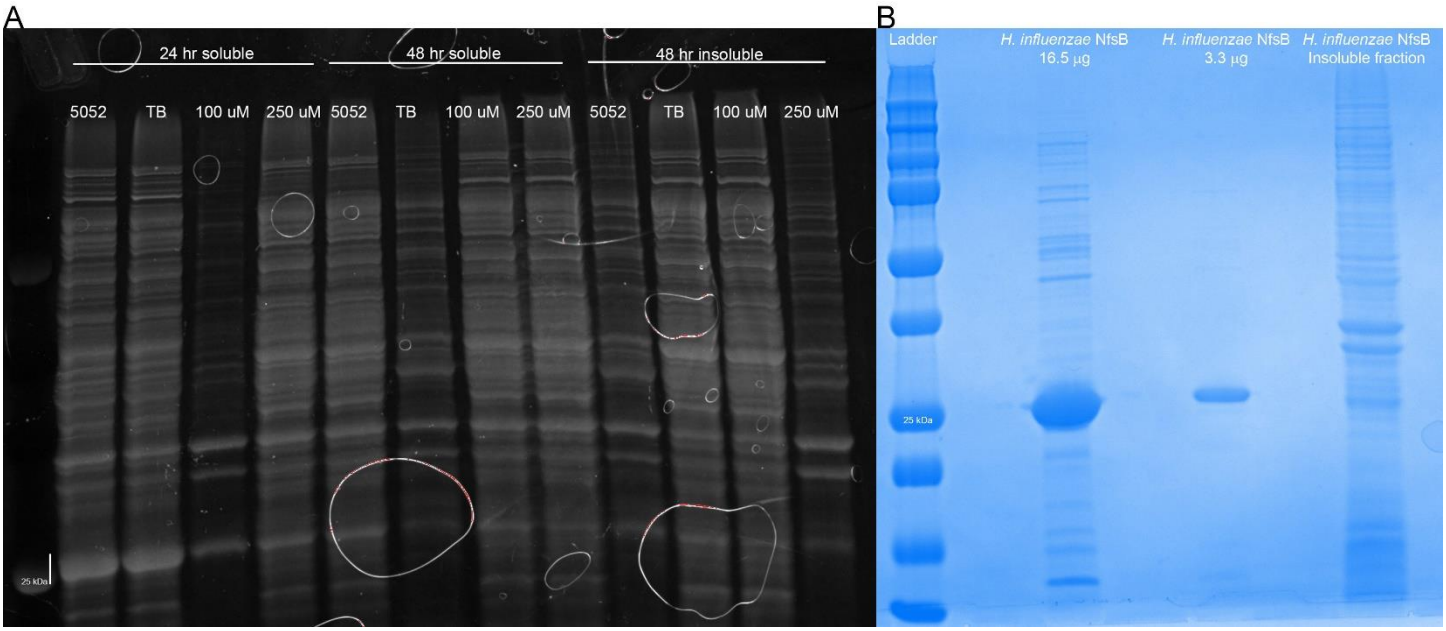
Supplemental table 1. Primers for gene amplification. Related to Star★Methods section METHODS DETAILS: Gene amplification and cloning.

Organism/Source	Citation	Forward primer	Reverse primer
H. influenzae DSM 11121	(Smith et al., 2007)	ATGACTCAACT TACTCGTGAAC AAG	TTACCCCACCC ATTTCACC
S. pyogenes HSC5	(Egami et al., 1951)	ATGAAATTTCT AGAGTTAAACA AAAAACG	CTATCTTCTTTC GATGACTTCAT CAAC
E. coli BL21(DE3)	(Smith and Worrel, 1953)	ATGGATATCAT TTCTGTCGCCTT AAAG	TTAGACTTCGGT TAAGGTGATGT TTTG
E. cloacae ATCC 13047	(Bryant and DeLuca, 1991)	ATGGATATTATT TCTGTCGC	TTAGCACTCAG TCACAATCGT
S. enterica ATCC 700720	(Yanto et al., 2010)	ATGGATATCGT TTCTGTCGCCTT	TTAAACTTCCGT CAGTGTGGTT
C. acetobutylicum ATCC 824	(O'Brien and Morris, 1971; Onderdonk et al., 1979)	ATGCTTAAAGA GATAGAAGAAA G	TCACCATTTTTC CATATGAATTA
cat from pZE31	(Lutz and Bujard, 1997)	ATGGAGAAAAA AATCACTGGAT ATACC	TTACGCCCCGC CCTG

Supplemental table 2. Michaelis-Menten kinetics constants of *H. influenzae* NfsB reduction reactions. Related to figure 6.

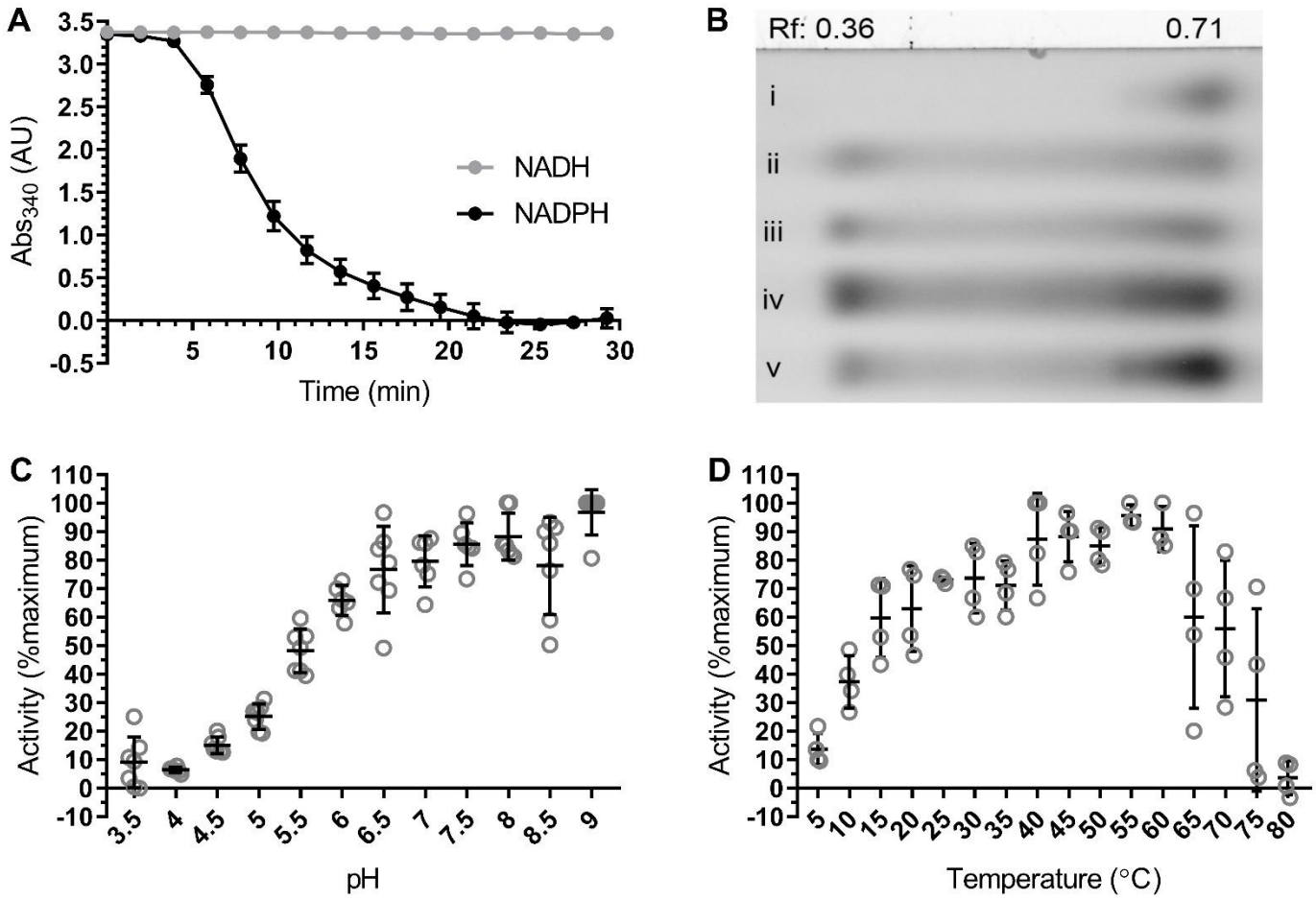
Substrate	k_{cat} (s^{-1})	95% CI	K_M (μM)	95% CI	k_{cat}/K_M (mM^{-1} s^{-1})
Chloramphenicol	10.2	9.42 - 11.0	491	409 - 593	20.7
Thiamphenicol	*nd*	*nd*	*nd*	*nd*	*nd*
Florfenicol	*nd*	*nd*	*nd*	*nd*	*nd*
Metronidazole	0.337	0.268 - 0.435	73.8	36.9 – 143	4.57
Nitrofurantoin	9.43	7.50 - 11.7	38.8	15.1 - 83.7	242
Menadione	12.1	9.74 - 15.3	152	85.4 – 277	79.3
3-Nitrophenol	4.31	3.97 - 4.66	69.7	50.7 - 94.4	61.8
4-Nitrophenol	≥0.03	*nd*	≥750	*nd*	*nd*

nd: Not determined

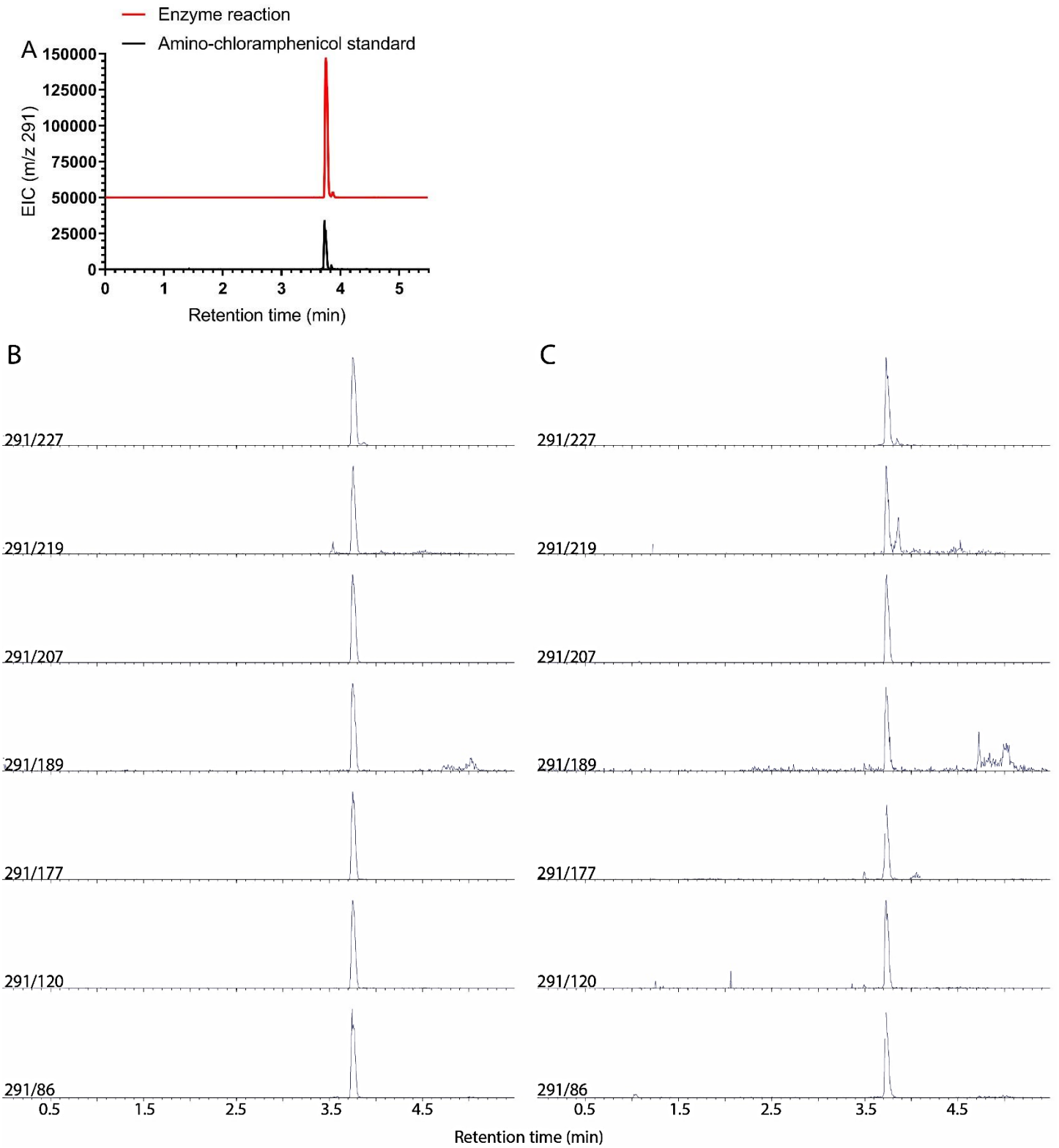


Supplemental figure 1. Expression and purification of *H. influenzae* NfsB from *E. coli*. Related to Figure 5.

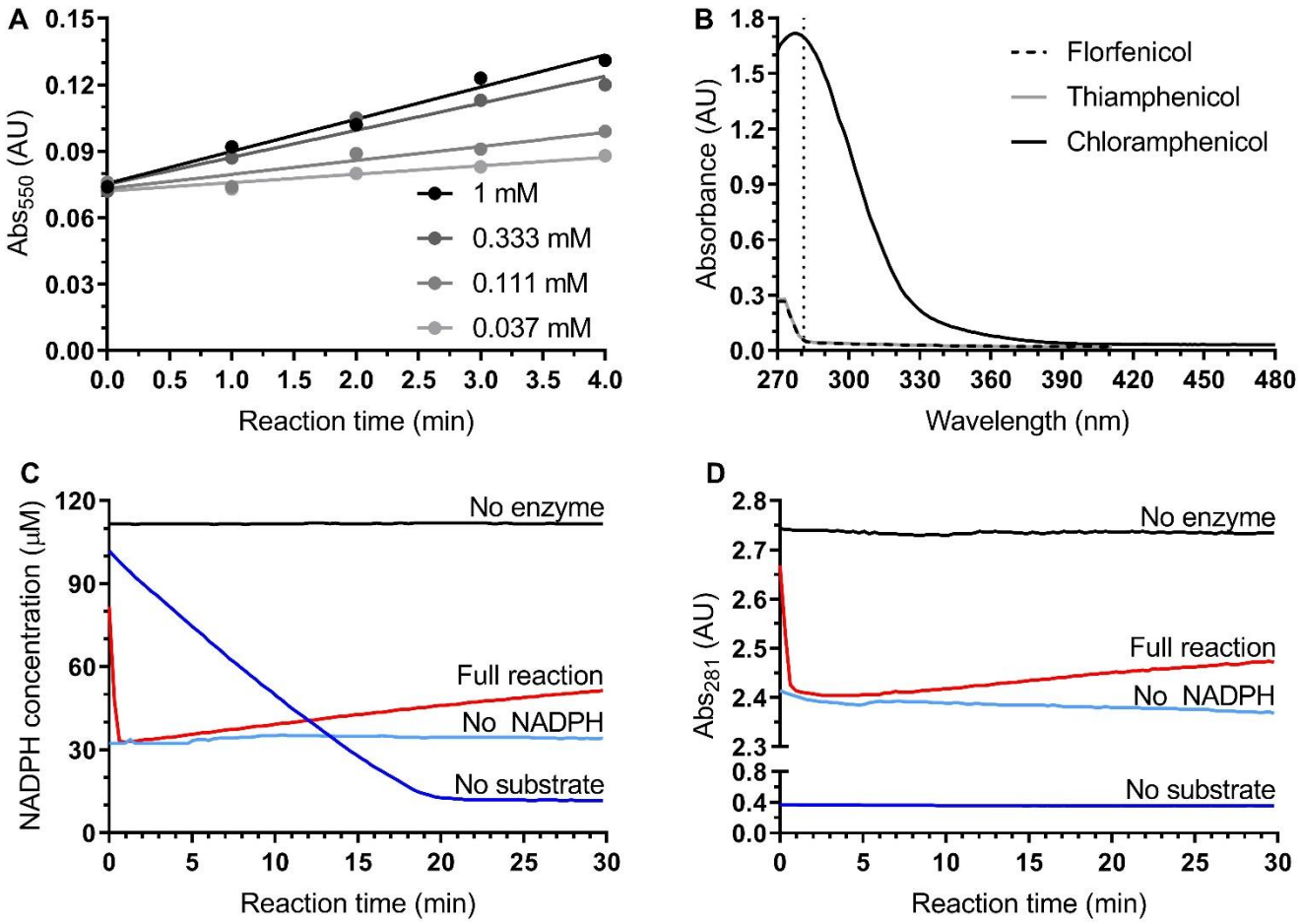
(A) Sypro Ruby SDS-PAGE gel analysis of culture conditions for optimal production of soluble *H. influenzae* NfsB from *E. coli*. 5052 corresponds to Studier auto-induction media ZYM-5052, TB stands for terrific broth, and 100 µM and 250 µM correspond to TB with the indicated concentrations of IPTG added for induction. The 25 kDa ladder is labelled on the left and a vertical white line indicates the expected migration distance for the protein of interest. (B) Coomassie stained SDS-PAGE gel of final purified *H. influenzae* NfsB at two dilutions as well as undiluted insoluble protein fraction. The 25 kDa marker on the ladder is highlighted. See also figure 5.



Supplemental figure 2. *H. influenzae* NfsB physiological requirements. Related to Figure 5.
(A) Assay to determine preferred cofactor for *H. influenzae* NfsB. Reactions compared 1 mM NADP against 1 mM NADPH and monitored cofactor oxidation at 340 nm. Data represents mean values of three replicates and error bars display the standard deviation. (B) TLC analysis of (i) riboflavin, (ii) flavin mononucleotide, (iii) extract from denatured NfsB, (iv) co-spot of flavin mononucleotdie and NfsB extract, and (v) co-spot of riboflavin and NfsB extract. Solvent consisted of 5:2:3 butanol:acetic acid:water and TLC was visualized under UV light. (C) Graph of normalized reaction velocities against pH with seven replicates and standard deviation. Outliers were removed by Grubbs test with $\alpha = 0.2$. (D) Graph of normalized Bratton-Marshall derivitizable compounds produced by reactions quenched after four minutes at the given temperature. Displayed are the mean and standard deviations of four replicates, with outliers removed by Grubbs test with $\alpha = 0.2$. See also figure 5.

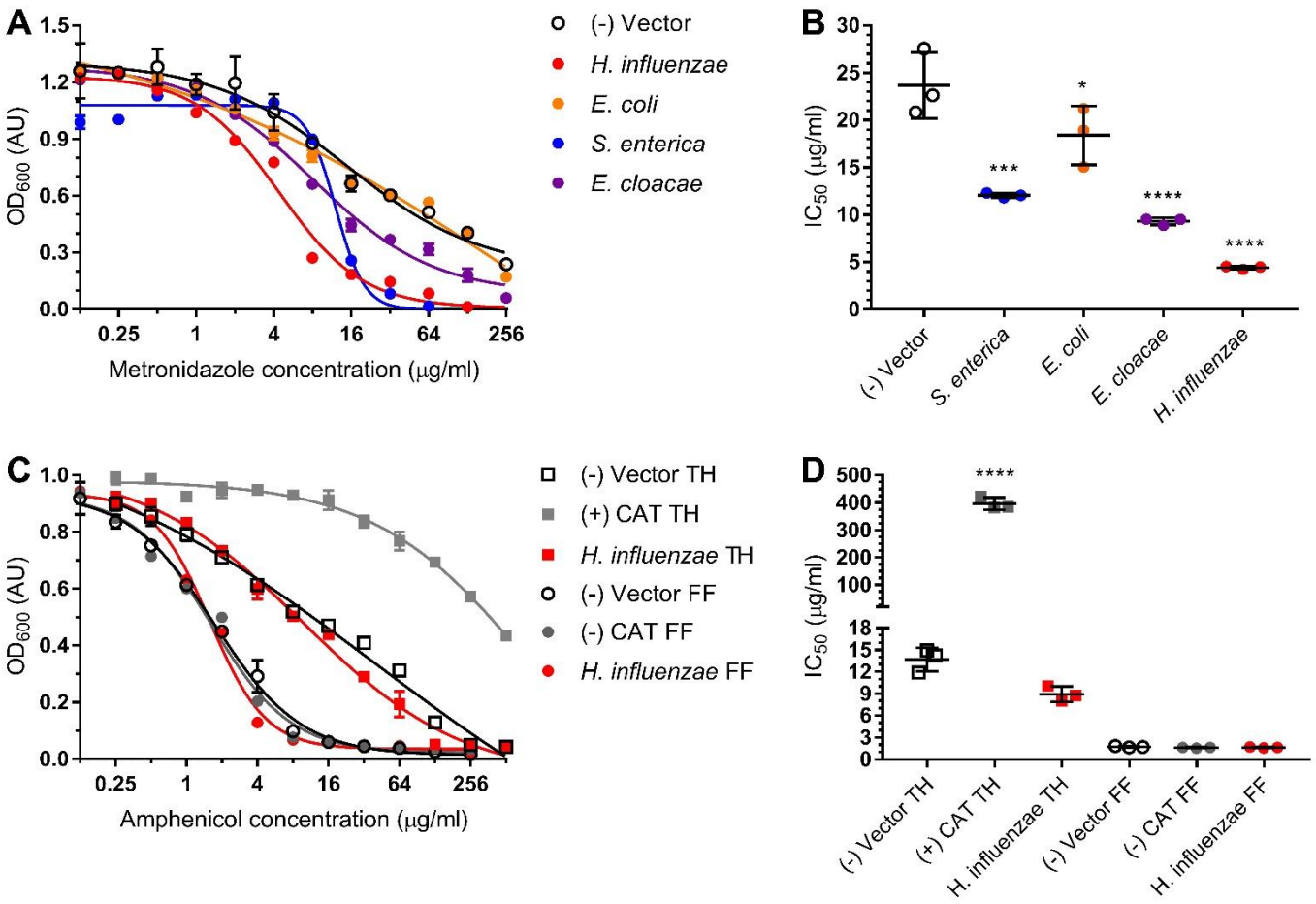


Supplemental figure 3. LCMS analysis of *H. influenzae NfsB* reactions. Related to Figure 5.
(A) LCMS extracted ion count analysis (291 m/z, corresponding to amino-chloramphenicol) of representative reaction (red) compared to amino-chloramphenicol standard (black). (B-C) Multiple-reaction monitoring (MRM) LCMS of (B) amino-chloramphenicol standard and (C) reaction product with the indicated precursor and product ions (*e.g.* 291 m/z precursor, 86 m/z product). See also figure 5.



Supplemental figure 4. Alternative methods for following the *H. influenzae* NfsB reduction of chloramphenicol. Related to Figure 6.

(A) Bratton-Marshall detection of aryl amines of *H. influenzae* NfsB reaction with 1 mM, 0.333 mM, 0.111 mM, and 0.037 mM chloramphenicol after 1 minute, 2 minutes, 3 minutes, and 4 minutes. (B) UV-vis spectra of 1 mM solutions of chloramphenicol, thiamphenicol, and florfenicol in 50 mM Tris HCl at pH 8. The dotted vertical line corresponds to 281 nm. Reaction monitoring for (C) oxidation of NADPH to NADP or (D) reduction of the chloramphenicol nitro group in full enzyme reactions (red), and reactions individually lacking enzyme (black), chloramphenicol (blue) or NADPH (teal). See also figure 6.



Supplemental figure 5. Reductase expression alters susceptibility of *E. coli* to metronidazole but not nitro-lacking amphenicols. Related to figures 2 and 7.

(A) Dose-response curves of microbroth dilution assays for *E. coli* expressing predicted reductase gene homologs in the presence of chloramphenicol with (B) corresponding 50% inhibitory concentrations (IC_{50}) calculated from the curve fit. All points are averages of triplicate experiments with standard deviation error bars. Statistical significance was calculated with respect to the vector control by ordinary one-way ANOVA with Dunnett's correction for multiple comparisons. Adjusted p-value displayed as $p \leq 0.0001$ (****), $p < 0.001$ (***), and $p < 0.05$ (*). Abbreviation: CAT, chloramphenicol acetyltransferase. See also figure 2 and figure 7.