically, as the prototypical aerobic ribonucleotide reductase (RNR) complex, NrdA and NrdB, requires molecular oxygen to generate the catalytic tyrosyl radical involved in its reaction mechanism (Stubbe & Riggs-Gelasco, 1998). In contrast to *E. coli* and other facultative anaerobes, gonococci do not contain a class II or class III anaerobic RNR. By encoding only an aerobic RNR, it remains unclear how gonococci can grow anaerobically at all, though several alternative possibilities have been proposed (Reviewed in (Clark, 2009)). A third requirement of anaerobic bacteria is the ability to synthesize unsaturated fatty acids (UFAs). Many aerobic organisms, from bacteria to humans, rely on oxygen-dependent desaturase enzymes to this end (Schweizer & Choi, 2011), while *E. coli* and other microorganisms capable of anaerobic growth encode oxygen-independent machinery for anaerobic UFA production (Feng & Cronan, 2009). Intriguingly, gonococci encode no homologs to any known mechanism of UFA synthesis, either aerobically or anaerobically.

In this study we identify a novel enzyme involved in anaerobic UFA synthesis in *N. gonorrhoeae*. As the mechanism of anaerobic UFA biosynthesis in many anaerobes remains unknown (White *et al.*, 2005, Zhu *et al.*, 2009), coupled with the widespread occurrence of the gene implicated in this study, the findings presented herein are likely to have a broad significance.

RESULTS AND DISCUSSION

Mutation of NGO1024 inhibits anaerobic growth in *N. gonorrhoeae*

In a previous study that identified gonococcal genes differentially expressed in response to anaerobiosis, expression of the open reading frame NGO1024, encoding a protein in the nitropropane dioxygenase family, was found to be anaerobically induced (Isabella & Clark, 2011). As an obligate human pathogen, this would seem unusual, as gonococci do not inhabit an environment where nitropropane or other toxic nitroalkanes would ever be encountered. Furthermore, it would seem counterintuitive that expression of a dioxygenase, which requires molecular oxygen for function, would be induced in the absence of oxygen (Gorlatova *et al.*, 1998, Gadda & Francis, 2010). We therefore selected NGO1024 for further analysis, as we reasoned that it may encode a protein of novel function.

NGO1024 was insertionally inactivated and screened for growth defects. Surprisingly, disruption of NGO1024 resulted in a complete inability of gonococcal cells to grow anaerobically (Figure 1A). There was no apparent defect in a Δ NGO1024 mutant when the cells were grown aerobically or microaerobically with or without nitrite (Figure 1B). Denitrification is the only physiological pathway known to be required for anaerobic growth of gonococci. The fact that a Δ NGO1024 mutant was still able to undergo nitrite-dependent growth microaerobically indicated that the inhibition of growth under anaerobic conditions was not caused by deficiency in this pathway. Also, the fact that growth of a Δ NGO1024 mutant was inhibited anaerobically but not microaerobically strongly suggested that the protein encoded by NGO1024 did not require molecular oxygen for function and was therefore unlikely to be a dioxygenase.

Unsaturated fatty acid supplementation complements the growth inhibition of Δ NGO1024

NGO1024 contains distant homology to *fabK*, a gene encoding an isoform of enoyl-ACP reductase found in many gram positive bacteria, though NGO1024 does not contain a complete FabK domain (White *et al.*, 2005, Marchler-Bauer *et al.*, 2009). Because of the distant similarity to FabK, we reasoned that NGO1024 may potentially play a role in fatty acid biosynthesis. Little is known about the fatty acid biosynthetic pathway in *N. gonorrhoeae* other than that which can be inferred by its homologs in *E. coli* (Table 1). Nothing is known about unsaturated fatty acid biosynthesis in this organism, as gonococci do not encode homologs to FabA or FabB, the proteins responsible for oxygen-independent UFA biosynthesis in *E. coli* and many other bacteria, or FabM, an isomerase involved in UFA biosynthesis in Streptococcal species (Fozo & Quivey, 2004, Feng & Cronan, 2009, Marrakchi *et al.*, 2002). In addition, gonococci do not contain homologs to any of the known oxygen-dependent fatty acid desaturases found in *Bacillus* spp. and other gram positive bacteria, mycobacteria, *Pseudomonas aeruginosa*, or *Saccharomyces cerevisiae* (Schweizer & Choi, 2011).

Of the 12 sequenced *Neisseria* species (NCBI), 9 encode a homolog to NGO1024, and none of these 9 species encode FabA or FabB homologs. Interestingly, the Neisserial species that did not encode a NGO1024 homolog, *N. elongata*, *N. bacilliformis* and *N. sp. oral taxon 014 F0314*, all encoded homologs to both FabA and FabB (Marchler-Bauer *et al.*, 2009). This observation led us to believe that NGO1024 could be involved in UFA biosynthesis. The addition of the UFAs myristoleate (14:1 ω 7c), palmitoleate (16:1 ω 7c), cis-vaccenate (18:1 ω 7c), oleate (18:1 ω 9c), or eicosenoate (20:1 ω 9c) to culture media restored anaerobic growth of a Δ NGO1024 mutant to varying degrees, with palmitoleate showing the greatest level of growth restoration (Figure 2). It is important to note that exogenous fatty acids are toxic to *N. gonorrhoeae* (Miller *et al.*, 1977, Bergsson *et al.*, 1999), as are non-ionic detergents commonly used to solubilize these hydrophobic molecules. For these reasons, low levels of UFAs were used in the supplementation, which is likely the reason why complete complementation outgrowth equivalent to the wild-type strain) was not achieved. Regardless, complementation with exogenous fatty acids was specific for UFAs, as supplementation with the saturated fatty acid palmitate (16:0) had no effect on the anaerobic growth defect of a Δ NGO1024 mutant (Figure 2). This experiment provided overwhelmingly strong evidence that NGO1024 is involved in the biosynthesis of UFAs. Gonococci do not encode enzymes allowing for the catabolism of fatty acids (NCBI), so the only other feasible alternative explanation, that gonococci are using exogenous fatty acids as a carbon source in the absence of NGO1024, is highly unlikely. In addition, if exogenous fatty acids were being used as a carbon source, we would have expected that palmitate would have also reversed the anaerobic growth defect in Δ NGO1024. For its apparent role in anaerobic UFA biosynthesis, we will refer to NGO1024 as *ufaA* for the remainder of this report.

Aerobic and anaerobic fatty acid profiles of wild type and *ΔufaA* strains

Previous studies of *ufaA* expression in gonococci revealed that this gene was expressed at extremely low levels aerobically (Isabella & Clark, 2011). We wished to determine if there were any changes in the fatty acid profile of aerobically grown *ΔufaA* cells. Gas chromatography was used to analyze the fatty acid content of gonococcal cell membranes. The fatty acid content of aerobically grown *ΔufaA* cells was essentially identical to that of wild type strain F62. Both strains contained a 1:1 ratio of saturated to unsaturated fatty acids (Table 2). This suggests that *UfaA* is not essential for UFA synthesis under aerobic conditions. We next sought to determine if there was a change in the fatty acid profile of wild type cells when grown anaerobically. The cell membranes of anaerobically grown gonococci contained a higher ratio of saturated to unsaturated fatty acids (SAFA/UFA = 1.6, Table 2). While the proportion of palmitoleate (16:1 ω 7c) was not notably different between aerobically and anaerobically grown cells, the proportion of *cis*-vaccenate (18:1 ω 7c) was reduced more than two-fold anaerobically, with a compensatory increase in the proportion of saturated fatty acids. This is the first evidence demonstrating that *N. gonorrhoeae* is capable of altering its membrane fatty acid composition in response to anoxic conditions. As an obligate human pathogen with a limited niche and strict requirement for growth at 37°C, the observed change in the membrane profile seen here is further evidence supporting that the mechanism of UFA synthesis in gonococci is different aerobically and anaerobically.

Palmitoleate supplementation was necessary to grow *ΔufaA* cells anaerobically for membrane analysis. While supplementation resulted in a slight increase in the proportion of UFAs in anaerobically grown wild type cells, the membranes of anaerobically grown *ΔufaA* cells contained very little UFAs, with a SAFA/UFA of 5.0 (Table 2). Also noteworthy is the small but reproducible proportion of 16:1 ω 5c produced in all cell membrane samples except for the anaerobically grown, palmitoleate-supplemented *ΔufaA* mutant. This is indicative of a lack of *de novo* UFA production in anaerobically grown *ΔufaA* cells. The UFAs that are present are likely only those derived from the media. Because low concentrations of palmitoleate were used in the supplementation to prevent toxicity, the availability of UFAs would be expected to be the anaerobic growth-limiting factor. When the exogenous UFA supply is exhausted, the cells would continue to divide until their membranes reach a minimum level of unsaturation that is necessary to maintain cell viability. The low level of UFAs in the membranes of anaerobic/supplemented *ΔufaA* cells would suggest that this is indeed the case. Taken together, these data demonstrate that gonococci encode an as yet unidentified UFA biosynthetic pathway utilized during aerobic conditions as well as a novel anaerobic mechanism of UFA biosynthesis that involves the *UfaA* protein.

FabA and FabB from *E. coli* do not complement a *ΔufaA* mutant

Gonococci encode all of the proteins necessary for the biosynthesis of saturated fatty acids (Table 1). The bacterial type II pathway for fatty acid biosynthesis has been extensively studied, and will not be discussed in detail here (see reviews, (White *et al.*, 2005, Chan & Vogel, 2010, Zhang *et al.*, 2006, Campbell & Cronan, 2001)). *FabA* and *FabB* are essential proteins in *E. coli* and comprise the only mechanism in this organism to make UFAs (White *et al.*, 2005). *FabA* acts as bifunctional dehydratase/isomerase capable of introducing a *trans* double bond into the 10-carbon fatty acid biosynthetic intermediate (3-hydroxydecanoyl-ACP $\rightarrow$ *trans*-2-decanoyl-ACP, dehydratase function), followed by isomerization of this *trans* double bond to the *cis* conformation (*cis*-3-decanoyl-ACP) (Feng & Cronan, 2009). *FabF*, the 3-ketoacyl-ACP synthase involved in saturated fatty acid chain elongation, is incapable of extending the *cis* double bond-containing products of *FabA*; another enzyme with homology to *FabF*, *FabB*, is required to complete this reaction (Feng & Cronan, 2009). In streptococcal species, a slightly different mechanism exists. In these organisms, the *FabM* protein is capable of isomerizing the *trans* double bond from the product of the dehydratase

FabZ to the *cis* conformation (*trans*-2-decanoyl-ACP → *cis*-3-decanoyl-ACP). In *fabM*-containing organisms, FabF is capable of extending *cis*-3-decanoyl-ACP (Marrakchi *et al.*, 2002). FabF can also extend *cis*-3-decanoyl-ACP in some organisms without *fabM* (Wang & Cronan, 2004, Zhu *et al.*, 2009, Morgan-Kiss & Cronan, 2008), leading to the conclusion that *fabB* is not always an essential gene for classical anaerobic UFA biosynthesis in some organisms, though its function is.

A recent study evaluated the role of FabF and FabZ proteins in the synthesis of UFAs in *Clostridium acetobutylicum* in order to determine if these proteins could functionally replace FabA and FabB in *E. coli*, as had been reported for FabF and FabZ proteins in *Enterococcus faecalis* (Zhu *et al.*, 2009, Wang & Cronan, 2004). This study concluded that although *C. acetobutylicum* FabF could functionally replace *E. coli* FabB, this organism's FabZ lacked the isomerase activity of FabA, and that therefore *C. acetobutylicum* must introduce the double bond UFAs by use of a novel and unknown enzyme (Zhu *et al.*, 2009). Due to the fact that this organism's FabF was capable of extending *cis*-3-decanoyl-ACP, it was hypothesized that the missing enzymatic step in the UFA biosynthesis pathway was encoded by a novel gene with the function of *fabA* or *fabM* (Zhu *et al.*, 2009). We used gonococcal UfaA as a query to BLAST the *acetobutylicum* genome and located a *ufaA* homolog (e^{-40}) immediately upstream of the *C. acetobutylicum* fatty acid biosynthesis operon (NCBI). Although we did not experimentally determine that this homolog was actually a part of that operon, less than 40-bp separates the 3' end of this gene from the 5' end of a gene cluster that encodes the entire pathway of saturated fatty acid synthesis (NCBI)(Campbell & Cronan, 2001), suggesting that *C. acetobutylicum* may also use UfaA in its mechanism of UFA synthesis. Because, like *N. gonorrhoeae*, *C. acetobutylicum* contains a *ufaA* homolog and a single *fabF* gene, and because the fatty acid profiles are similar between these two organisms (Lepage *et al.*, 1987), we reasoned that the gonococcal mechanism of anaerobic UFA synthesis may be completed through the UfaA and FabF proteins in a fashion analogous to *E. coli* FabA and FabB. If UfaA was involved in performing the same reaction as FabA or FabM, expression of these proteins in gonococci should complement the anaerobic growth defect.

Gonococcal strains carrying LacI-repressed, IPTG-inducible chromosomal copies of *ufaA*, *E. coli fabA*, or *S. mutans fabM* were constructed in the *ΔufaA* mutant (Mehr & Seifert, 1998). Induction of *fabA* or *fabM* was found to be extremely toxic when expressed aerobically. For this reason, IPTG was supplied by disk diffusion, which would allow for a gradient of IPTG concentration (*i.e.* gene induction) extending from the center of the plate on which these strains were grown (Figure 3A). If any amount of these proteins were able to complement the *ufaA* mutation, we should see a ring of growth at some distance from the IPTG disk. Anaerobically, induction of no gene other than *ufaA* itself was able to complement the *ufaA* mutation. Expression of an engineered bicistronic *E. coli fabAB* transcript in the *ΔufaA* mutant was also found to be highly toxic when expressed aerobically, and was also unable to complement the anaerobic growth deficiency (Figure 3B). Expression of *E. coli fabB* alone had no toxic effect aerobically, but was also unable to restore anaerobic growth.

Expression of *fabA* or *fabM* would be expected to produce *cis*-3-decanoyl-ACP. One possible explanation for the toxicity observed when expressing these genes in gonococci is that the terminal enoyl reductase, FabI, is unable to effectively compete with FabA and FabM for substrate, leading to a lethal defect in saturated fatty acid biosynthesis. However, previous expression studies of these proteins in *E. coli* would suggest that this is not the case. In *E. coli*, FabM was shown to be incapable of competing with FabI for substrate and was found to be unable to complement a *fabA* mutation for this reason (Marrakchi *et al.*, 2002). In contrast, overproduction of FabA in *E. coli* actually increases the level of saturated

fatty acids, as the bulk of the excess *cis*-3-decanoyl-ACP formed is isomerized back to *trans*-2-decanoyl-ACP and utilized by FabI; FabA-catalyzed reactions are freely reversible and FabB is the limiting step in UFA synthesis (Feng & Cronan, 2009). It is possible that *cis*-3-decanoyl-ACP itself is toxic. This may also explain why expression of *fabAB* did not complement the gonococcal defect in UFA synthesis. In any case, taken together, these data imply that *cis*-3-decanoyl-ACP is not utilized for UFA synthesis, which also suggests that the chemistry involved in gonococcal UFA synthesis is distinct from that of the classical anaerobic pathway. Moreover, the pattern of aerobic and anaerobic UFA synthesis observed in the gonococcus is inconsistent with facultative anaerobes that utilize the classical anaerobic UFA synthesis pathway. *E. coli* and Streptococcal species utilize only the anaerobic pathway under both aerobic and anaerobic growth conditions. *P. aeruginosa* utilizes the classical anaerobic pathway in addition to two oxygen-dependent fatty acid desaturases, however, mutation of FabA in this organism resulted in a marked reduction in both growth rate and cellular UFA content when cells were grown aerobically (Cronan, 2006). Our data, including the fact that *ufaA* expression is induced anaerobically (Isabella & Clark, 2011), is consistent with the existence of separate and independently functioning aerobic and anaerobic UFA synthesis mechanisms in *N. gonorrhoeae*.

UfaA expression in *E. coli*

We wished to determine if *ufaA* could complement an *E. coli* temperature sensitive *fabA* mutant at the non permissive temperature (42°C). Inducible expression of *ufaA* from a high or low copy vector, aerobically or anaerobically, did not permit growth at 42°C (data not shown). We believe that this lack of complementation was most likely due to the requirement of additional gonococcal factors that were not present in *E. coli*. To understand what these factors may be, we must investigate the other known mechanisms of UFA synthesis.

In contrast to the classical anaerobic UFA biosynthetic reactions, which occur concurrently with fatty acid biosynthesis, the aerobic desaturases create double bonds on fully elongated saturated fatty acids (White *et al.*, 2005, Subramanian *et al.*, 2010). The $\Delta 5$ -desaturase from *B. subtilis* (Des) and $\Delta 9$ -desaturases from *P. aeruginosa* (DesA and DesB) are among the best characterized oxygen-dependent fatty acid desaturases in bacteria (Chazarreta-Cifre *et al.*, 2011, Zhu *et al.*, 2006). The mechanism of aerobic desaturation requires a specific electron transport chain where electrons are passed from NAD(P)H to various electron donors, to the iron-dependent, membrane-bound desaturase, where a double bond is then introduced into a CoA *P. aeruginosa* or phospholipid (*B. subtilis*) linked fatty acid with a concomitant reduction of oxygen to water (Chazarreta-Cifre *et al.*, 2011, Diaz *et al.*, 2002, Aguilar & de Mendoza, 2006). The precise route of electron transfer to the desaturase is an unknown component of the pathway in many bacteria. However, the route of electron transfer in *B. subtilis* $\Delta 5$ -Des was recently discovered and reported to include a ferredoxin and two flavodoxins (Chazarreta-Cifre *et al.*, 2011).

UfaA contains an FAD/FMN binding domain, suggesting that this protein is capable of electron transfer and can utilize NAD(P)H as an electron donor like other members of the nitropropane dioxygenase family (Marchler-Bauer *et al.*, 2009, Saito *et al.*, 2008, Gorlatova *et al.*, 1998). BLAST analysis of gonococcal UfaA against the NCBI protein database reveals that many UfaA homologs exist, and of every UfaA homolog examined thus far, all contain four conserved cysteine residues in the C-terminal end, which could be involved in the coordination of a redox-active cofactor capable of electron transfer, such as a [4Fe-4S] cluster, as is predicted by the NCBI conserved domain database (Marchler-Bauer *et al.*, 2009). The UfaA protein contains no motifs to suggest that it would be localized to the membrane.

Although entirely speculative at this point, UfaA could act within a pathway analogous to the aerobic desaturases, requiring a functional electron transport chain in the formation of a double bond, but unlike the aerobic desaturases, where the electron donors are unknown, NAD(P)H can donate electrons directly to UfaA. In the case of UfaA, it could be the route of electron transfer to a terminal electron acceptor that is unknown, as oxygen is unavailable. In our previous transcriptome studies, we were surprised to observe the anaerobic induction of *etfD*, encoding electron transfer flavoprotein (ETF) ubiquinone oxidoreductase (EtfD) (Isabella & Clark, 2011). In other organisms, subunits Etf α and Etf β of the ETF pathway form a heterodimer capable of accepting electrons from several NADH dehydrogenases involved in the β -oxidation of fatty acids. Electrons are then delivered by Etf $\alpha\beta$ to membrane-bound EtfD, which in turn transfers these electrons to the ubiquinone pool (Ishizaki *et al.*, 2005, Weidenhaupt *et al.*, 1996). It is a mystery why the ETF pathway would be encoded within the gonococcal genome at all, let alone be anaerobically induced, given the fact that gonococci are incapable of β -oxidizing fatty acids. And although the ETF pathway can accept electrons from a few NADH dehydrogenases not involved in fatty acid catabolism, gonococci do not contain homologs to any of these enzymes (NCBI) (Ishizaki *et al.*, 2005, Herrmann *et al.*, 2008). The ETF pathway could link electron transfer to the ubiquinol-utilizing nitric oxide reductase, NorB (NGO1275) (Zumft, 2005), meaning that if UfaA passes electrons through the ETF pathway, this could constitute an anaerobic, nitric oxide-dependent UFA synthesis mechanism.

Unfortunately, we were unable to construct a mutant in any component of the ETF pathway in gonococci to determine if this resulted in a phenotype similar to *ΔufaA*, even under aerobic conditions, suggesting that the gonococcal ETF pathway is essential. In addition, we were unable to complement an *E. coli fabA* temperature sensitive mutant at the non permissive temperature when *ufaA* was co-expressed with *etfβα* (NGO1935 and NGO1936; NCBI) and *etfD* (NGO1396) both aerobically and anaerobically with nitrate supplementation (data not shown). This does not necessarily imply that the ETF system is not somehow involved in the mechanism of UFA synthesis, as we do not know, [1] if these proteins were functional in *E. coli* (*i.e.* these proteins require cofactors, post-translational processing, or transport to the membrane that is not being provided by *E. coli*), [2] if the expression system was adequate, [3] if additional electron transfer proteins are involved (in anaerobic bacteria, it has been suggested that additional ferredoxins are involved in the ETF reaction mechanism (Herrmann *et al.*, 2008)), or [4] if additional enzymes are involved in the pathway of UFA synthesis that come before or after the involvement of UfaA. If UfaA is part of a pathway that requires the completion of an electron transport chain as a mechanism for UFA synthesis, it would not be difficult to envision that the ETF system could be involved, as it presents an ideal mechanism to link electron transfer to a terminal acceptor. For this reason we feel that even with the lack of complementation in *E. coli*, involvement of the ETF system should not be discounted at this point.

UfaA homologs are widespread in bacteria

FabK, nitroalkane oxidase (Nmo), and UfaA homologs all contain a 2-nitropropane dioxygenase-like conserved domain (cd4730) and fall within COG2070 (cluster of orthologous group, NCBI), suggesting that these proteins share a common evolutionary origin (Marchler-Bauer *et al.*, 2009). The use of gonococcal UfaA as a query against the NCBI microbial protein database revealed the existence of many UfaA homologs across the bacterial kingdom. The sequences of many of these homologs were used in conjunction with the sequences of other characterized members of the nitropropane dioxygenase-like superfamily to generate a phylogenetic tree (Figure 4) (Dereeper *et al.*, 2008). UfaA homologs (Figure 4, box I) encompass a distinct lineage from that of Nmo and FabK homologs (Figure 4, box III and IV respectively). The UfaA lineage is comprised mostly, if

not entirely, of organisms that are facultative or obligate anaerobes, and none of the organisms within this lineage were found to contain a FabA homolog (NCBI).

The UfaA lineage can further be broken down into two major clades (Figure 4, box *Ia* and *Ib*). Clade *Ia* contains UfaA homologs with the greatest level of similarity to gonococcal UfaA (e values ranging from e^{-89} to 0.0, NCBI). Organisms within this clade are all members of the β -proteobacteria division with the exception of two *Acidithiobacillus* species, which are members of the γ division. During our search for UfaA homologs, *Bordetella petrii* was found to contain a UfaA homolog with a high level of similarity to gonococcal UfaA (61% identity, 75% similarity, expect= $2e^{-124}$, NCBI). This corroborates UfaA function in this organism, as *B. petrii* is the only species in this genus known to be capable of anaerobic growth, while all other *Bordetella* species are reported to be obligate aerobes (no other *Bordetella* species was found to contain a UfaA homolog) (von Wintzingerode *et al.*, 2001). This finding, coupled with the extremely high level of UfaA sequence conservation within this clade, has led us to speculate that UfaA homologs in clade *Ia* are very likely to function in anaerobic UFA synthesis, as demonstrated in gonococci. The *ufaA* gene appears to be stochastically located within bacterial genomes, occurring either as a monocistron or organized into apparent dissimilar operonic arrangements between different organisms (NCBI). However, *ufaA* appears to be located at the 3' end of an apparent operon with the anaerobic ribonucleotide reductase genes (*nrdDG*) in some of the facultative anaerobes in clade *Ia* (*B. petrii*, *Lutiella nitroferum*, *Thiobacillus denitrificans*, and *Achromobacter* spp., NCBI), potentially indicating that *ufaA* regulation could be responsive to anaerobiosis and not to its product or substrate, which in turn may explain the inability to correlate protein function with genetic context.

UfaA homologs in clade *Ib* contained less similarity to gonococcal UfaA compared to members of clade *Ia* (Similarity ranged from 49–55%, and e values ranged from e^{-30} to e^{-52} , Figure 4, box *Ib*, NCBI). This clade is comprised of bacteria in the δ and ϵ -proteobacteriadivision as well as Firmicute species, and includes microaerobic (*Campylobacter* spp. and *Helicobacter* spp.) and obligate anaerobic (*Clostridia* spp.) bacteria of clinical significance. As with clade *Ia*, the chromosomal location of *ufaA* homologs in clade *Ib* was stochastic, with a notable exception of *Clostridium* spp. In the majority of *Clostridium* species that contained a UfaA homolog (most were not included in Figure 4), the *ufaA* gene was located at the 5' end of the *fab* operon (NCBI). In some species *ufaA* was oriented in the same direction as the *fab* operon, and in other species immediately divergent from the 5' end of this operon, suggesting that *ufaA* could possibly be a member of, or be co-regulated with, the *fab* operon (NCBI). If *ufaA* is regulated by oxygen availability in facultative anaerobes, examination of the genetic context of *ufaA* in obligate anaerobes such as *Clostridia* spp. may give the best indication of protein function, where oxygen would not be involved in regulation of a constitutive cellular process. Much mystery has surrounded the mechanism of UFA synthesis in *Clostridium* species (Campbell & Cronan, 2001, Zhu *et al.*, 2009), and indeed the location of the *ufaA* gene in the *C. acetobutylicum* genome was a major reason why we initially pursued UFA synthesis as a function for gonococcal UfaA. However, much work remains to be completed in order to determine if homologs within this lineage are truly involved in UFA synthesis.

A unique α -proteobacteria-specific lineage of 2-nitropropane dioxygenase-like proteins was also discovered (Figure 4, box *II*). The proteins in this lineage contain less similarity to UfaA homologs, but were also distinct from Nmo and FabK homologs. This is likely not an α -proteobacterial-specific FabK lineage, as many of these organisms encode *fabK* homologs with much greater similarity to *Clostridium* and *Streptococcus* *fabK* at a separate locus. This lineage could constitute an α -proteobacterial-specific lineage of nitroalkane oxidases or UfaA homologs, or could potentially represent a lineage of proteins with a completely novel

function. Interestingly, with the exception of the two *Acidithiobacillus* spp. mentioned above, *ufaA* appears to be completely absent from the γ division of proteobacteria, where *fabA* homologs are widespread (Wang & Cronan, 2004).

Concluding remarks

It is fascinating to note that a *fabA* pseudogene exists in the gonococcal genome NGO1350, NCBI). This ORF is missing much of the 5' end of characterized *fabA* genes, and our transcriptome studies have shown that this pseudogene is not expressed (Isabella & Clark, 2011). This could be indicative of a 5' deletion that also removed a promoter element. A second FabF/FabB homolog is also annotated in the gonococcal genome, and lies downstream of the *fabA* pseudogene (NGO1352, NCBI). This gene, also shown not to be expressed, contains a frameshift mutation that has destroyed the 3-ketoacyl synthase domain (Marchler-Bauer *et al.*, 2009, Isabella & Clark, 2011). Thus it appears that gonococci have inactivated the *fabA* and *fabB* genes, presumably in favor of other mechanisms of UFA biosynthesis. We have provided evidence demonstrating that gonococci encode separate aerobic and anaerobic UFA synthesis mechanisms, with the anaerobic mechanism involving the UfaA protein. The *Neisseria* appear to be unique in that none of these species encode a homolog to any known enzyme involved in aerobic fatty acid desaturation, which is in contrast to other facultative anaerobes containing a *ufaA* homolog, which generally did contain homologs to the $\Delta 9$ -desaturases from *Pseudomonas* species or the $\Delta 5$ -desaturase from *Bacillus* species (NCBI). In any case, as an obligate human pathogen, the fact that gonococci encode an anaerobic-specific UFA synthesis mechanism furthers our assertion that anaerobic growth is a physiologically significant state for this organism and should be considered when examining the host/pathogen interaction.

We have yet to discover any bacterial genome that encodes both a *fabA* and a *ufaA* homolog (including organisms omitted from Figure 4 in the interest of space). Many *ufaA*-containing organisms are known to be capable of anaerobic growth, while for others it can be presumed based on analysis of their genomes (*i.e.* the presence of genes encoding an anaerobic ribonucleotide reductase or alternative respiratory chains such as denitrification). We have provided evidence that the chemistry involved in anaerobic gonococcal UFA synthesis may be distinct from that of the classical anaerobic UFA synthetic pathway. We have also searched the NCBI microbial protein database and discovered that UfaA is highly conserved, and is encoded within the genomes of organisms that have presented a mystery in their mechanism of UFA synthesis for some time. Unfortunately, we were unsuccessful in collecting any data that would give us a biochemical understanding of the UfaA reaction mechanism. However, based on the presumed structure of the protein, the toxicity associated with expression of proteins involved in classical UFA synthesis in gonococci, and a lack of ability of *ufaA* to complement an *E. coli fabA* mutant, it may be likely that a UFA biosynthetic pathway involving UfaA requires the completion of a specific electron transport chain, similar to the aerobic desaturation pathway, and probably requires additional proteins not encoded by *E. coli*. UfaA could be, [1] a desaturase, by itself or as a subunit in conjunction with other proteins, [2] it could be required to activate or modify a desaturase enzyme, or [3], it could be involved in transfer of electrons to or from a desaturase to an electron transport chain. We encourage other researchers to continue this work and hope that our initial identification of the UfaA protein will help to get things started in that direction.

EXPERIMENTAL PROCEDURES

Growth of gonococcal strains

All gonococcal strains were derived from strain F62 and were grown on Difco™ GC medium base (Becton, Dickinson and Co., Sparks, MD) plates with 1% Kellogg's

supplement (GCK) (Kellogg *et al.*, 1963). For plates to be used for anaerobic culture, GC medium was boiled for 5 min following autoclave sterilization in order to remove residual oxygen. After boiling, the media was immediately moved to a Coy anaerobic chamber (Coy Laboratory Products, Grass Lake, MI) maintained at an atmosphere of 90% N₂, 5% H₂, and 5% CO₂. Media was allowed to cool anaerobically before the addition of Kellogg's supplement and 2 mM nitrite. These anaerobically poured plates remained in the anaerobe chamber until inoculation. Aerobic and microaerobic growth curves were performed in GCP broth (proteose peptone #3 (Difco, 15 g), soluble starch (1 g), KH₂PO₄ (4 g), K₂HPO₄ (1 g), NaCl (5 g) L⁻¹ dH₂O) supplemented with 1% Kellogg's, and 0.042% sodium bicarbonate (GCK). Aerobic growth curves were performed in 10 mL cultures grown in 50 mL flasks shaking at 250 rpm. Microaerobic growth curves were performed in 25 mL cultures grown in 50 mL flasks shaking at 85 rpm, with or without the addition of 5 mM nitrite. Gonococci are incapable of anaerobic growth in broth culture, therefore, quantitation of anaerobic cell growth required that cells be harvested from plate cultures. To perform this quantitation, 0.2 µm pore size, 47 mm diameter Nucleopore™ polycarbonate Track-Etch membranes (Whatman) were sterilely transferred to anaerobically prepared plates. Overnight plate cultures of aerobically grown gonococci were suspended in GCP to an OD₆₀₀ of ~0.5 and serially diluted tenfold to 10⁻⁸ and plated to determine cell number. A 100 µL volume of the 10⁻⁴ dilution was spread on the polycarbonate membranes (Cell input; generally ranged from 3000–6000 cfu). Following anaerobic incubation at 37°C for 24 h, plates were removed from the anaerobe chamber and polycarbonate filters were sterilely transferred into 10 mL of GCP broth, briefly vortexed to resuspend cells, serially diluted tenfold, and plated to determine the quantity of cells from the filter (cell output). Anaerobic cellular outgrowth is reported as cell output/cell input. For UFA supplementation, UFAs were supplied to the growth media at a concentration of 2.0 µg ml⁻¹.

PCR

Genomic DNA from gonococcal strain F62, *E. coli* strain MC1061, or *Streptococcus mutans* UA159 was isolated for use as a PCR template. Synthesized oligonucleotide primers were purchased from Eurofins or Invitrogen. All DNA amplification used for the construction, complementation, and screening of mutants was performed with iProof high fidelity polymerase (Bio-Rad). Primer sequences used in this study are listed in Table 3.

Gonococcal transformation

Gonococci were transformed naturally. Light suspensions of piliated cells were prepared in 1 ml of GCK broth containing 0.042% NaHCO₃ and 10 mM MgCl₂ (Kellogg *et al.*, 1963). Purified plasmid DNA or linear ligation fragments were added and 100 µl of the suspensions were plated on GCK plates and incubated 6–9 hours at 37 °C. Cells were then harvested from the plates and streaked on GCK plates containing erythromycin (Sigma) at 2 µg ml⁻¹ or kanamycin (Calbiochem) at 150 µg ml⁻¹ for selection of clones. Clones typically took 2 days to become visible on antibiotic plates. Clones were screened for the presence of chromosomal modification using PCR.

Construction and complementation of a gonococcal *ΔufaA* mutant

To construct a *ΔufaA* mutant, the amplified PCR products of P1/P2 and P3/P4 (Table 3) were digested with XhoI and HindIII, respectively, and ligated (T4 DNA ligase, Invitrogen) to an XhoI/HindIII double digested kanamycin resistance cassette (*aph-3*) containing a gonococcal uptake sequence (Elkins *et al.*, 1991). This ligation mix was used to transform gonococcal strain F62. To complement the *ΔufaA* mutant (Figure 1), the *ufaA* gene was amplified using primers P5/P7, which included a 380 bp region upstream of the *ufaA* translation start site containing the previously identified promoter elements (Isabella & Clark, 2011), digested with PacI and FseI, and ligated to PacI/FseI-digested gonococcal

complementation vector, pGCC4 (Mehr & Seifert, 1998). This ligation mix was used to transform chemically competent *Escherichia coli* DH10B. Transformants were selected on LB plates (Bacto-tryptone (Difco, 10g), yeast extract (Difco, 5g), NaCl (10g), Bacto-agar (Difco, 15g) L⁻¹) containing kanamycin at 50 µg ml⁻¹. Plasmid was isolated from individual clones and checked for the presence of insert by restriction mapping. Plasmid that contained the proper insert was used to transform the *ΔufaA* mutant. Though pGCC4 results in the integration of the *lacI* repressor and an IPTG inducible promoter, no IPTG was added for *ΔufaA* complementation reported in Figure 1, and *ufaA* expression is considered to be under control of its own promoter. For inducible expression of *ufaA*, *fabA*, *fabM*, and *fabB* in the *ΔufaA* mutant, the amplified PCR products of P6/P7, P8/P9, P10/P11, and P12/P13, respectively (Table 2), were cloned into pGCC4 under control of the *lac* promoter and used to transform *ΔufaA*. To construct a *fabAB* fragment, the *fabA* and *fabB* genes were separately amplified, purified, and treated with T4 polynucleotide kinase (NEB) to add phosphates to ends of the PCR products. After subsequent purification, the *fabA* fragment was cut with *PacI* and the *fabB* fragment was cut with *FseI*. These fragments were used in a triple ligation with *PacI*/*FseI* digested pGCC4 to generate an IPTG-inducible *fabAB* transcript. IPTG (Invitrogen) was supplied by disk diffusion using 50 µl of 0.05M IPTG per disk.

Preparation of cells for fatty acid profiling

Plate-grown gonococcal cultures were prepared identically to that described for cell quantitation (above), except cells were plated directly on agar plates without the use of a polycarbonate filter. Cells were harvested with sterile swabs into 20 mL of ice cold TBS (50 mM Tris, 150 mM NaCl, pH 7.4) and spun down at 6000 × g (4°C). Cells were subsequently washed four times in 1 mL of ice cold TBS. After the final wash, cell pellets were flash frozen and shipped on dry ice. Fatty acid analysis was performed by Microbial ID© (<http://microbialid.com>, Newark, DE).

Phylogenetic tree construction

“One Click” phylogenetic analysis was completed online using the default settings of the Phylogeny.fr algorithm (<http://www.phylogeny.fr/>)(Dereeper *et al.*, 2008). The Gblocks program was used to eliminate poorly aligned positions and divergent regions. Sequences to be analyzed were retrieved from the NCBI microbial protein database http://www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). To retrieve UfaA sequences, the gonococcal UfaA protein was used as a query against all divisions of bacteria. Sequences were considered to be UfaA homologs if they were more similar to UfaA than other members of the 2- nitropropane dioxygenase-like superfamily of proteins, FabK and Nmo. FabK and Nmo homologs were added to the phylogenetic analysis to show that UfaA homologs made up a distinct lineage. Streptococcal FabK was used as a query against several divisions of bacteria to retrieve FabK homolog sequences (Marrakchi *et al.*, 2003). *Streptomyces anochromogenes* Nmo, which has been crystallized (Li *et al.*, 2011), and two characterized Nmo proteins with 3-nitropropionic acid oxygenase activity from *Burkholderia phytofirmans* (Nishino *et al.*, 2010), were used as queries to retrieve Nmo homolog sequences from other bacterial divisions.

Molecular biology techniques

Cloning and PCR techniques were performed in accordance to standard protocols (Ausubel, 1987, Ausubel, 1992, Sambrook, 1989). All restriction enzymes were purchased from New England Biolabs or Invitrogen. Plasmid preparations were obtained with QIAprep miniprep kits, and DNA fragments were purified with QIAquick PCR Purification or QIAquick Gel Extraction kits.

Acknowledgments

This study was supported by Public Health Service grant R21 AI 080912 from the National Institute of Allergy and Infectious Disease. In addition, we thank Stephen Spence and Brendaliz Santiago for their technical assistance, Robert Quivey for his gift of *S. mutans* gDNA and UFAs, and John Cronan for his gift of *E. coli* strain CY57.

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Figure 1. Mutation of NG1024 inhibits anaerobic growth in gonococci

A) The Δ NG1024 mutant is shown to be incapable growth after 24 hr of anaerobic incubation on 2 mM nitrite plates. Single copy complementation of NG1024 in the gonococcal genome restores the anaerobic growth capacity. Data is representative of 4 independent trials for each strain $\pm$ SD. B) No growth defect is observed in a Δ NG1024 mutant when grown aerobically or anaerobically with or without nitrite. Open markers indicate cells grown aerobically, grey markers indicate cells grown microaerobically, and black markers represent cells grown microaerobically with the addition of 5 mM nitrite. Diamonds (Wild type F62), Squares (Δ NG1024). Data is representative of 3 independent growth curves for each strain in each growth condition $\pm$ SD.

Figure 2. Supplementation with unsaturated fatty acids partially restores anaerobic growth in a Δ NG1024 mutant

The UFAs myristoleate (14:1 ω 7c), palmitoleate (16:1 ω 7c), oleate (18:1 ω 9c), *cis*-vaccenate (18:1 ω 7c), and eicosenoate (20:1 ω 9c) partially restores the anaerobic growth defect in a Δ NG1024 mutant while the SAFA, palmitate (16:0) has no effect. Fatty acids were added to the anaerobic growth medium at a concentration of 2.0 μ g mL⁻¹. White bars indicate the outgrowth of wild type F62, and grey bars indicate the outgrowth of *AufaA* mutant. Data presented is representative of at least 3 independent trials $\pm$ SD.

Figure 3. Expression of *fabA* and *fabM* does not restore anaerobic growth in a *ΔufaA* mutant
 Gonococcal cells were grown with and without IPTG supplied by disk diffusion, both aerobically and anaerobically. Numbered sectors refer to following strains: A) (i) Wild type F62, (ii) *ΔufaA*, pGCC4 (chromosomal integration of empty vector), (iii) *ΔufaA*, *ufaA*⁺, (iv) *ΔufaA*, *fabA*⁺, (v) *ΔufaA*, *fabM*⁺, B) (i) Wild type F62, (ii) *ΔufaA*, (iii) *ΔufaA*, *fabAB*⁺, (iv) *ΔufaA*, *fabB*⁺ (+) indicates IPTG-inducible chromosomal integration of indicated gene(s).

Figure 4. Widespread presence of UfaA homologs in bacteria

Phylogenetic distribution of selected members of COG2070 based on amino acid sequences of UfaA, FabK, and Nmo. Box I represents the UfaA lineage, box II represents an α -proteobacterial-specific COG2070 lineage, box III represents the Nmo lineage, and box IV represents the FabK lineage.

Table 1Fatty acid biosynthetic genes in *N. gonorrhoeae*.

Gene Name	Enzyme Activity ^a	Gonococcal Gene ^b
<u>Acetyl-CoA Carboxylase</u>		
<i>accA</i>	Carboxyl-transferase subunit	NGO0821
<i>accB</i>	Biotin carboxyl carrier protein (BCCP)	NGO0045
<i>accC</i>	Biotin carboxylase	NGO0044
<i>accD</i>	Carboxyl-transferase subunit	NGO0249
<u>Saturated Fatty Acid Synthesis</u>		
<i>acpP</i>	apo-ACP	NGO1351
<i>fabD</i>	Malonyl-CoA:ACP transacylase	NGO2166
<i>fabF</i>	3-Ketoacyl-ACP synthase II	NGO1763
<i>fabG</i>	3-Ketoacyl-ACP reductase	NGO2163
<i>fabH</i>	3-Ketoacyl-ACP synthase III	NGO2168
<i>fabI</i>	Enoyl-ACP reductase I	NGO1666
<i>fabK</i>	Enoyl-ACP reductase II <i>S. pneumonia</i> , (Marrakchi <i>et al.</i> , 2003)	None
<i>fabL</i>	Enoyl-ACP reductase III <i>B. subtilis</i> , (Heath <i>et al.</i> , 2000)	None
<i>fabV</i>	Enoyl-ACP reductase <i>V. cholerae</i> , (Massengo-Tiasse & Cronan, 2008)	None
<i>fabZ</i>	3-hydroxyacyl-ACP dehydrase	NGO1804
<u>Unsaturated Fatty Acid Synthesis</u>		
<i>fabA</i>	3-Hydroxydecanoyl- ACP dehydrase/ isomerase <i>E. coli</i> , (Feng & Cronan, 2009)	None
<i>fabB</i>	3-Ketoacyl-ACP synthase I <i>E. coli</i> , (Feng & Cronan, 2009)	None
<i>fabM</i>	Enoyl-CoA hydratase <i>S. pneumonia</i> , (Marrakchi <i>et al.</i> , 2002)	None
<i>desA</i>	aerobic fatty acid desaturase <i>P. aeruginosa</i> , (Zhu <i>et al.</i> , 2006)	None
<i>desB</i>	aerobic fatty acid desaturase <i>P. aeruginosa</i> , (Zhu <i>et al.</i> , 2006)	None
<i>des</i>	aerobic fatty acid desaturase <i>B. subtilis</i> , (Mansilla & de Mendoza, 2005)	None

^aFor all genes without a homolog in *N. gonorrhoeae*, the organism in which that gene has been characterized is given.

^bAccording to annotation NCBI annotation.

Table 2

Aerobic and anaerobic fatty acid profiles of F62 and a *ΔufaA* mutant.

Fatty Acid ^c	+O ₂		-O ₂ ^a	
	F62	<i>ΔufaA</i>	F62	<i>ΔufaA</i>
Saturated:				
12:0	6.36 ± 0.37	5.79 ± 0.21	7.56 ± 0.18	7.14 ± 0.25
3OH-12:0	4.31 ± 0.42	4.06 ± 0.20	4.86 ± 0.02	4.76 ± 0.18
14:0	2.88 ± 0.29	2.50 ± 0.24	5.62 ± 0.44	4.74 ± 0.47
15:0	0.22 ± 0.04	0.20 ± 0.01	0.42 ± 0.04	0.41 ± 0.01
16:0	33.46 ± 0.51	33.55 ± 0.66	38.17 ± 0.21	35.13 ± 0.74
18:0	1.06 ± 0.35	0.93 ± 0.66	1.76 ± 0.35	1.52 ± 0.36
Unsaturated:				
16:1 ω7c	32.92 ± 0.40	32.26 ± 0.51	29.27 ± 2.32	34.21 ± 0.38
16:1 ω5c	1.11 ± 0.06	1.18 ± 0.06	0.33 ± 0.04	0.33 ± 0.01
18:1 ω7c	13.73 ± 1.02	15.61 ± 1.10	6.19 ± 0.31	7.22 ± 0.27
18:1 ω5c	0.20 ± 0.02	0.25 ± 0.02	ND	ND
Total SAFA	48.29 ± 1.64	47.02 ± 1.15	58.38 ± 0.43	53.69 ± 0.80
Total UFA	47.97 ± 1.34	49.29 ± 0.69	35.79 ± 2.07	41.95 ± 0.82
SAFA/UFA	1.0	1.0	1.6	1.3

Abbreviations: (ND) Not detected, (SAFA) saturated fatty acids, (UFA) unsaturated fatty acids, (ω) omega, (c) carbon – denotes where *cis* double bond is located with respect to the omega end of the carbon chain. All samples were recovered from plate-grown cells.

^a Gonococci grown in an anaerobic chamber on plates containing 2 mM nitrite.

^b Gonococci grown on 2 mM nitrite plates supplemented with palmitoleic acid at 2.0 μg mL⁻¹.

^c Fatty acids listed are those that could be positively assigned by gas chromatograph data and that had an abundance of greater than 0.1 % in at least one sample. Data presented are representative of 3 independent trials for each sample ± SD.

Table 3

Bacterial strains, constructs, and primers used in this study.

Contracts	Relevant genotype or properties	Source or reference	
<i>Plasmids</i>			
pGCC4	Gonococcal complementation vector, <i>lacI</i> , P _{<i>lac</i>} , Kn ^r , Erm ^r	(Mehr & Seifert, 1998)	
pVI140	pGCC4/ <i>ufaA</i> (P5/P7 fragment)	This Study	
pVI141	pGCC4/ <i>ufaA</i> , IPTG inducible (P6/P7 fragment)	This Study	
pVI142	pGCC4/ <i>fabA</i> , IPTG inducible (P8/P9 fragment)	This Study	
pVI143	pGCC4/ <i>fabM</i> , IPTG inducible (P10/P11 fragment)	This Study	
pVI144	pGCC4/ <i>fabB</i> , IPTG inducible (P12/P13 fragment)	This Study	
PVI145	pGCC4/ <i>fabAB</i> , IPTG inducible	This Study	
<i>E. coli strains</i>			
DH10B	F [−] <i>endA1 recA1</i>	Laboratory Collection	
CY57	Temperature sensitive <i>fabA</i> mutant	(Clark <i>et al.</i> , 1983)	
<i>N. gonorrhoeae strains</i>			
F62	<i>pro</i> [−]	Laboratory Collection	
RUG7950	F62 with Δ <i>ufaA</i> mutation	This Study	
RUG7951	RUG7950 transformed with pVI140	This Study	
RUG7952	RUG7950 transformed with pVI141	This Study	
RUG7953	RUG7950 transformed with pVI142	This Study	
RUG7954	RUG7950 transformed with pVI143	This Study	
RUG7955	RUG7950 transformed with pVI144	This Study	
RUG7956	RUG7950 transformed with pVI145	This Study	
<i>Primers</i>			
Name	Region Amplified	DNA source ^a	Sequence ^b
P1	5' <i>ufaA</i> forward	F62	5'- ATTCGTTACCATATCTTTACCTACCC
P2	5' <i>ufaA</i> XhoI reverse	F62	5'- AAAACT CTCGAG CTTGCACGATGGGAATAAGG
P3	3' <i>ufaA</i> HindIII forward	F62	5'- TATAA AGCTT GGACGCTGACATCAGGTAG
P4	3' <i>ufaA</i> reverse	F62	5'- CAGCACGCGACAGATTTGGTTAC
P5	5' <i>ufaA</i> PacI forward-1	F62	5'- GCAAT TTAATTA ATATCTTTACCTACCC
P6	5' <i>ufaA</i> PacI forward-2	F62	5'- CTAA ATTAATTA AAACCATTTTTTCATGA
P7	3' <i>ufaA</i> FseI reverse	F62	5'- TATAG GCCGGCCG GGAACACAACCTG
P8	5' <i>fabA</i> PacI forward	DH10B	5'- TTCA ATTAATTA AGGCTTACAGAGAACATGG
P9	3' <i>fabA</i> FseI reverse	DH10B	5'- TGGCG GGCCGGCC AGTAATGGCCTGATTCTGTCTC
P10	5' <i>fabM</i> PacI forward	UA159	5'- TAATC TTAATTA ATTAGAGATGGAGAAATAAGAAGATGG
P11	3' <i>fabM</i> FseI reverse	UA159	5'- TTTT GGGCCGGCC TATACTTACACTTTTAACAGAG
P12	5' <i>fabB</i> PacI forward	DH10B	5'-TGTG TTAATTA ACTTACTCTATGTGCGAC
P13	3' <i>fabB</i> FseI reverse	DH10B	5'-AATA AGGCCGGCCG CATTGGCGCGTAAC

^aRefers to strain: F62 (*N. gonorrhoeae*), UA159 (*S. mutans*), DH10B (*E. coli*)^bRestriction sites displayed in bold