Discovery and Characterization of a Nitroreductase Capable of Conferring Bacterial Resistance to Chloramphenicol

Highlights
- Bacteria can resist chloramphenicol via reduction, but the causal genes are unknown
- nfsB nitroreductase expression can confer chloramphenicol resistance in E. coli
- H. influenzae NfsB quickly reduces chloramphenicol to amino-chloramphenicol in vitro
- Metronidazole potentiates chloramphenicol activity against nitro reducing E. coli

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In Brief
Crofts et al. report the characterization of bacterial genes that confer chloramphenicol resistance by nitroreduction. The most effective gene, H. influenzae nfsB, encodes an enzyme that efficiently reduces the chloramphenicol nitro group in vitro. The authors found that resistance via reduction could be countered using the anaerobic antibiotic metronidazole.
Widespread antibiotic resistance has led to the reappraisal of abandoned antibiotics including chloramphenicol. However, enzyme(s) underlying one form of chloramphenicol resistance, nitroreduction, have eluded identification. Here we demonstrate that expression of the *Haemophilus influenzae* nitroreductase gene *nfsB* confers chloramphenicol resistance in *Escherichia coli*. We characterized the enzymatic product of *H. influenzae* NfsB acting on chloramphenicol and found it to be amino-chloramphenicol. Kinetic analysis revealed reduction of diverse substrates including the incomplete reduction of 5-nitro antibiotics metronidazole and nitrofurantoin, likely resulting in activation of these antibiotic pro-drugs to their cytotoxic forms. We observed that expression of the *H. influenzae* nfsB gene in *E. coli* results in significantly increased susceptibility to metronidazole. Finally, we found that in this strain metronidazole attenuates chloramphenicol resistance synergistically, and *in vitro* metronidazole weakly inhibits chloramphenicol reduction by NfsB. Our findings reveal the underpinnings of a chloramphenicol resistance mechanism nearly 70 years after its description.

**INTRODUCTION**

Antibiotic resistance is a growing threat to modern medicine and is expected to be responsible for an increasing number of deaths worldwide in the future (O’Neil, 2014). Compounding this threat is the ubiquity of cryptic resistance mechanisms present in the environment (Crofts et al., 2017) and the declining development of new antibiotics (Kinch et al., 2014; Payne et al., 2007; Pye et al., 2017). One solution to this problem has been to reconsider the use of antibiotics previously abandoned due to compromised safety or efficacy profiles (Wright, 2017). In particular, the rise of resistance specifically to *β*-lactams, the most widely used class of antimicrobials worldwide, has changed the cost-benefit analysis for multiple other classes of antibiotics that were formerly concluded to be too toxic for general use. For example, colistin and daptomycin are now important clinical antibiotics of last resort despite prior abandonment due to toxicity issues. Their potential toxicity is outweighed by the lethality of otherwise virtually pan-resistant infections (Civljak et al., 2014; Li et al., 2006; The Pew Charitable Trusts, 2016; Wright, 2017).

It has been suggested that amphenicols, antibiotics such as chloramphenicol (Figure 1A), thiamphenicol, and florfenicol, which have lost or failed to gain approval for human use due to potential toxicity, may follow this same path for readoption (Civljak et al., 2014; Dinos et al., 2016; Rahim et al., 2015).

Chloramphenicol (Figure 1A), co-discovered and marketed by Parke-Davis beginning in the 1940s, is a natural product antibiotic produced by the bacterium *Streptomyces venezuelae* with antibiotic activity against Gram-positive and Gram-negative bacteria (Ehrlich et al., 1947; Smith et al., 1948). It is stable under a range of conditions (Ehrlich et al., 1947) and was the first natural product antibiotic to be economically produced synthetically (Ehrlich et al., 1947; Schwarz et al., 2004; Wright et al., 2014). Chloramphenicol is orally available and can cross the blood-brain barrier and other sites that are difficult to reach, making it a potent therapy for bacterial meningitis and tissue infections (Dinos et al., 2016; Schwarz et al., 2004).

Despite their attractiveness as antibiotics, the clinical use of amphenicols has largely ceased in more economically developed countries as a result of chloramphenicol’s epidemiological linkage to hematological toxicity (Shu et al., 1987; Wallerstein et al., 1969). Potentially because of this, chloramphenicol remains active against a variety of bacterial pathogens. While chloramphenicol does not show greater efficacy against routine infections compared with current standard treatments, its use may be justifiable against multi-drug resistance organisms (Eliakim-Raz et al., 2015) as chloramphenicol retains substantial activity against Gram-positive organisms, especially methicillin-resistant...
amino-chloramphenicol. Since this first observation, the importance of an electron-withdrawing constituent at this position was demonstrated. The resulting compound, amino-chloramphenicol (Figure 1C), lacked antibacterial activity against Gram-negative organisms (Dinos et al., 2016). While many of these organisms are known to be promiscuous reducers of aromatic nitro groups, homologs of these genes are found across diverse bacterial taxa, including in many members of the intestinal microbiome (Roldán et al., 2008). Nitro-reduction via these enzymes occurs in two electrons at a time, often stopping at the production of a hydroxylamino group after a four-electron reduction (Roldán et al., 2008), though some enzymes, such as the NfsB homologs from E. coli, Enterobacter cloacae, and Salmonella enterica, have been demonstrated to reduce aromatic nitro groups completely to amines (LinWu et al., 2009; Yanto et al., 2010) (Figure 1D).

Figure 1. Structure of Chloramphenicol and Its Bacterial Modifications
(A–C) Chemical structure of (A) chloramphenicol, (B) chloramphenicol inactivated following acetylation, and (C) chloramphenicol inactivated following nitroreduction. (D) Products of stepwise chloramphenicol reduction, from left to right: chloramphenicol, nitroso-chloramphenicol, hydroxylamino-chloramphenicol, and amino-chloramphenicol.

Staphylococcus aureus in more developed countries, and moderate activity against Gram-negative organisms (Civljak et al., 2014; Lim et al., 2016; Nitzan et al., 2010; Sood, 2016).

The most widespread chloramphenicol resistance mechanisms are via well-annotated functions such as enzymatic inactivation (e.g., via chloramphenicol acetyltransferase, Figure 1B), efflux pump removal, and ribosome protection (Dinos et al., 2016; Long et al., 2006; Schwarz et al., 2004). One of the first reports of bacterial modification of chloramphenicol came only two years after its discovery. The authors described multiple species of bacteria, including Escherichia coli, having the ability to fully reduce the nitro group of chloramphenicol and in this way resist its bacteriostatic effects (Smith and Worrel, 1949). The resulting compound, amino-chloramphenicol (Figure 1C), lacked antibacterial activity, an observation borne out by studies demonstrating the importance of an electron-withdrawing constituent at this position of the molecule (Dinos et al., 2016). Since this first observation, additional studies have replicated the finding that a variety of bacteria can reduce the nitro group of chloramphenicol, resulting in altered susceptibility (Egami et al., 1951; Merkel and Steers, 1953; O’Brien and Morris, 1971; Onderdonk et al., 1979; Smith and Worrel, 1950, 1953; Smith et al., 2007). While many of these bacteria demonstrated a susceptible phenotype in vitro, there is evidence that in a host this strategy leads to phenotypic resistance in the presence of serum chloramphenicol concentrations reaching almost 3-fold the in vitro minimal inhibitory concentration (MIC) (Onderdonk et al., 1979). Furthermore, based on the phylogenetic distribution of these taxa, chloramphenicol nitroreduction is likely not limited to these individually studied organisms. Similarly, amino-chloramphenicol has been detected in the feces of conventional rats, but not in their germ-free counterparts (Glazko et al., 1952; Wal et al., 1983), and this metabolite has been documented in samples from dogs, non-human primates, and humans as well (Glazko, 1987a; Holt et al., 1995; Kunin et al., 1959). Despite its apparent ubiquity and potential medical importance, the mechanism underlying chloramphenicol reduction has never been described and no genes or enzymes having this activity have been documented.

We set out to identify bacterial genes that may be effectors of this activity. The oxygen-insensitive family of nitroreductases, such as those encoded by the nfsA or nfsB genes in E. coli, are known to be promiscuous reducers of aromatic nitro groups, and homologs of these genes are found across diverse bacterial taxa, including in many members of the intestinal microbiome (Roldán et al., 2008). Nitro-reduction via these enzymes occurs in two electrons at a time, often stopping at the production of a hydroxylamino group after a four-electron reduction (Roldán et al., 2008), though some enzymes, such as the NfsB homologs from E. coli, Enterobacter cloacae, and Salmonella enterica, have been demonstrated to reduce aromatic nitro groups completely to amines (LinWu et al., 2009; Yanto et al., 2010) (Figure 1D). We found that expression of some of these genes in E. coli led to altered susceptibility/resistance to chloramphenicol. In vitro characterization of the enzyme corresponding to the most active gene, the Haemophilus influenzae nfsB homolog, revealed an ability to reduce chloramphenicol to amino-chloramphenicol, as well as an ability to at least partially reduce several other aromatic nitro-substrates. Finally, we made initial strides into counteracting this cryptic resistance mechanism, showing that the commonly used antibiotic metronidazole both inhibits chloramphenicol reduction in vitro and synergistically kills chloramphenicol-reducing E. coli in the presence of chloramphenicol. We report the isolation and characterization of genes/enzymes that confer chloramphenicol resistance via a reductive mechanism and consider the implications of our findings for future amphenicol use in the clinic, as well as potential links to chloramphenicol toxicity.

RESULTS

Nitroreductase Gene Expression Can Alter E. coli Chloramphenicol Susceptibility

In choosing which candidate genes to assay for chloramphenicol reduction activity, we began our search by focusing (fortuitously) on homologs of the oxygen-insensitive type I nitroreductase family. We choose to focus at first on oxygen-insensitive
enzymes due to the ability of this class to reduce aromatic nitro groups to a stable amino product, rather than to unstable intermediates as has been observed with many type II oxygen-sensitive enzymes (Roldán et al., 2008). The most relevant members of this class of enzyme are the well-studied E. coli NfsA and NfsB enzymes (Zenno et al., 1996a, 1996b). Because a Lactococcus lactis homolog of E. coli NfsA (CinD) has been reported to be unable to reduce chloramphenicol in vitro (Mermod et al., 2010), we further chose to focus on predicted NfsB homologs originating from bacterial taxa that have been phenotypically associated with chloramphenicol reduction (Table S1) including E. coli, S. enterica, E. cloacae, H. influenzae, Clostridium acetobutylicum, and Streptococcus pyogenes. We cloned a target gene from each organism into a constitutive expression vector for expression in E. coli. Because amino-chloramphenicol (Figure 1C) does not show antibacterial properties (Smith and Worrel, 2007), we utilized microbroth dilution susceptibility testing to determine whether any of the strains showed altered susceptibility to chloramphenicol as a result of reductase expression (Figure 2A). Our negative control, E. coli carrying an empty vector, was susceptible to chloramphenicol, with a calculated 50% inhibitory concentration (IC$_{50}$) of 2.12 ± 0.19 μg/mL (Figure 2B) and an observed MIC of 8 μg/mL across three replicates. E. coli expressing a canonical chloramphenicol resistance gene, chloramphenicol acetyltransferase or cat (Schwarz et al., 2004), was highly resistant with an IC$_{50}$/MIC greater than 256 μg/mL. Two of the cloned reductases imparted a significant increase in chloramphenicol resistance, as measured by IC$_{50}$, when expressed in E. coli: the nfsB homologs from H. influenzae (IC$_{50}$ 21.5 ± 1.1 μg/mL) and E. cloacae (IC$_{50}$ 6.24 ± 1.1 μg/mL) (Figure 2B). E. coli strains expressing these genes were also the only strains to show at least a two-step jump in MIC compared with vector control, with the H. influenzae nfsB-expressing strain showing growth up to 64 μg/mL chloramphenicol, and the E. cloacae nfsB-expressing strain showing growth up to 32 μg/mL chloramphenicol.

Because we observed variation in the ability of nfsB expression to confer resistance to chloramphenicol, we set out to determine whether activity could be predicted by amino acid sequence. We focused on the amino acid sequences of the six reductases tested in E. coli (Figure 2), two other well-studied nitroreductases (E. coli NfsA [Zenno et al., 1996a] and L. lactis CinD [Mermod et al., 2010]), and nine sequences corresponding to nfsB homologs from bacterial taxa previously tested for their ability to reduce chloramphenicol in culture (Smith et al., 2007). For a broader comparison, a selection of bacterial proteins annotated in UniProt as NfsB homologs were compared as well. The resulting maximum-likelihood tree (Figure 3) is organized largely by phylogeny but with evidence for clustering by function as well.

This is evident in the clustering of the Pasteurellales family, where Haemophilus, Neisseria, and Pasteurella genera appear to cluster as a group by phylogeny. Within this group, the Neisseria NfsB homologs cluster with Haemophilus NfsB homologs while the Pasteurella homolg is further removed despite its closer phylogenetic relatedness to Haemophilus. This subgrouping instead matches predicted ability to reduce chloramphenicol based on previous phenotypic studies (Smith et al., 2007). On the other hand, the E. cloacae NfsB clusters with its phylogenetic relatives, E. coli and S. enterica, although it appears to have significantly greater chloramphenicol reduction activity (Figure 2). Because our analyses did not establish any sequence markers for predicting chloramphenicol reduction, we chose to focus on further characterizing the H. influenzae NfsB homolog, the reductase with greatest apparent activity.

**Chloramphenicol-Reducing E. coli Cultures Can Degrade Chloramphenicol to Non-inhibitory Levels and Rescue Susceptible Strains**

One report of chloramphenicol reduction occurring in the context of a rat infection model noted the survival of susceptible bacteria despite chloramphenicol treatment with confirmed serum levels well above their MIC (Onderdonk et al., 1979). To test our system for this phenotype, we performed a series of experiments on the E. coli strain expressing the H. influenzae nfsB
gene. Our first approach used a chloramphenicol challenge assay in which we measured culture lag-phase duration in the presence of increasing concentrations of chloramphenicol. The utility of this lag-phase assay has previously been demonstrated for chloramphenicol (Tao et al., 2012). We observed that while our control E. coli strain did not show substantial growth in the presence of any concentration of chloramphenicol (Figure 4A), the H. influenzae nfsB-expressing strain grew at several concentrations (Figure 4B), in line with previous observations of chloramphenicol reduction. We found a clear correlation between the time it took the resistant strain to reach half-maximal growth (Figure 4D), suggests complete reduction of the nitro group to an amine as partially reduced intermediates could potentially spontaneously oxidize back to chloramphenicol in a futile cycle (Dingsdag and Hunter, 2018; Roldán et al., 2008).

We therefore set out to characterize the H. influenzae nfsB chloramphenicol product in vitro. We cloned the H. influenzae nfsB gene into an inducible system for heterologous expression and purification in E. coli. After optimization (Figure S1A) we obtained an enzyme of the expected denatured size of ~25 kDa (Figure S1B). We found that H. influenzae nfsB (UniProt: Q57431) requires NADPH as a cofactor and does not utilize NADH (Figure S2A) and tightly binds a flavin cofactor, most likely flavin mononucleotide (FMN) (Figure S2B). The enzyme shows activity across a wide range of pH values, with highest activity recorded between pH 6.5 and pH 9 (Figure S2C), and shows activity in our assay at temperatures between 25°C and 55°C (Figure S2D). Based on these results, assays were performed at 25°C in 50 mM Tris-HCl buffer at pH 8 with NADPH and FMN.

To determine whether chloramphenicol is reduced completely to amino-chloramphenicol, we utilized liquid chromatography coupled with mass spectrometry (LC-MS). We first verified...
enzymatic activity by following chloramphenicol degradation over the course of time. Two reactions were initiated by adding enzyme or buffer to solutions containing chloramphenicol and NADPH, following which paired aliquots were quenched every 2.5 min for quantitative analysis of chloramphenicol concentrations. LC-MS analysis confirmed loss of chloramphenicol in the enzyme reactions but not in the control reaction (Figures 5A and 5B).

For confirmation that the product of this reaction was amino-chloramphenicol, a preparative scale reaction with an NADPH regeneration system was incubated for 22.5 hr, extracted with ethyl acetate, and the resulting products analyzed by LC-MS. Extraction of ion m/z corresponding to amino-chloramphenicol and chloramphenicol revealed peaks at the same retention time as standards (Figures 5C and S3A), with the first peak exhibiting a mass spectrum and retention time consistent with amino-chloramphenicol (Figure 5D). Verification of amino-chloramphenicol as a product of _H. influenzae nfsB_ reduction of chloramphenicol was completed by comparison of product with standard by multiple-reaction monitoring (MRM) mass spectrometry fragment analysis (Figures S3B and S3C).

**Nitrated Compounds and Chloramphenicol, but Not Other Amphenicols, Are _H. influenzae nfsB_ Substrates**

Nitroreductase enzymes generally show wide substrate specificity (Akiva et al., 2017; Pitsawong et al., 2014; Roldán et al., 2008), so we next set out to more thoroughly characterize the substrate range of the _H. influenzae_ nfsB enzyme and its kinetics. We first attempted to measure crude chloramphenicol reduction kinetics via the Bratton-Marshall derivatization test (Bratton and Marshall, 1939), using time-quenched reaction samples as inputs. The Bratton-Marshall assay is sensitive and specific for aromatic amines but not other aromatic nitrogenous substituents, and is especially apt for detecting amino-chloramphenicol (Bratton and Marshall, 1939; Glazko, 1987b; Smith et al., 2007). Reaction progress curves of Bratton-Marshall-positive products at different concentrations of chloramphenicol indicated enzymatic production of amino-chloramphenicol and suggested that the _H. influenzae_ nfsB enzyme follows Michaelis-Menten kinetics (Figure S4A). Nitroreductase reactions can be followed in real time spectrophotometrically by measuring the oxidation of NAD(P)H to NAD(P) (Mermod et al., 2010). To confirm that _H. influenzae_ nfsB oxidation of NADPH...

Figure 4. Chloramphenicol Resistance in _E. coli_ Expressing _H. influenzae nfsB_ Occurs via a Drug-Modification Mechanism

(A and B) Lag-phase assays of _E. coli_ expressing (A) empty vector or (B) _H. influenzae nfsB_ grown in the presence of increasing concentrations of chloramphenicol ranging from 0 μg/mL to 64 μg/mL. (C) Linear regression of incubation time required to reach half-maximal OD_600_ plotted against chloramphenicol concentration. The slope of the fit line is 3.12 ± 0.11 with an R^2^ of 0.998. (D) Growth of chloramphenicol-susceptible _E. coli_ in chloramphenicol medium pre-conditioned by empty vector or reductase-expressing _E. coli_ strains. All experiments were performed in triplicate with error bars representing SD when present. Significance at ****p < 0.0001 was calculated based on triplicate experiments using a one-way ANOVA test with Dunnett’s correction for multiple comparisons, and reflects a comparison with vector control.
corresponds to chloramphenicol reduction, we followed the same reaction wells at a wavelength specific to NADPH (beyond the absorbance range of chloramphenicol, Figure S4B) and at a wavelength specific to the chloramphenicol nitro group (at the isosbestic point of NADPH/NADP), as determined by comparison of UV-visible spectra of chloramphenicol and the nitro group free amphenicols thiamphenicol and florenicol (Figure S4B). The resulting progress curves (Figures S4C and S4D) match closely, confirming NADPH monitoring as an appropriate proxy for nitroreduction.

We next measured the kinetics of *H. influenzae* NfsB reduction of chloramphenicol, as well as other potential aromatic nitro-substrates. We began by comparing reduction of chloramphenicol with reduction of thiamphenicol and florenicol. Because thiamphenicol and florenicol do not contain nitro groups, we expected to see no activity with these substrates. Of the three amphenicols, only the reaction containing chloramphenicol showed evidence of enzymatic activity, demonstrating classic Michaelis-Menten kinetics (Figure 6A). NfsB and other nitroreductase enzymes are the targets of the 5-nitro class of antibiotics, including metronidazole and nitrofurantoin. These antibiotics are pro-drugs and become bactericidal when reductases act on their aromatic nitrogen groups, leading to the production of highly reactive hydroxylamines (Dingsdag and Hunter, 2018). We assayed the activity of *H. influenzae* NfsB against these two compounds and confirmed that both are substrates, with nitrofurantoin in particular showing rapid turnover rates (Figure 6B).

Finally, we also assayed 3- and 4-nitrophenol as well as menadione, a quinone lacking nitro groups that is often nonetheless reduced by NfsB nitroreductases (Zenno et al., 1996b). All three of these compounds also acted as substrates, with menadione showing relatively high activity and 4-nitrophenol showing relatively low activity (Figure 6C). Michaelis-Menten kinetics for each substrate are reported in Table S2.

Subsequently, we performed Bratton-Marshall endpoint assays on reactions with each nitro-containing substrate (substrates listed above except thiamphenicol, florenicol, and menadione), as well as enzyme and cofactor controls. As expected, the control lacking NADPH showed very little production of Bratton-Marshall-derivatized material, while the full
chloramphenicol reaction showed a significant amount. In contrast, the 5-nitro antibiotics showed no significant production compared with the substrate control reaction, while the two nitrophenol compounds showed intermediate levels (Figure 6D).

**Metronidazole Inhibits Chloramphenicol Reduction by H. influenzae NfsB In Vitro and Synergizes with Chloramphenicol to Inhibit E. coli Expressing nfsB**

During the course of our in vitro experiments we were surprised to see that the H. influenzae NfsB enzyme reduces chloramphenicol to amino-chloramphenicol (Figures 5 and S3) and also uses metronidazole as a substrate (Figure 6C), but does not reduce the metronidazole nitro group all the way to an amino group (Figure 6D). Because partial reduction of the aromatic nitro group underlies the bactericidal activity of 5-nitro class of antibiotics such as metronidazole (Dingsdag and Hunter, 2018), we next assayed E. coli expressing nfsB homologs from H. influenzae and E. cloacae, as well as from S. enterica and E. coli, for altered susceptibility to metronidazole. Metronidazole susceptibility is usually limited to anaerobic bacteria (Dingsdag and Hunter, 2018), as illustrated by the high IC50 (23.7 ± 2.0 µg/mL) of our vector control. All four E. coli strains expressing nfsB homologs showed significantly increased susceptibility to metronidazole compared with the vector control, with the strains expressing the H. influenzae and E. cloacae nfsB genes showing the most sensitivity (Figures S5A and S5B).

Based on the antibiotic susceptibility evidence, H. influenzae nfsB expression is advantageous in the presence of chloramphenicol, providing resistance through reduction (Figure 2). However, in the presence of metronidazole nfsB expression is disadvantageous, as E. coli does not normally activate metronidazole to its toxic form but appears to do so under these conditions (Figures S5A and S5B). Our kinetics data (Table S2) also suggest that, based on K_M measures, metronidazole should be able to compete with chloramphenicol substrate binding. We therefore hypothesized that chloramphenicol and metronidazole might act synergistically when used in combination against chloramphenicol-reducing bacteria. We found that by increasing
the concentration of metronidazole, the chloramphenicol susceptibility of the H. influenzae nfsB expression E. coli strain increased markedly (Figure 7A).

Our follow-up analysis using a checkerboard-type assay to measure interactions between the antibiotics demonstrated that chloramphenicol and metronidazole do indeed act synergistically in E. coli expressing H. influenzae nfsB, with the lowest fractional inhibitory concentration indices (FICI) score reaching 0.727 ± 0.080 (Figure 7B), significantly lower than E. coli with empty vector (FICI = 2.30 ± 0.73). We further studied this interaction in vitro by measuring the rate of chloramphenicol reduction in the presence of increasing concentrations of metronidazole or dicoumarol, a known inhibitor of NfsB enzymes (Roldán et al., 2008). In vitro we observed that both substrates have the potential to inhibit chloramphenicol reduction, though with metronidazole showing an IC50 of ~1 mM (Figure 7C). The hypothesized interplay between resistance via reduction and pro-drug activation via reduction is summarized in Figure 7D.

**DISCUSSION**

We present results from a series of cross-validating experiments that demonstrate the ability of H. influenzae NfsB to reduce the nitro group of chloramphenicol. We provide the identification and characterization of an enzyme with chloramphenicol reductase activity, beginning with its ability to confer resistance to chloramphenicol when expressed in E. coli (Figure 2). While resistance was not as thorough as that seen in E. coli expressing the cat gene, resistance conferred by H. influenzae nfsB allowed for growth well beyond the EUCAST clinical breakpoint for E. coli of 8 μg/mL. Similarly, expression of the nfsB homolog from E. cloacae was sufficient to confer increased resistance to chloramphenicol in E. coli, again above the clinical breakpoint for defining resistance.

We were curious as to whether we could detect a signal in the amino acid sequence of the H. influenzae NfsB enzyme that could predict its heightened ability to resist chloramphenicol.
but were unable to do so (Figure 3), suggesting that structural studies may be warranted. Nevertheless, the phylogenetic distance between H. influenzae and E. cloaca NfsB proteins suggests to us that chloramphenicol reduction may be relatively widely spread. Chloramphenicol is a natural product antibiotic that has been recovered from across the globe (Ehrlich et al., 1947; Gottlieb and Bhattacharyya, 1949) and is detectable in the soil at low concentrations (Berendsen et al., 2013), suggesting that nitroreduction may be a feasible resistance mechanism against environmental levels of the drug.

We also found compelling evidence for utility of this resistance mechanism in the face of clinical therapy. In addition to growing in the presence of chloramphenicol concentrations above resistance breakpoints (Figure 2), we found in a different assay that E. coli expressing H. influenzae nfsB appears capable of detoxifying increasing levels of chloramphenicol given enough time (Figure 4). This finding is in agreement with a literature report (Onderdonk et al., 1979) that during infection, Bacteroides fragilis strains that showed in vitro susceptibility to chloramphenicol were capable of resisting treatment through reduction. It is also possible, therefore, that during treatment with chloramphenicol commensal or pathogenic bacteria may find a selective advantage in reducing the nitro group of chloramphenicol.

In vitro, the H. influenzae NfsB enzyme is capable of reducing the chloramphenicol nitro group completely to an amine, as well as potentially the nitro groups of other nitrophenols (Figures 5 and 6). Complete reduction of aromatic nitro groups is a rare feature among described NfsB homologs (Pitsawong et al., 2014; Roldán et al., 2008) that may have further industrial or medical applications (Copp et al., 2017). The kinetics of chloramphenicol reduction (Table S2), with a $k_{cat}/K_{m}$ of 20.71 mM$^{-1}$ s$^{-1}$, are slower than many canonical antibiotic resistance enzymes, such as diffusion-limited β-lactamases, but fall within the range of kinetics observed for average enzymes (Bar-Even et al., 2011). The NfsB enzyme from H. influenzae appears to have promiscuous substrate binding, based on the variety of substrates it is capable of turning over and their $K_{M}$s, ranging from roughly 40 μM to 500 μM. In this respect it is similar to other nitroreductase enzymes which, as a class, show substrate promiscuity and relatively weak substrate binding (Akiva and DeLucca, 1991; Pitsawong et al., 2014; Race et al., 2005; Roldán et al., 2008). Of the substrates tested, H. influenzae NfsB showed the greatest activity with nitrofurantoin and lowest reliably measured activity with metronidazole, with $k_{cat}/K_{m}$ measures approximately an order of magnitude above and below that measured for chloramphenicol, respectively. Despite their wide difference in kinetics, reactions containing neither nitrofurantoin nor metronidazole showed evidence for the production of aromatic amino groups (Figure 6D). Partial reduction of these compounds is the key to their antibiotic activity, but full reduction can provide resistance (Roldán et al., 2008). In the case of H. influenzae NfsB, reduction is sufficient to provide resistance to chloramphenicol, but might only serve to more fully activate the antibacterial activity of metronidazole and nitrofurantoin.

In exploring this idea further, we found that expression of H. influenzae nfsB and other nfsB homologs in E. coli leads to significantly increased susceptibility to metronidazole, a drug that is usually only used to treat anaerobes (Figures SSA and SSB). We further observed that metronidazole potentiates chloramphenicol resistance in the H. influenzae nfsB-expressing strain and that in fact the two antibiotics synergistically inhibit the growth of this strain (Figures 7A and 7B). We hypothesized that this synergy may be the result of two activities. The first of these relates to our observation that metronidazole is not fully reduced to form an amino group, but does act as an NfsB substrate. Therefore it is likely that while NfsB protects the cell against chloramphenicol it also activates metronidazole to its lethal form, counteracting any survival benefit. The second activity that we believe might be relevant is substrate inhibition. While metronidazole is a poor substrate compared with chloramphenicol as measured by $k_{cat}$ (0.337 s$^{-1}$ versus 10.17 s$^{-1}$) it is much better when measured by $K_{M}$ (74 μM versus ~500 μM) (Table S2). We hypothesized that metronidazole may therefore be able to compete with chloramphenicol as a substrate, a hypothesis borne out by our measurements of chloramphenicol reduction in the presence of titrated metronidazole (Figure 7C). A schematic of chloramphenicol/metronidazole synergy is presented in Figure 7D, wherein metronidazole directly kills H. influenzae-expressing E. coli via its reduction-mediated activation to a toxic form and indirectly inhibits growth of this strain by competing with chloramphenicol, resulting in diminished resistance to it.

Finally, it has been hypothesized that chloramphenicol nitroreduction by host-associated bacteria underlies the development of a rare, serious side effect of chloramphenicol, namely aplastic anemia (Holt, 1967). The lack of mechanistic understanding of chloramphenicol-induced aplastic anemia is largely responsible for the failure of many developed countries to utilize amphenicols in the clinic, including potentially superior second- and third-generation compounds. For example, florfenicol, which lacks a nitro group, is immune to inactivation by chloramphenicol acetyltansferases (Dinos et al., 2016) (Figures S5C and S5D) but is not used clinically due to fears that its use in humans may result in development of aplastic anemia. The identification of a bacterial enzyme capable of chloramphenicol reduction provides the prerequisite for testing the aplastic anemia hypothesis and potentially shedding light on the safety of nitro group free amphenicols.

**SIGNIFICANCE**

Chloramphenicol was first identified as a potent antibiotic more than 70 years ago, and since then a number of bacterial resistance mechanisms against it have been identified. However, the enzyme underlying the first reported mechanism of chloramphenicol resistance, reduction of the nitro group to an amine, has remained elusive despite numerous reports of bacteria showing this activity. We describe an example of an enzyme with this activity, the oxygen-insensitive nitroreductase NfsB from Haemophilus influenzae. Expression of the gene for this enzyme, or its homolog from Enterobacter cloacae, is sufficient to confer resistance to chloramphenicol in Escherichia coli. In vitro characterization of the purified enzyme revealed its ability to fully reduce aromatic nitro groups on a variety of substrates and partially reduce the nitro groups of metronidazole and nitrofurantoin, two antibiotics that gain their ability to kill bacteria following reduction. We found that expression of the H. influenzae nitroreductase gene in E. coli augments the killing power of metronidazole and potentiates the efficacy of chloramphenicol, likely
through both direct and indirect mechanisms. Finally, identification of this enzyme closes a long-open chapter in chloramphenicol resistance and potentially provides a clue to the mechanistic basis of one of chloramphenicol’s serious side effects, aplastic anemia, that has prevented adoption of promising chloramphenicol analogs in the clinic.

**STAR METHODS**

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- **SUPPLEMENTAL INFORMATION**

Supplemental Information includes five figures and two tables and can be found with this article online at [https://doi.org/10.1016/j.chembiol.2019.01.007](https://doi.org/10.1016/j.chembiol.2019.01.007).

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**AUTHOR CONTRIBUTIONS**


**DECLARATION OF INTERESTS**

The authors declare no competing interests.

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**REFERENCES**


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CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Gautam Dantas (dantas@wustl.edu).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

*Escherichia coli* DH10B and BL21(DE3), *Haemophilus influenzae* KW20 (ATCC 51907), *Salmonella enterica* LT2 (ATCC 700720), *Enterobacter cloacae* 96-3 (ATCC 13047), and *Clostridium acetobutylicum* NCIB 8052 (ATCC 824) were cultured respectively (1) aerobically at 37°C in LB, (2) aerobically at 37°C in DSMZ *Haemophilus medium* (per liter: 21 g Mueller-Hinton broth, 5 g yeast extract, supplemented with 15 mg each of NAD and hemine), (3) aerobically at 37°C in Mueller-Hinton broth, (4) aerobically at 37°C in Mueller-Hinton broth, or (5) anaerobically at 37°C in ATCC modified reinforced Clostridial broth (per liter: 10 g tryptose, 10 g beef extract, 3 g yeast extract, 5 g dextrose, 5 g NaCl, 1 g soluble starch, 0.5 g L-cysteine HCI, 3 g sodium acetate, and 4 ml of 0.025% resazurin). For routine growth, *E. coli* strains were incubated aerobically at 37°C in LB media or in Mueller-Hinton media for antibiotic susceptibility tests with kanamycin sulfate at 50 μg/ml when appropriate. Strain identities were verified through successful amplification of target reductase genes confirmed by Sanger sequencing (see Methods Details: Gene Amplification and Cloning).

METHODS DETAILS

**Gene Amplification and Cloning**

Genomic DNA from grown cultures was extracted and purified using a Wizard Genomic DNA purification kit (Promega, No. A1120) according to manufacturer’s instructions. *Streptococcus pyogenes* Genomic DNA was kindly provided to us by the Caparon lab at Washington University in St. Louis. Reductase and cat genes were amplified from genomic DNA, or the pZE31 vector (*Lutz and Bujard, 1997*) for cat, with primer pairs specified in Table S1 using Platinum Pfx polymerase (ThermoFisher Scientific, No. 11708039) or Q5 hotstart 2X master mix (New England Biosciences, No. M0494L) according to manufacturer’s guidelines. Inserts were purified by QIAquick PCR purification kit (Qiagen, No. 28104) and ligated into modified pZE21(*Lutz and Bujard, 1997*) or pET-28b(+) (with N-terminal 6X-his tag) vectors by blunt-ended ligation using Fast-Link DNA ligation kit (Epicentre, No. LK0750H) or 2X Gibson Assembly master mix (New England Biosciences, No. E2611S). Constructs were next transformed into chemically competent *E. coli* DH10B cells (pZE21 vectors) or *E. coli* BL21(DE3) (pET-28b(+) vectors) by heat shock at 42°C followed by recovery for 30 minutes at 37°C in SOC media (Invitrogen, No. 46-0821) and plating on to LB agar with 50 μg/ml kanamycin. Colonies were picked into LB with kanamycin, grown overnight at 37°C with aeration and plasmids extracted by QIAprep Spin Miniprep kit (Qiagen, No. 27104). Insert integrity was finally verified by Sanger sequencing (Genewiz). All strains were stored long-term at -80°C as stocks in 15% glycerol in LB.

**Microbroth Serial Dilution Assays**

Microbroth serial dilution assays were used to determine IC50 values for amphenicols and metronidazole in a modification of EUCAST standard protocol. 96-well plates (COSTAR, 3595) were prepared with 100 μl volumes of antibiotics in Mueller-Hinton broth with kanamycin arranged in two-fold dilutions by column, with concentrations ranging from 512 μg/ml to 0.5 μg/ml for chloramphenicol, 512 μg/ml to 0.25 μg/ml for metronidazole and florfenicol, or 1024 μg/ml to 0.5 μg/ml for thiamphenicol (concentrations are two-fold greater than final concentrations).

Overnight Mueller-Hinton with kanamycin starter cultures of *E. coli* DH10B strains expressing reductase genes on pZE21 vectors were inoculated into fresh Mueller-Hinton with kanamycin at a density of 0.1 AU measured at 600 nm (OD600). Diluted cultures were added to the prepared antibiotic-containing 96-well plates in 100 μl aliquots for an initial OD600 and volume of 0.05 AU and 200 μl respectively, with each strain/antibiotic combination prepared in triplicate. After inoculation, 96-well plates were sealed with Breath-Easy membranes (Sigma-Aldrich, Z380059) and incubated with shaking at 220 rpm at 37°C for 18 to 24 hours. Growth was measured at 600 nm in a Powerwave HT microplate spectrophotometer (Biotek, Inc.). Dose-response curves were plotted and analyzed using GraphPad Prism version 7.01 for Windows (GraphPad Software, La Jolla California, USA). Triplicate data were fit to the following four parameter Hill equation:

\[
Y = \frac{\text{Bottom} + (\text{Top} - \text{Bottom})}{1 + \left(\frac{\text{IC50}}{\text{HorizontalSlope}}\right)^{\text{VerticalSlope}}}
\]

IC50 p-values were calculated by One-way ANOVA with Dunnett’s correction for multiple comparisons. MIC was determined by taking the lowest concentration that resulted in culture growth measuring less than 0.1 AU at 600 nm. Chloramphenicol resistant/susceptible breakpoint of 8 μg/ml is according to EUCAST standards for *E. coli*.
Phylogenetic Analysis of Reductase Proteins

Amino acid sequences were downloaded and aligned using ClustalW (Thompson et al., 1994) in Mega7 (Kumar et al., 2016) using default parameters with manual trimming. Un-targeted NfsB homologs were downloaded from the UniProt database (UniProt Consortium, 2015). The resulting alignment was used to construct a Maximum Likelihood tree with default parameters and 100 rounds of bootstrapping in Mega7. The tree was visualized in the tree viewing program FigTree (Rambaut, 2008).

Lag-Phase Chloramphenicol Modification Assay

Triplicate 100 μl aliquots of LB with kanamycin and 0 μg/ml, 8 μg/ml, 16 μg/ml, 32 μg/ml, 64 μg/ml, or 128 μg/ml chloramphenicol were prepared in a 96-well plate (COSTAR, 3595). Overnight triplicate cultures of empty vector or H. influenzae nfsB-expressing E. coli cultures were grown in LB with kanamycin and diluted 10-fold into fresh LB with kanamycin and incubated for 3 hours at 37°C in order to enter exponential phase. Exponentially growing cultures were diluted to an OD_{600} of 0.1 AU and added in 100 μl volumes to the 96-well plate. Plates were sealed as before and incubated in a Powerwave HT microplate spectrophotometer (Biotek, Inc.) at 37°C with constant shaking (medium setting, rpm not available) for 96 hours with monitoring every 30 minutes. Growth data were fit to the Hill equation as above to determine how many hours of incubation were required to reach half-maximal growth. A plot of chloramphenicol concentration against time to reach 50% growth was prepared and projected to 96 hours.

Pre-conditioned Media Assay

E. coli containing empty pZE21 vector or vector-expressed H. influenzae nfsB were inoculated into LB with kanamycin and 8 μg/ml chloramphenicol and incubated overnight at 37°C alongside a cell-free (+) chloramphenicol control and an empty vector (-) chloramphenicol control. Following overnight growth cell growth were removed from cultures by first pelleting at 16,000 rcf for 10 minutes, followed by filtration of the supernatant through a 0.22 μm filter. Cell-free supernatants were split into three aliquots as technical replicates and inoculated with E. coli containing empty vector (to provide resistance to residual kanamycin) prior to incubating overnight at 37°C. Following incubation growth was measured as before and OD_{600} values were compared using a one-way ANOVA test with Dunnett’s correction for multiple comparisons to determine level of chloramphenicol removal in the pre-conditioned media.

H. influenzae NfsB Expression and Purification

E. coli BL21(DE3) containing pET-28b(+) with H. influenzae nfsB open reading frame downstream of a 6-histidine tag was inoculated into 3 ml Studier auto-inducing media ZYM-5052 (Studier, 2005) with kanamycin and grown overnight with aeration at 37°C. 750 μl aliquots of grown culture were added to a 750 ml culture of ZYM-5052 with 100 μg/ml kanamycin and to three 750 ml cultures of Terrific broth (TB, Fisher Scientific, No. BP9729-600) supplemented with 2 mM MgSO₄ in 2.8 L Fernbach flasks. Cultures were shaken at 350 rpm at 37°C in a MaxQ 5000 (Thermo Scientific) temperature-controlled incubator until OD_{600} of ~1 was reached (~3-hour incubation). The incubator temperature was reduced to 25°C and the TB flasks were induced with 0 μM, 100 μM, or 250 μM IPTG with shaking for 48 hours with aliquots taken at 24 hours as well. Cell aliquots taken at 24 hours and 48 hours were pelleted at 8,000 rcf, resuspended in phosphate buffered saline (PBS), and 100 μl aliquots were lysed by sonication using a Bioruptor Pico (Diagenode) at 4°C for 10 minutes with 30 seconds on and 30 seconds off. Lysates were centrifuged at 21,000 rcf to separate soluble and insoluble fractions and analyzed for soluble enzyme production via SDS-PAGE analysis with Sypro Ruby gel stain (ThermoFisher, No. S12001). While 24 hour incubation in ZYM-5052 gave the best results, the 48 hour incubations for all four flasks were combined and cells pelleted at 8,000 rcf for 20 minutes using a Sorvall Legends XTR centrifuge (Thermo Scientific) in a Fiberlite F14-6x250 LE rotor (Thermo Scientific) before removal of supernatants and freezing of wet cell pellets overnight at -80°C.

Cell pellets were weighed and allowed to thaw on ice. All following steps were performed at 4°C or on ice. The cell pellets were resuspended to 20% w/v in ice-cold loading buffer consisting of (per liter) 8.709 g K₂HPO₄ (50 mM), 29.22 g NaCl (500 mM), 351 μl β-mercaptoethanol (5 mM), 0.68 g imidazole (10 mM), and 100 ml glycerol (10% v/v) at pH 8 with addition of lysozyme at 1 mg lysozyme/g wet cell mass. Suspensions were incubated on ice for 30 minutes then lysed by sonication on a Branson Sonifier 250 instrument (Branson Ultrasonics) with microtip adjusted to reach ca. 30% output on 50% duty until suspension viscosity and color were reduced. Insoluble matter was clarified from the suspensions by centrifugation for 30 min at 24,446 rcf at 4°C in a Sorvall Legends XTR centrifuge (Thermo Scientific) in a Fiberlite F14-6x50 cy rotor (Thermo Scientific). Supernatants were collected and stored at 4°C until purification.

Purification of the His-tagged enzyme was performed using an Ni-NTA nickel resin (BioRad, No. 7800800) and a low-pressure chromatography system (BioRad, Econo system) coupled to a fraction collector (BioRad, No. 7318122) as previously described (Crofts et al., 2018). Fractions containing purified enzyme were identified by their yellow color and verified by SDS-PAGE analysis and staining with Bio-Safe Coomassie stain (BioRad, No. 1610796) according to manufacturer’s instructions. Fractions containing pure recombinant H. influenzae NfsB were pooled and concentrated via centrifugation through a 10 kDa molecular weight cut-off filter (Amicon, No. UFC901024) according to manufacturer’s instructions. The ca. 1 ml concentrate was washed three times as above with storage buffer consisting of (per liter) 8.709 g K₂HPO₄ (50 mM), 8.766 g NaCl (250 mM), 1 ml of 1 M dithiothreitol (1 mM) and 50 ml of glycerol (5% v/v) at pH of 7.5. Purified enzyme concentration was determined by Qubit Protein Assay kit (Thermo Scientific, No. Q33211) and the mass concentration was converted to molar concentration using molecular weights predicted by EXPASY (Gasteiger et al., 2003). Aliquots of 55 μl were stored at -80°C.
Chloramphenicol Reduction Assay Optimization

A preliminary in vitro assay based on reported reductase conditions (Mermod et al., 2010; Smith et al., 2007) demonstrated that the H. influenzae NfsB enzyme could reduce chloramphenicol (see below for optimized conditions). We used these baseline conditions to determine the effect of reduced cofactor on activity. A pre-reaction mixture was prepared containing 50 mM Tris-HCl pH 8, 2 mM chloramphenicol, 10 μM flavin mononucleotide (FMN), and 1 μM H. influenzae NfsB. Six 100 μl aliquots were transferred to a 96-well plate (COSTAR, 3595) to which was added 100 μl of 2 mM NADH (Grainger, No. 31GA86) or NADPH (Cayman chemical, No. 9000743) in 50 mM Tris-HCl pH 8. Oxidation of NAD(P)H to NAD(P)⁺ was monitored at 340 nm (Mermod et al., 2010) for 30 min at 25°C in a Powerwave HT microplate spectrophotometer (Biotek, Inc.) with minimal kinetic interval.

In order to determine the identity of the native flavin cofactor, a 50 μl aliquot of enzyme was heated at 70°C for 20 minutes to denature the enzyme and release the tightly bound flavin (Zenno et al., 1996b). The yellow supernatant was removed following centrifugation at 21,000 rcf for 20 min and analyzed via thin layer chromatography using silica gel 60 F₂₅₄ (Millipore Sigma, No. 105794) alongside standards and extract/standard co-spots in a solvent system consisting of 5:2:3 1-butanol:acetic acid:H₂O (Sherma and Fried, 2003). The plate was visualized and photographed under UV-vis at 366 nm in a Gel-Doc XR+ (BioRad). The observed retention factors of the standards were consistent with the literature.

To determine optimal pH for H. influenzae NfsB activity, 100 mM Tris/citrate buffer with 10 μM FMN and 2 mM chloramphenicol was prepared. 400 μl aliquots of buffer were prepared at pH values ranging from 3 to 9 and aliquoted as seven technical replicates in 100 μl volumes in a 96-well plate (COSTAR, 3595). A second solution containing 1 μM enzyme and 6 mM NADPH was prepared and added in 100 μl volume to all buffer wells. Reactions were monitored at 340 nm as before and the progress curve slopes were compared to determine relative activity compared to the most active samples in GraphPad Prism (GraphPad Software, La Jolla California, USA). Due to the highly variable nature of the assay, Grubbs test with α=0.2 for outlier detection was used to remove potentially inaccurate samples.

To determine appropriate temperature ranges for enzyme activity, substrate mixes of 50 mM Tris-HCl pH 8, 1.25 mM NADPH, 1.25 mM chloramphenicol, and 6.25 μM FMN were aliquoted 40 μl at a time into PCR tubes (four replicates for each temperature to be tested). Substrate mixes were pre-incubated in a thermocycler at the test temperature for 1 minute following which 10 μl of enzyme solution (2.5 μM in 50 mM Tris-HCl pH 8) was added. Reactions were allowed to incubate for 4 minutes at which point they were quenched by addition of 50 μl of 20% trichloroacetic acid (TCA) and transfer to ice. Reaction progress was measured in quenched samples by derivatization of amino-chloramphenicol by the Bratton-Marshall method (Bratton and Marshall, 1939; Smith et al., 2007). Briefly, 12.5 μl of the following solutions were added sequentially with 10 minute incubations at room temperature: 0.1% sodium nitrite, 0.5% ammonium sulfamate, and 0.05% Bratton-Marshall reagent (N-(1-Naphthyl)ethylenediamine dihydrochloride, 20 minutes). The reactions were then transferred to half-area 96-well plates (Corning, No. 3696) and Abs₅₅₀ measurements were taken using a Powerwave HT microplate spectrophotometer (Biotek, Inc.) to quantify production of arylationamines. Absorbance at 550 nm was normalized as percent maximum for each reaction and outliers were excluded using a Grubbs test with α=0.2. All following reactions were performed in 50 mM Tris-HCl at pH 8 and at 25°C unless otherwise stated.

Chloramphenicol Modification LCMS Assay

Enzymatic loss of chloramphenicol during NfsB reduction was monitored by liquid chromatography tandem mass spectrometry (LCMS) as follows. Two 700 μl aliquots of substrate buffer consisting of 2 mM NADPH, 10 μM FMN, and 600 μM chloramphenicol were prepared. An equal volume of Tris buffer or 1 μM enzyme in Tris buffer was added to each aliquot at t=0 and 250 μl was immediately removed and quenched with 12 μl of 88% formic acid. Additional 250 μl aliquots were quenched at 2.5 min, 5 min, 7.5 min, and 10 min.

Samples were sent to the Metabolomics Facility at Washington University for LCMS analysis where they were diluted 5-fold in methanol. An equal volume of 10 μg/ml thiophenicol as internal standard was added to each sample before loading onto an ACE Excel Super C18 column (3 μm, 50 x 4.6 mm) on an Applied Biosystems Sciex 4000QTRAP tandem mass spectrometer. Samples were run with multiple reaction monitoring (MRM) in negative mode. Chloramphenicol standards were used to prepare calibration curves in duplicate for quantification.

Amino-Chloramphenicol Detection Assay

The product of H. influenzae NfsB reduction of chloramphenicol in the presence of excess reducing equivalence was analyzed by targeted LCMS. Amino-chloramphenicol HCl salt was purchased from Toronto Research Chemicals (No. A622670) as a standard. An NADPH regeneration system (Xenotech, No. K5000) was used according to the manufacturer’s protocols to provide the equivalent of 700 μM to 900 μM NADPH concentrations during an overnight reaction. The reaction consisted of the regeneration system, 2 mM chloramphenicol, 15 μM FMN, and ca. 1.5 μM enzyme dissolved in 35 ml of 50 mM Tris-HCl buffer. Following incubation for 22.5 hours at room temperature the reaction was extracted three times with 10 ml of ethyl acetate. The combined organic layers were extracted three times with 2.5 ml of 0.1 M aqueous HCl. The aqueous extracts were combined, frozen at -80°C, and lyophilized to dryness. The dried yellow product was dissolved in 750 μl water and checked for potential presence of amino-chloramphenicol by Bratton-Marshall derivatization and TLC analysis against an amino-chloramphenicol standard (silica, 8:2 dichloromethane:methanol, visualized with iodine vapour).

LCMS analyses were performed at the Metabolomics Facility at Washington University on an Applied Biosystems Sciex 4000QTRAP tandem mass spectrometer coupled to a Shimadzu 20AD HPLC system. Metabolites were separated using an ACE
Excel 3 super C18 column (3 μm, 50 x 4.6 mm) running 0.03% diethylamine and 20 mM hexafluoro-2-propanol in water, pH 8.5 (solvent A) against methanol (solvent B). The full mass scan (Q3 scan) and MS2 scans of m/z 291 (amino-chloramphenicol) and m/z 321 (chloramphenicol) were acquired for the samples as well as amino-chloramphenicol and chloramphenicol standards. Data processing was conducted with Analyst 1.5.2 (Applied Biosystems).

**Enzyme Substrate Specificity and Activity**

Reactions to examine substrate activity and specificity were set-up by preparing substrate mixes containing 2 mM NADPH and 10 μM FMN and each substrate in triplicate. Concentrations (at twice the final reaction concentration) ranged from 3 mM to 46.875 μM (chloramphenicol, thiamphenicol, florfenicol, 3-nitrophenol, and 4-nitrophenol), 750 μM to 46.875 μM (metronidazole), and 1.5 mM to 46.875 μM (menadione) by 2-fold steps and were prepared as 50 μl aliquots in a half-area 96-well plate (Corning, No. 3696). Enzyme solution was prepared at 1 μM, and 50 μl of enzyme solution was added to all wells with mixing, followed by monitoring of the reaction progression in a Powerwave HT microplate spectrophotometer (Biotek, Inc.) with minimal kinetic interval at 340 nm for NADPH oxidation (chloramphenicol, thiamphenicol, florfenicol, metronidazole, menadione, and 3-nitrophenol), or at other wavelengths when spectra interfered substantially with measurements at 340 nm (420 nm for nitrofurantoin, 450 nm for 4-nitrophenol). In some cases (metronidazole and menadione) an optimal wavelength could not be found resulting in the exclusion of high-substrate concentration data points where spectral overlap was too high.

Initial rate velocities in absorbance change, found by taking the slope during the linear stage of the reactions, were converted to μM s⁻¹ using automatic pathlength correction and the following extinction coefficients for the substrate being monitored (determined empirically in triplicate if needed): NADPH ε₄₅₀= 6220 M⁻¹ cm⁻¹ (Mermod et al., 2010), nitrofurantoin ε₄₂₀= 7970 M⁻¹ cm⁻¹, and 4-nitrophenol ε₄₅₀= 3211 M⁻¹ cm⁻¹. Triplicate data points were plotted in GraphPad Prism (GraphPad Software, La Jolla California, USA) and fit to Michaelis–Menten curves for kcat and Km determination using the following equation:

\[ Y = Et \times kcat \times X / (Km + X) \]

Where Et corresponds to enzyme concentration (0.5 μM). For each fit the estimated 95% confidence interval as well as Km were plotted on the graph while kcat values were recorded elsewhere.

In order to monitor chloramphenicol nitro group reduction three assays were performed. In the first assay, 100 μl reactions arrayed in 8 replicates containing 50 mM Tris-HCl buffer at pH 8, 1 mM NADPH, 50 μM FMN, 5 μM enzyme, and 250 μM substrate were allowed to react for 30 minutes at room temperature along with control reactions lacking substrate or NADPH. Substrates included chloramphenicol, metronidazole, nitrofurantoin, 3-nitrophenol, and 4-nitrophenol. Following incubation, reactions were quenched with an equal volume of 20% TCA and analyzed by Bratton-Marshall derivatization as performed earlier. The absorbance at 550 nm was plotted to demonstrate accumulation of amino-chloramphenicol over time, with increased accumulation at higher chloramphenicol concentrations. In the third assay, 200 μl reactions in a UV transparent 96-well plate (Corning, No. 3635) containing 0.5 mM NADPH, 0.5 mM chloramphenicol, and 0.25 mM metronidazole, or with each ingredient missing, were monitored at 340 nm for NADPH oxidation to NAD⁺ and at 282 nm (the isosbestic point of NADPH/NAD⁺) for loss of the chloramphenicol nitro group with minimal interval on a Powerwave HT microplate spectrophotometer (Biotek, Inc.).

**Checkerboard Assay to Detect Synergy**

Triplicate 96-well plates (COSTAR, 3595) were prepared containing 100 μl of Mueller-Hinton broth with kanamycin and mixtures of chloramphenicol and metronidazole. Chloramphenicol ranged in concentrations from 512 μg/ml to 0.25 μg/ml by 2-fold jumps and metronidazole ranged in concentrations from 1024 μg/ml to 0.125 μg/ml by 4-fold jumps, with each chloramphenicol concentration existing as a mixture with each metronidazole concentration. *E. coli* expressing *H. influenzae nfsB* or containing empty vector were inoculated into Mueller-Hinton broth with kanamycin and incubated overnight with aeration at 37°C. The resulting cultures were diluted in Mueller-Hinton broth with kanamycin to and OD₆₀₀ of 0.1 AU and added as 100 μl aliquots to the wells of the prepared antibiotics plates. Plates were sealed as before and incubated at 37°C with shaking for 20 to 24 hours before measuring OD₆₀₀ as above. Chloramphenicol growth inhibition at each metronidazole concentration was analyzed using GraphPad Prism (GraphPad Software, La Jolla California, USA) as before. Fractional inhibitory concentration indices (FICI scores) were calculated following growth by scoring wells with OD₆₀₀ less than 0.1 AU as inhibited. FICI scores of <1 were taken as evidence for synergistic relationships, near 1 as additive, and >1 as antagonistic.

**In Vitro Inhibition of Chloramphenicol Reduction**

Substrate buffer was prepared with 2 mM NADPH, 10 μM FN, and 982 μM (twice the measured Km). Inhibitors metronidazole and dicoumarol were prepared in substrate buffer and added in triplicate to a UV transparent 96-well plate (Corning, No. 3635) in 50 μl aliquots with metronidazole ranging in concentration from 4096 μg/ml to 2 μg/ml and dicoumarol ranging in concentration from 1024 μg/ml to 1 μg/ml by 2-fold jumps. *H. influenzae NfsB* was prepared at 1 μM in Tris buffer and 50 μl aliquots were added with mixing prior to monitoring of chloramphenicol reduction at 281 nm on a Powerwave HT microplate spectrophotometer (Biotek,
Inc.) with minimal interval. Velocities were normalized against wells containing no inhibitor, and inhibition dose-response curves were determined as before using GraphPad Prism (GraphPad Software, La Jolla California, USA) to find IC\textsubscript{50} concentrations.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

For all assays, replicate descriptions are available in the corresponding methods section as well as in the specific figure legend. Statistical analyses were carried out using GraphPad Prism with experiment-specific details available in the corresponding methods section and figure legend.