Nitric oxide disrupts bacterial cytokinesis by poisoning purine metabolism

Jessica Jones-Carson1,2, Atsushi Yahashiri3, Ju-Sim Kim1, Lin Liu1, Liam F. Fitzsimmons1, David S. Weiss3*, Andrés Vázquez-Torres1,4*

INTRODUCTION

Nitric oxide (NO) is an essential effector of the host response against diverse eukaryote and prokaryote pathogens (1). NO is bactericidal against microorganisms such as Burkholderia pseudomallei that overwhelmingly rely on oxidative phosphorylation to satisfy their energetic requirements (2). However, bacteriostasis is the dominant manifestation of NO toxicity against most bacteria. The combined effects on oxidative phosphorylation, amino acid biosynthesis, central metabolism, and DNA replication and repair contribute to the profound growth arrest noted in bacteria undergoing nitrosative stress (3). Although cytokinesis is a crucial component of bacterial cell growth, it is currently unknown whether bacterial cell division is adversely affected during nitrosative stress. The first event in bacterial cell division is the guanosine 5′-triphosphate (GTP)–dependent assembly of a tubulin-like protein named FtsZ into a collection of short filaments that accumulate in a ring-like structure at the mid-cell (Fig. 1A) (4–6). This structure, called the “Z ring,” contains a handful of proteins such as the actin homolog FtsA that promote FtsZ assembly and link FtsZ filaments to the cytoplasmic membrane. After a lag period, the Z ring recruits more than 20 additional proteins, including the peptidoglycan (PG) synthase Ftsl and its regulators FtsQ and FtsN to form a mature structure called the “septal ring” or “divisome.” Movement of FtsZ filaments by treadmilling around the circumference of the cell guides the incorporation of new glycan strands (7–10), which, together with the processing of septal PG by a collection of cell wall hydrolases, ultimately produces two daughter cells. FtsZ treadmilling is fueled by GTP hydrolysis and occurs when new FtsZ-GTP monomers add to one end of a filament while FtsZ-GDP (guanosine 5′-diphosphate) monomers dissociate from the other. Turnover of septal FtsZ is rapid, with a t1/2 (half-time) of about 10 s (11). As a consequence, even existing divisomes soon disassemble if FtsZ polymerization is impeded (12), for example, in the sequestration of FtsZ monomers during the SOS response to DNA damage (13–15).

RESULTS

Exploratory experiments revealed that exposure of growing Escherichia coli cells to NO resulted in an almost immediate cessation of growth and division. Because NO treatment collapses the proton motive force (PMF) (16) and loss of the PMF causes rapid disassembly of FtsA in both Bacillus subtilis and E. coli (17), we tested whether disassembly of FtsA might explain how NO blocks division. To test this hypothesis, we grew an E. coli strain that expresses an ftsA-gfp fusion in LB-glycerol broth, fixed the cells with paraformaldehyde to preserve divisome architecture, and visualized green fluorescent protein (GFP) fluorescence by microscopy. As expected, about 50% of midlog-phase E. coli exhibited a fluorescent band at the midcell indicative of FtsA-GFP localization (Fig. 1, B and C). Addition of 50 μM of the PMF inhibitor carbonyl cyanide-3-chlorophenylhydrazone (CCCP) or 750 μM of the NO donor spermine NONOate before fixation reduced the fraction of cells exhibiting septal localization of FtsA-GFP to about 5% within 2 min (Fig. 1, B and C). Some of the fluorescent FtsA-GFP signal lost from the division site appeared as puncta at various locations around the cell. Rapid disassembly of FtsA-GFP was also observed after the addition of the alternative NO donor propylamine propylamine NONOate (250 μM) (PAPA NONOate) to midlog-phase E. coli grown in LB without glycerol (fig. S1A).

Similar experiments with an ftsZ-gfp strain revealed that NO destabilizes FtsZ (Fig. 1D and fig. S1A). Disappearance of Z rings was fast and lasted for at least 30 min (Fig. 1D). We verified disappearance of endogenous FtsZ rings in E. coli by immunofluorescence (fig. S1B) and demonstrated by Western blotting that NO does not simply cause proteolytic destruction of FtsZ or FtsZ-GFP (fig. S1C). NO appears to destroy dynamically active Z rings because fixing cells as little as 15 s before the addition of the NO donor prevented the NO-mediated destruction of Z rings (fig. S1D). Rapid disassembly of FtsZ and FtsA is expected to induce disassembly of the entire

1Department of Immunology and Microbiology, University of Colorado School of Medicine, Aurora, CO, USA. 2Division of Infectious Diseases, University of Colorado School of Medicine, Aurora, CO, USA. 3Department of Microbiology and Immunology, Carver College of Medicine, The University of Iowa, Iowa City, IA, USA. 4Veterans Administration Eastern Colorado Health Care System, Aurora, CO, USA. *Corresponding author. Email: andres vazquez torres@cuanschutz.edu (A.V.-T); david weiss@uiowa.edu (D.S.W.)
Fig. 1. Rapid disappearance of FtsA and FtsZ rings in *Escherichia coli* undergoing nitrosative stress. (A) Overview of *E. coli* cell division. Division starts with GTP-dependent assembly of the FtsZ ring, which is subject to both positive and negative regulation. (B) Representative micrographs of *E. coli* EC447 (ftsA-gfp) grown to midlog phase in LB-glycerol. Where indicated, the specimens were treated with 50 µM carbonyl cyanide-m-chlorophenylhydrazone (CCCP) or 750 µM spermine NONOate (sNO) for 2 min before fixation. The micrographs are representative of specimens from three experiments. (C) The percent of bacterial cells containing FtsA-GFP (green fluorescent protein) in septa or puncta was recorded in a total of 644 to 960 cells from three separate experiments. ***P < 0.001 compared to septa in control bacteria. (D) Organization of Z rings in FtsZ-GFP–expressing *E. coli* (EC448) treated for 1 to 30 min with 750 µM sNO. Untreated cells were used as controls.

Fig. 2. Effects of carbon source on early stages of cell division. (A and C) Bacterial growth was recorded by following optical density at 600 nm (OD₆₀₀) after the cultures were treated with PAPA NONOate (pNO) or CCCP at the indicated times (arrows). Representative micrographs of *E. coli* EC448 (ftsZ-gfp) or EC447 (ftsA-gfp) grown in MOPS–casamino acids (CAA) (B) or MOPS-glucose (GLC) (D) minimal media containing isopropyl-β-D-thiogalactopyranoside (IPTG) to induce the gfp fusions. Where indicated, cultures were treated with 250 µM pNO or 50 µM CCCP for 5 min before fixation. The data are representative of three experiments.
divisome, and we verified that this is indeed the case for ZipA, FtsQ, FtsI, and FtsN (fig. S1A). An interesting exception was DamX, which was retained at the division site during nitrosative stress. (fig. S1A). Follow-up studies suggest that DamX is retained by tight binding of its C-terminal sporulation-related repeat (SPOR) domain to septal PG (fig. S2A) (18, 19).

We extended these studies to two additional organisms: Salmonella enterica serovar Typhimurium, which is a close relative of E. coli, and B. subtilis, a Gram-positive species quite distantly related to E. coli. In Salmonella, both NO and CCCP destroyed Z rings within ~1 min (fig. S3A). In B. subtilis, CCCP delocalized FtsZ more gradually over a period of >10 min, as reported previously (17), whereas NO treatment destroyed B. subtilis Z rings in <2 min (fig. S3B). Collectively, these findings demonstrate that NO inhibits the earliest step in cell division in E. coli, Salmonella, and B. subtilis—assembly of FtsZ into a ring-like collection of dynamic filaments at the division site.

We next evaluated the mechanism by which NO inhibits FtsZ ring formation in E. coli. Initial experiments indicated that NO destroys Z ring formation independently of the SOS and stringent response programs or effects on the Min system (fig. S4). Insights into the mechanism by which NO arrests early steps in cell division came from manipulating energy metabolism by growing E. coli in glucose or casamino acids. Addition of 250 μM PAPA NONOate or 50 μM CCCP halted growth immediately regardless of the source of carbon and energy (Fig. 2, A and C), but at the level of divisome assembly, the outcomes were profoundly different. In casamino acids, PAPA NONOate and CCCP delocalized FtsZ and FtsA rapidly (Fig. 2B), similar to what was observed in LB, but in glucose, both proteins persisted at the division site for at least 20 min (Fig. 2D and figs. S5 and S6). NO also failed to delocalize FtsA when E. coli was grown in an alternative glucose minimal medium based on E salts (fig. S7). These findings were extended to ZipA, FtsQ, FtsI, FtsN, and DamX (fig. S5, A and B). FtsN localization was less sensitive to NO challenge in MOPS–casamino acids than LB, possibly reflecting differences in the abundance or turnover of denuded glycans. We conclude that glycolytic metabolism makes the divisome largely immune to nitrosative stress despite marked repression of bacterial cell growth and cessation of division.

Glucose fuels growth largely by generating adenosine 5′-triphosphate (ATP) via substrate-level phosphorylation at the payoff phase of glycolysis and fermentation of pyruvate to acetate, whereas carbon from amino acids supports growth by generating reducing power in the tricarboxylic acid (TCA) cycle that powers oxidative phosphorylation at the respiratory chain (20). The addition of 250 μM PAPA NONOate

---

**Fig. 3. Carbon source modulates the energetics of bacterial cells undergoing nitrosative stress.** Effect of NO on respiration ([A] and PMF ([B] of E. coli grown in MOPS-GLC or MOPS-CAA. O2 consumption was measured polarographically, whereas the PMF was estimated fluorometrically by the accumulation of 3,3′-dipropylthiadicarbocyanine iodide (DiSC3(5)). Selected samples were treated for 5 min with 250 μM pNO, 750 μM sNO, or 50 μM CCCP. ****P < 0.0001 versus untreated controls, as calculated by one-way analysis of variance (ANOVA). A.U., arbitrary units. ([C]) Thin-layer chromatography (TLC) analysis of nucleoside triphosphates (NTPs) extracted from E. coli cells treated with sNO, pNO, or CCCP for 5 min. UTP, uridine 5′-triphosphate; CTP, cytidine 5′-triphosphate; PPi, inorganic pyrophosphate; ppGpp, guanosine 5′-diphosphate 3′-diphosphate. ([D]) ATP was measured in cytoplasmic extracts by luciferase-dependent chemiluminescence. ****P < 0.0001 versus untreated controls, as calculated by one-way ANOVA. ([E]) Localization of FtsZ-GFP was evaluated by fluorescence microscopy. **P < 0.001 versus wild-type (WT) control (Ctrl). The data in (A) are representative of three independent experiments, whereas the data in (B), (D), and (E) are means ± SD from four independent observations. Strain EC448 was used for (A), (C), and (D). Strain MC4100 was used for (B). Strains EC3749 and EC3751 were used for (E). ns, not significant.
inhibited most aerobic respiration in \textit{E. coli}, regardless of whether the bacteria were grown in glucose or casamino acids (Fig. 3A), suggesting that the inability of NO to destroy Z rings in glucose-grown \textit{E. coli} cannot be explained by differences in respiratory activity. Moreover, CCCP and two NO donors depolarized the cytoplasmic membrane to similar extents regardless of whether \textit{E. coli} was grown in glucose or casamino acids (Fig. 3B). One implication of our findings is that FtsA localization tolerates a substantial drop in PMF, in contrast to an earlier report that septal localization of FtsA requires a membrane potential (17). Further studies will be needed to understand this discrepancy because our experiments and the previous study used different strains and growth conditions.

FtsZ and FtsA use GTP and ATP, respectively [reviewed in (4–6, 21)]. As shown in \textit{Salmonella} (20), it is possible that the differential effect that NO has on Z ring formation in \textit{E. coli} grown in glucose or casamino acids could stem from different degrees of ATP and GTP depletion.

---

**Fig. 4. Inositol monophosphate dehydrogenase is a target of NO-mediated cytostasis.** (A) Total GuaB protein in \textit{E. coli} MC4100 expressing pWSK29:guaB-3xFLAG (strain AV17035) grown in MOPS-GLC or MOPS-CAA was measured by Western blot. Where indicated (+), the cells were treated with 250 \( \mu \)M pNO. The DnaK chaperone was used as internal control. GuaB/DnaK ratios were calculated by densitometry. (B) The redox state of GuaB cysteine residues was determined by Western blotting after alkylation of thiol groups with AMS. Oxidized cysteine residues are not amenable to derivatization with AMS, and thus, the higher the number of reduced cysteine residues in GuaB, the higher the molecular weight. (C) IMPDH enzymatic activity in log-phase \textit{E. coli} grown in MOPS-GLC or MOPS-CAA media. Some cultures were treated with increasing concentrations of pNO for 5 min before determination of enzymatic activity. IMPDH enzymatic activity, corrected for protein concentrations, is expressed as mean ± SD; \( n = 4 \). (D) IMPDH activity of recombinant GuaB protein after treatment with pNO. (E) TLC analysis of nucleotides extracted from \textit{E. coli} MC4100 expressing pBAD18 (strain AV17086) or pGUAB (strain AV17072) grown in MOPS supplemented with CAA and 0.2% \( \text{l-} \)arabinose. Bacteria were treated with 0 to 100 \( \mu \)M pNO for 1 min before extraction. Autoradiogram is representative of three independent experiments. (F) Quantification of the GTP pool by ImageJ analysis (\( N = 3 \)). (G) The localization of FtsZ-GFP in log-phase \textit{E. coli} EC448 grown in MOPS-CAA media containing 10 \( \mu \)M IPTG and 0.2% \( \text{l-} \)arabinose. Where indicated, the specimens were treated with 150 \( \mu \)M pNO. The micrographs are representative of specimens from four to six experiments. (H) The percentage of cells with FtsZ rings was quantified from cells imaged in (G). (I) Model for NO-dependent inhibition Z ring formation in respiring \textit{E. coli}. Nitrosylation of quinol oxidases (\textit{Qnl Ox}) poisons respiration, collapses the PMF, and prevents ATP synthesis via oxidative phosphorylation and, thus, conversion of GDP to GTP. Concomitantly, NO inhibits GTP synthesis by modifying cysteine residues in IMPDH, the first committed step for de novo GTP synthesis. ***\( P < 0.001 \); ****\( P < 0.0001 \).
To test this idea, we labeled nucleotides with $^{32}$P orthophosphate in *E. coli* grown in MOPS minimal media supplemented with glucose or casamino acids. Treatment of *E. coli* grown in casamino acids with 250 $\mu$M PAPA NONOate, 750 $\mu$M spermine NONOate, or 50 $\mu$M CCCP resulted in a profound depletion of uridine 5′-triphosphate, cytidine 5′-triphosphate, ATP, and GTP (Fig. 3C). In contrast, glucose-grown *E. coli* challenged with NO or CCCP retained more ATP and GTP than controls grown in casamino acids (Fig. 3C). Independent measurements of intracellular ATP using firefly luciferase confirmed the thin-layer chromatography (TLC) findings (Fig. 3D). Cumulatively, our investigations support the idea that the effects of NO on the nucleotide pool depend on the pathway generating the ATP (20) and raise the interesting possibility that NO inhibits FtsZ ring formation by blocking nucleoside triphosphate (NTP) biosynthesis. Consistent with this hypothesis, NO did not destroy Z rings in a ΔnusA ΔnudG E. coli strain that lacks both NASH dehydrogenases of the electron transport chain and is therefore forced to fulfill most energy requirements through fermentation, even when amino acids serve as the source of carbon and energy (Fig. 3E).

Nitrosylation of quinol oxidases and repression of aerobic respiration deplete cells of ATP directly and of GTP indirectly since GTP is derived by transfer of the γ-phosphate from ATP to GDP. In addition, NO treatment could impair de novo GTP biosynthesis by inactivating guaB-encoded inosine 5′-monophosphate dehydrogenase (IMPDH). This enzyme catalyzes the first step of de novo biosynthesis of guanine nucleotides and has a catalytically active cysteine residue that can be oxidized by hypochlorous acid (22). We therefore investigated the potential for IMPDH to contribute to GTP depletion during nitrosative stress. Western blot analysis showed that glucose-grown *E. coli* contain about seven times more IMPDH protein than controls grown in casamino acids (Fig. 4A). Wild-type and ΔnudG E. coli grown in MOPS–casamino acids media expressed similar levels of GuaB (Fig. 5A). Derivatization of cysteine residues with the thiol reagent 4-acetamido-4′-maleimidylstilbene-2,2′-disulfonic acid (AMTS) in *E. coli* 1 min after NO treatment showed modifications of IMPDH cysteine residues (Fig. 4B). IMPDH enzymatic activity was inhibited in vivo after the addition of 250 $\mu$M PAPA NONOate to *E. coli* grown in LB broth (Fig. 5B). Extracts from glucose grown cells had about two times more IMPDH enzyme activity compared to controls grown in casamino acids (Fig. 4C). Moreover, E. coli grown in casamino acids contained substantially less IMPDH after NO treatment than controls grown in glucose (Fig. 4C). NO inhibited the IMPDH enzymatic activity of recombinant protein in a concentration-dependent manner (Fig. 4D and fig. S8, C and D).

If inhibition of IMPDH contributes to depletion of GTP pools during nitrosative stress, then overproduction of IMPDH may protect against NO-dependent inhibition of FtsZ assembly. To test this possibility, we cloned *guaB* into an arabinose-inducible pBAD expression plasmid. *E. coli* expressing pGUAB contained higher basal levels of GTP than controls harboring the pBAD empty vector (Fig. 4, E and F). Addition of NO reduced the amount of GTP and destroyed the Z rings in both strains; however, pGUAB-expressing cells retained higher levels of GTP and more Z rings (~35% versus ~15% of the cells) after NO treatment than controls bearing the pBAD vector (Fig. 4, G and H). Induction of pGUAB also increased the amount of ATP, whereas the concentration of pyrophosphate was consistently decreased (fig. S8, E and F). These data demonstrate that IMPDH expression levels are inversely associated with the susceptibility of Z rings to NO.

DISCUSSION

Our investigations have identified inhibition of NTP biosynthesis as the molecular mechanism by which NO destroys early steps in bacterial cell division. NO causes rapid disassembly of FtsZ rings by inhibiting nucleoside biosynthesis via dissipation of the PMF and poisoning of IMPDH in de novo purine biosynthesis (Fig. 4I). The two mechanisms work cumulatively, so impairing them individually has less of an effect on GTP pools and early steps in cell division than impairing both pathways simultaneously. A constant supply of GTP is required to sustain the divisome because FtsZ polymers turn over very rapidly as they traverse the division site by virtue of their guanosine triphosphatase–dependent remodeling activity (7–11). NO does not destroy early steps of cell division in glycolytic cells because these bacteria obtain a sizable fraction of their purine nucleosides by favoring substrate-level phosphorylation and expressing enhanced IMPDH levels. Analogous glycolytic adaptations may provide a continuous supply of NTPs for undeterred growth of tumor cells despite a prevailing nitrosative environment (23). However, glycolysis is not sufficient to prevent growth arrest in bacteria undergoing nitrosative stress. The depolarization induced by NO in glycolytic cells directly interferes with multiple processes that depend on the PMF, including proper localization of some morphogenic proteins (17), translocation of PG precursors across the cytoplasmic membrane by MtuJ (24), and invagination of the outer membrane by the Tol-Pal complex (25, 26).

MATERIALS AND METHODS

**Media**

The growth media used for this study include lysogeny broth, LB (27), MOPS minimal media (28), and E salts [MgSO$_4$ (0.2 g/liter), Ca$_3$H$_7$O$_7$·H$_2$O (0.2 g/liter), K$_2$HPO$_4$ (10 g/liter), and Na(NH)$_2$PO$_4$·4H$_2$O (3.5 g/liter)]. Plates contained agar at 15 g/liter. Where indicated, LB was supplemented with 0.4% glycerol, and minimal media were supplemented with 0.4% d-glucose or 0.4% casamino acids. Antibiotics were used at the following concentrations: ampicillin, 200 $\mu$g/ml for plasmids and 25 $\mu$g/ml for chromosomal alleles; kanamycin at 40 $\mu$g/ml; and spectinomycin, 100 $\mu$g/ml for plasmids and 35 $\mu$g/ml for chromosomal alleles. CCCP was obtained from Sigma-Alrich and used at 50 or 100 $\mu$M. The NO donors spermine NONOate and propylene propylamine NONOate were from Cayman Chemicals (Ann Arbor, MI) and used at 750 $\mu$M and 100 to 250 $\mu$M, respectively. In some experiments, isopropyl β-D-1-thiogalactopyranoside (IPTG), L-arabinose, D-glucose, or D-xylene was used to modulate gene expression at concentrations indicated below.

**Strains and plasmids**

Table S1 lists all strains used in this study and, in the case of newly constructed strains, described how they were obtained. Plasmid transformation, P1-mediated transduction, chromosomal integration of plasmids using λ InCh or CRIM methods, and eviction of antibiotic cassettes by using pCP20 were done according to published procedures (29–31). Table S2 lists plasmids and DNA oligonucleotides used in this study. The construction of new plasmids is described below. Constructs made using polymerase chain reaction (PCR) were confirmed by DNA sequencing. Plasmid pDSW238 [P$_{204}$::gfp-ftsN, Amp$^\beta$] was constructed by PCR amplification of ftsN from pJC2 (32) using primers GFP-N-5′ and GFP-N-3′. The 988-base pair (bp) product was digested with Eco RI and Hind III and ligated into the same sites.
of pDSW207 (33). To construct pDSW1377 [P204::Tgfp-damXspoR, q80att, SpcR], plasmid pDSW997 (34) was digested with Sph I and Sca I. The 3452-bp fragment that carried lacF and P204::Tgfp-damXspoR was ligated into pIC69 (32) that had been cut with Sph I and Hinc II. Plasmid pDSW1379 [P204::Tgfp-ftsQspoR, q80att, SpcR] was constructed similarly using a 3421-bp Sph I–Sca I fragment from pDSW992 (34). Plasmid pDSW1856 [P204:ftsZ-gfp, att, KanR] is a 3.4-kb PCR product encoding lacF and P204:ftsZ-gfp, was amplified from plasmid pDSW230 (33) using primers P2150 and P2151. Gibson assembly was used to insert the PCR product pAH120 that had digested with Sph I and Nhe I. Plasmid pGUAB [Pbad-guaB-3xFLAG, AmpR] was constructed by PCR amplification of guaB from E. coli strain MC4100 using primers pGUAW FB and pGUAB REV. The resulting 1549-bp product was digested with Nde I and Xho I and ligated into the same sites of pBAD18 (35). Plasmid pWSK29::guaB-3xFLAG (AmpR) was constructed by PCR amplification of guaB together with its native promoter using primers pWSK29 guaB FWD and pWSK29 guaB REV. The PCR template was MC4100 chromosomal DNA. The 1821-bp product was digested with Xho I and Bam HI and ligated into the same sites of pWSK29 (36). Plasmid pET-22b::guaB [Petr::guaB-His6, AmpR] was constructed using primers pET-22b gauB REV and pET-22b gauB FWD to amplify gauB from MC4100 chromosomal DNA. The 1481-bp product was digested with Nde I and Xho I and then ligated into the same sites of pET-22b (+). A derivative of pET-22b::guaB that produces inactive GuaB protein was constructed using site-directed mutagenesis to change codon 305 from Cys to Ala. The guaB that produces inactive GuaB protein was constructed using 22b:: DNA. The 1481-bp product was digested with Nde I and Xho I and the primers pBAD-guaB FWD and pBAD-guaB REV. The resulting 1549-bp product was digested with Nde I and Xho I and ligated into the same sites of pBAD18 (35). Plasmid pWSK29::guaB-3xFLAG (AmpR) was constructed by PCR amplification of guaB together with its native promoter using primers pWSK29 guaB FWD and pWSK29 guaB REV. The PCR template was MC4100 chromosomal DNA. The 1821-bp product was digested with Xho I and Bam HI and ligated into the same sites of pWSK29 (36). Plasmid pET-22b::guaB [Petr::guaB-His6, AmpR] was constructed using primers pET-22b gauB REV and pET-22b gauB FWD to amplify gauB from MC4100 chromosomal DNA. The 1481-bp product was digested with Nde I and Xho I and then ligated into the same sites of pET-22b (+). A derivative of pET-22b::guaB that produces inactive GuaB protein was constructed using site-directed mutagenesis to change codon 305 from Cys to Ala. The mutation was introduced using PfDNA polymerase (QuikChange II Kit, Agilent, Santa Clara, CA) and the primers gauB C305A FWD and gauB C305A REV.

**Assays for septal localization of GFP fusion proteins**

*E. coli* gfp fusion strains were grown overnight at 30° or 37°C in LB broth supplemented with ampicillin (25 μg/ml) or spectinomycin (35 μg/ml). The bacteria were subcultured 1:200 in LB or LB glycerol broth, and the expression of the gfp fusion was induced with IPTG: 2.5 μM for gfp-ftsN and gfp-ftsI; 5 μM for ftsZ-gfp and gfp-ftsQ; 10 μM for gfp-damX; 50 μM for zapA-gfp; and 25 to 100 μM for ftsA-gfp. Cultures were grown in a shaker incubator at 30° or 37°C to an optical density at 600 nm (OD600) of 0.2 to 0.4, at which time 100 to 250 μM PAPA NONOate, 750 μM spermine NONOate, or 50 to 100 μM CCCP was added to selected cultures and incubation was continued. At various times, the cells were fixed directly in growth medium as described (37). The specimens were refrigerated until visualization. The same procedures were used to assess localization in minimal media except that overnight cultures were diluted 1:200 into MOPS-glucose or MOPS-casamino acids containing IPTG. It was not necessary to supplement MOPS-glucose with amino acids because there was sufficient carryover from the LB.

These procedures were modified slightly to assess colocalization of FtsZ-mCherry and TGF-SPOR fusions. Overnight cultures contained chloramphenicol (30 μg/ml) and spectinomycin (35 μg/ml). Cultures were diluted 1:200 into LB containing chloramphenicol (30 μg/ml) and 50 μM IPTG. After 90 min, expression of ftsZ-mCherry was induced transiently by adding 1-aminobasine to 0.2% final concentration, followed 1 min later by d-glucose to 0.2% final concentration. Incubation was continued for about 1 hour to OD600 = 0.3, at which time 250 μM PAPA NONOate was added. Samples were fixed at various times.

In Salmonella, the ftsA-gfp fusion was induced with 100 μM IPTG. In *B. subtilis*, the ftsZ-gfp fusion was induced with 10 μM IPTG, and the yfp-zapA fusion was induced with 5 mM xylose.

**Immunolocalization of FtsZ**

Bacterial cultures growing in LB were fixed and processed for immunofluorescence as described (37), either immediately before or 10 min after addition of 250 μM PAPA NONOate. Lysozyme-permeabilized cells were incubated overnight at 4°C with a 1:5000 dilution of anti-FtsZ serum from rabbit HM969. The secondary antibody was goat anti-rabbit conjugated to Alexa Fluor 488 diluted 1:500 and incubated for 1 hour at room temperature.

**Microscopy**

In the laboratory of D.S.W., cells were immobilized using thin agarose pads (1%). Phase-contrast and fluorescence micrographs were recorded on an Olympus BX60 microscope equipped with a 100× UPlanApo objective (numerical aperture, 1.35). Filter sets for fluorescence imaging were from Chroma Technology Corp. (Battleboro, VT). The GFP filter set comprised a 450- to 490-nm excitation filter, a 495-nm dichroic mirror (long pass), and a 500- to 550-nm emission filter. The red fluorescent protein (RFP) filter set comprised a 538- to 582-nm excitation filter, a 595-nm dichroic mirror (long pass), and a 582- to 682-nm emission filter. Micrographs were captured with a Hamamatsu ORCA-Flash4.0 V2+ complementary metal-oxide semiconductor camera. Typical exposure times for GFP and RFP were 1 s. Adobe Illustrator or Olympus cellSens Dimension software were used to adjust image brightness and contrast, to crop images, and to assemble images into figures.

In the laboratory of A.Y.-T., small aliquots of fixed cells were dried onto Fisherbrand Superfrost Plus microscope slides to adhere the cells. Just before examination, the specimens were rehydrated and mounted with a coverslip. The distribution of fluorescently tagged proteins in the bacterial cells was examined with a Zeiss Axioplan 2 epifluorescence upright microscope. The images were captured digitally with a Cooke Sensicam QE high-resolution (1376 × 1024 resolution) black and white supercooled charge-coupled device camera using the SlideBook version 4.1.0 software (Intelligent Imaging Innovations Inc., Denver, CO). The digital images were assigned colors by the software interface.

**Detection of FtsZ by immunoblotting**

To assess the effect of nitrosative stress on FtsZ and FtsZ-GFP abundance, bacterial cultures growing in LB were harvested immediately before or 10 min after addition of 250 μM PAPA NONOate. Samples were processed as described (34), except that the primary antibody was either a 1:10,000 dilution of anti-FtsZ or a 1:1000 dilution of anti-GFP sera.

**Detection of GuaB by immunoblotting**

*E. coli* expressing gauB::3xFLAG from the low-copy plasmid pWSK29 were grown in LB at 37°C overnight and resuspended in MOPS medium supplemented with glucose or casamino acids. The cells were treated with 250 μM PAPA NONOate at an OD600 of 0.4 to 0.5. After 5 min of treatment at 37°C in a shaker incubator, the bacteria were harvested for Western blot analysis. Cells were disrupted by sonication, and protein concentrations were determined using bicinchoninic acid (BCA; Thermo Fisher Scientific). Samples were separated on 10% SDS–polyacrylamide gel electrophoresis (SDS-PAGE) gels, and the...
proteins were transferred onto nitrocellulose membranes by electro-transfer. Blots were probed with anti-FLAG monoclonal antibody (Sigma-Aldrich, Burlington, MA), followed by horseradish peroxidase (HRP)–conjugated anti-mouse immunoglobulin G (IgG) antibodies (GE Healthcare). Immunoblots were developed as recommended using the ECL Prime Western blotting detection system (GE Healthcare) and imaged with a ChemiDoc XRS imaging system (Bio-Rad, Hercules, CA). As a control, DnaK was detected with anti-DnaK monoclonal antibodies (MBL International, Woburn, MA), followed by HRP-conjugated anti-mouse IgG (GE Healthcare).

Evaluation of GuaB oxidation by alklylation with AMS
To determine in vivo thiol modification of GuaB cysteine residues, gauB::3xFLAG-expressing E. coli were grown in LB at 37°C overnight and diluted 1:100 in MOPS-glucose or MOPS–casamino acid media. Selected cultures were treated at midlog phase with 250 μM PAPA NONOate at 37°C for 5 min. Bacteria were treated with 15% TCA, and the pellets were resuspended in AMS buffer [1 M tris-HCl (pH 8.0), 1 mM EDTA, 0.1% SDS, and 15 mM AMS (Thermo Fisher Scientific)]. Samples were incubated at 37°C for 1 h in the dark and loaded onto nonreducing 6% SDS–PAGE gels (Bio-Rad, Hercules, CA). GuaB-3xFLAG proteins were detected by immunoblot analysis as described above.

Overexpression and purification of IMPDH
IMPDH was overproduced from a T7 vector in E. coli BL21 (DE3) and purified as follows. Cells grown in LB at 37°C to an OD600 of 0.5 to 0.7 were treated with 0.1 mM IPTG. After 3 h, the cells were harvested, disrupted by sonication, and centrifuged to obtain cell-free supernatants. 6His-tagged fusion proteins were purified using TALON metal affinity chromatography (Clontech, Mountain View, CA) according to the manufacturer’s protocols. Purified GuaB proteins were aliquoted inside a BACTRON anaerobic chamber (Shel Lab, Cornelius, OR). The purity and mass of the recombinant proteins were assessed by SDS–PAGE.

IMPDH activity
IMPDH enzyme activity was measured in E. coli grown in LB broth or MOPS-glucose or MOPS–casamino acids media. Cells cultured in LB overnight at 37°C were diluted 1:100 in MOPS media supplemented with 0.4% casamino acids. Where indicated, midlog-phase bacteria were treated with increasing concentrations of PAPA NONOate for 5 min with shaking, harvested by centrifugation, and resuspended in 100 mM tris–HCl (pH 8.0) and 10 mM KCl buffer. Cells were disrupted by sonication, and 20 μl was used to measure IMPDH activity. Protein concentrations were determined using BCA (Thermo Fisher Scientific). To determine in vitro IMPDH activity with recombinant GuaB, selected samples were pretreated with PAPA NONOate for 30 min at 37°C, and then, IMPDH activity was measured, as described previously (38). The reaction was performed in 100 mM tris–HCl (pH 8.0), 10 mM KCl, 1 mM NAD (Sigma-Aldrich, Burlington, MA), and 1 mM IMP (Sigma-Aldrich), with appropriate amounts of samples at 37°C. IMPDH activity was monitored by following the formation of xanthine monophosphate (ε = 5.4 mM−1 cm−1) at 290 nm using a Cary 50 Bio ultraviolet spectrophotometer (Agilent).

O2 consumption
E. coli were grown overnight in LB-glycerol broth at 37°C with shaking. Overnight cultures were diluted to an OD600 of 0.5 in a volume of 1 ml in MOPS minimal media containing either 0.4% glucose or 0.4% casamino acids. Where indicated, 100 to 250 μM PAPA NONOate was added to the bacterial cultures grown to an OD600 of 0.5. The samples were transferred to a multiport temperature-controlled chamber, and the consumption of O2 by the bacteria was measured over a 75-s period using an ISO-OXY-2 O2 sensor attached to an APOLLO 4000 free radical analyzer. To ensure the even distribution of gases in the chamber, the samples were constantly stirred. The data are expressed as micromolar of O2.
linear regression of ATP standards. Intracellular ATP concentrations of the original samples were calculated using the colony-forming units per microliter counts assuming a cell volume of 1 fl.

Statistical analysis
The data were analyzed using a Student’s paired t test. Determination of statistical significance between multiple comparisons was achieved using one- or two-way analysis of variance (ANOVA) followed by a Bonferroni post-test. Data were considered statistically significant when \( P < 0.05 \).

SUPPLEMENTARY MATERIALS
Supplemental material for this article is available at http://advances.sciencemag.org/cgi/content/full/6/9/eaaz0260/DC1

References
1. C. Nathan, M. U. Shlomo, Reactive oxygen and nitrogen intermediates in the relationship between mammalian hosts and microbial pathogens. Proc. Natl. Acad. Sci. U.S.A. 97, 8841–8848 (2000).
2. J. Jones-Carlson, J. R. Laughlin, A. L. Stewart, M. I. Voskuil, A. Vázquez-Torres, Nitric oxide–dependent killing of aerobic, anaerobic and persistent Burkholderia pseudomallei. Nitric Oxide 27, 25–31 (2012).
3. F. C. Fang, A. Vázquez-Torres, Reactive nitrogen species in host–bacterial interactions. Curr. Opin. Immunol. 60, 96–102 (2019).
4. D. P. Haeusser, W. Margolin, Spilvictule: Structural and functional insights into the dynamic bacterial Z ring. Nat. Rev. Microbiol. 14, 305–319 (2016).
5. T. den Blaauwen, L. W. Hamoen, P. A. Levin, The divisome at 25: The road ahead.
6. S. Du, J. Lutkenhaus, Assembly and activation of the Escherichia coli divisome. Mol. Microbiol. 105, 177–187 (2017).
7. A. W. Bisson-Filho, Y.-P. Hsu, G. R. Squyres, E. Kuru, F. Wu, C. Jukes, Y. Sun, C. Dekker, S. Holden, M. S. VanNieuwenhze, Y. V. Brun, E. C. Garner, Treadmilng by FtsZ filaments drives peptidoglycan synthesis and bacterial cell division. Science 355, 739–743 (2017).
8. S. X. Yang, Z. Lyu, A. Miguel, R. McQuillen, K. C. Huang, J. Xiao, GTPase activity–coupled treadmilng of the bacterial tubulin FtsZ organizes septal cell wall synthesis. Science 355, 744–747 (2017).
9. J. M. Monteiro, A. R. Pereira, N. T. Reichmann, B. M. Saraiva, P. F. Fernandes, H. Veiga, A. Tavares, M. Santos, M. T. Ferreira, V. Macário, M. S. VanNieuwenhze, S. R. Filipe, M. G. Pinho, Peptidoglycan synthesis drives an FtsZ-treadmilng-independent step of cytokinesis. Nature 554, 528–532 (2018).
10. A. J. Perez, V. Cesbron, S. L. Shaw, J. Bazan Villicana, H.-C. T. Tsui, M. J. Boersma, Z. A. Ye, Y. Tovpeko, C. Dekker, S. Holden, E. M. Winkler, Movement dynamics of divisome proteins and PBP2x FtsW in cells of Streptococcus pneumoniae. Proc. Natl. Acad. Sci. U.S.A. 116, 3211–3220 (2019).
11. D. E. Anderson, F. J. Gueiros-Filho, H. P. Erickson, Assembly dynamics of FtsZ rings in Bacillus subtilis and Escherichia coli and effects of FtsZ-regulating proteins. J. Bacteriol. 186, 5775–5781 (2004).
12. L. Romberg, P. A. Levin, Assembly dynamics of the bacterial cell division protein FTSZ: Pooled at the edge of stability. Annu. Rev. Microbiol. 57, 125–154 (2003).
13. A. Mukherjee, C. Cao, J. Lutkenhaus, Inhibition of FtsZ polymerization by SuA, an inhibitor of septation in Escherichia coli. Proc. Natl. Acad. Sci. U.S.A. 95, 2885–2890 (1998).
14. Y. Chen, S. L. Milam, H. P. Erickson, SuA inhibits assembly of FtsZ by a simple sequestration mechanism. Biochemistry 51, 3100–3109 (2012).

J. Jones-Carson et al., Sci. Adv. 2020; 6 : eaaz0260 26 February 2020
41. J. M. Schapiro, S. J. Libby, F. C. Fang, Inhibition of bacterial DNA replication by zinc mobilization during nitrosative stress. Proc. Natl. Acad. Sci. U.S.A. 100, 8496–8501 (2003).

42. E. Bi, J. Lutkenhaus, Cell division inhibitors SulA and MinCD prevent formation of the FtsZ ring. J. Bacteriol. 175, 1118–1125 (1993).

43. M. A. De Groote, E. H. Robinson, J. Löwe, Crystal structure of the SOS cell division inhibitor SulA and in complex with FtsZ. Proc. Natl. Acad. Sci. U.S.A. 100, 7889–7894 (2003).

44. S. Pichoff, J. Lutkenhaus, Escherichia coli division inhibitor MinCD blocks septation by preventing Z-ring formation. J. Bacteriol. 183, 6630–6635 (2001).

45. V. W. Rowlett, W. Margolin, The bacterial Min system. Curr. Biol. 23, RS53–RS56 (2013).

46. C. Chimerel, C. M. Field, S. Piñero-Fernandez, U. F. Keyser, D. K. Summers, Indole prevents Escherichia coli cell division by modulating membrane potential. Biochim. Biophys. Acta 1818, 1590–1594 (2012).

47. X. Duan, J. Yang, B. Ren, G. Tan, H. Ding, Reactivity of nitric oxide with the [4Fe–4S] cluster of dihydroxyacid dehydratase from Escherichia coli. Biochem. J. 417, 783–789 (2009).

48. A. R. Richardson, E. C. Payne, V. C. Thomas, L. A. Becker, W. W. Navarre, M. E. Castor, S. J. Libby, F. C. Fang, Multiple targets of nitric oxide reprograms nucleotide metabolism in its adaptation to nitrosative stress. Mol. Syst. Biol. 9, e00211–e00218 (2018).

49. S. Korshunov, J. A. Imlay, Detection and quantification of superoxide formed within the periplasm of Escherichia coli. J. Bacteriol. 188, 6326–6334 (2006).

50. H. Xiao, M. Kalman, K. Ikehara, S. Zemel, G. Glaser, M. Cashel, Residual guanosine 3’5’-bispyrophosphate-synthetic activity of relA null mutants can be eliminated by spoT null mutations. J. Biol. Chem. 266, 5980–5990 (1991).

51. D. Hanahan, Studies on transformation of Escherichia coli with plasmids. J. Mol. Biol. 166, 557–580 (1983).

52. J. C. Chen, D. S. Weiss, J.-M. Ghigo, J. Beckwith, Septal localization of FtsQ, an essential cell division protein in Escherichia coli. J. Bacteriol. 181, 521–530 (1999).

53. J.-M. Ghigo, D. S. Weiss, J. C. Chen, J. C. Yarrow, J. Beckwith, Localization of FtsL to the Escherichia coli septal ring. Mol. Microbiol. 31, 725–737 (1999).

54. K. B. Williams, A. Yahashiri, S. J. R. Arends, D. L. Popham, C. A. Fowler, D. S. Weiss, Nuclear magnetic resonance solution structure of the peptidoglycan-binding SPOR domain from Escherichia coli DamX: Insights into septal localization. Biochemistry 52, 627–639 (2013).

55. T. Baba, T. Ara, M. Hasegawa, Y. Takai, Y. Okumura, M. Baba, K. A. Datsenko, M. Tomita, B. L. Warner, H. Mori, Construction of Escherichia coli K-12 in-frame, single-gene knockout mutants: The Keio collection. Mol. Syst. Biol. 2, 641–649 (2006).

56. J. E. Peters, T. E. Thate, N. L. Craig, Definition of the Escherichia coli MC4100 genome by use of a DNA array. J. Bacteriol. 185, 2017–2021 (2003).

57. P. A. J. de Boer, R. E. Crossley, L. I. Rothfield, A division inhibitor and a topological specificity factor coded for by the minicell locus determine proper placement of the division septum in E. coli. Cell 56, 641–649 (1989).

58. L. R. Bullas, J. I. Ryu, Salmonella typhimurium LT2 strains which are r–m+ for all three chromosomally located systems of DNA restriction and modification. J. Bacteriol. 156, 471–474 (1983).

59. S. Ben-Yehuda, R. Losick, Asymmetric cell division in B. subtilis involves a spiral-like intermediate of the cytokinetic protein FtsZ. Cell 109, 257–266 (2002).

60. E. Galli, K. Gerdes, Spatial resolution of two bacterial cell division proteins: ZapA recruits ZapB to the inner face of the Z-ring. Mol. Microbiol. 76, 1514–1526 (2010).

Acknowledgments: We thank W. Margolin for insights into the role of ATP in FtsA function, K. Gerdes for the pEG4 plasmid, J. Lutkenhaus and S. Korshunov for an E. coli nuo ndh double mutant, D. Kao for a guaD mutant, M. Cashel for an E. coli relA spoT double mutant, P. d. Boer for an E. coli minB mutant, B. Jones for Salmonella LSU000, R. Bernard and D. Rudner for Bacillus strains, and the E. coli Genetic Stock Center for supplying mutants from the Keio collection. Funding: We are grateful to NIH, VA, and the University of Iowa for support to A.V.-T. (AI36520, AI54959, and BX02073), L.F.F. (T32 AI052066 and F31 AI118223), and D.S.W. (1R01GM125656, a Development Grant from The University of Iowa Department of Microbiology and Immunology). Author contributions: A.V.-T. and D.S.W. designed the study. D.S.W., A.Y., L.L., and J.-S.K. constructed strains and plasmids. J.J.-C., A.Y., J.-S.K., L.L., J.-S.K., and D.S.W. performed experiments. A.V.-T. and D.S.W. wrote the manuscript with help from the other authors. Competing interests: The authors declare that they have no competing interests. Data and materials availability: All data needed to evaluate the conclusions in the paper are present in the paper and/or the Supplementary Materials. Bacterial strains and plasmids are available from the authors. Additional data related to this paper may be requested from the authors.

Submitted 6 August 2019
Accepted 4 December 2019
Published 26 February 2020
10.1126/sciadv.aaz0260

Citation: J. Jones-Carson, A. Yahashiri, J.-S. Kim, L. Liu, L. F. Fitzsimmons, D. S. Weiss, A. Vázquez-Torres, Nitric oxide disrupts bacterial cytokinesis by poisoning purine metabolism. Sci. Adv. 6, eaaz0260 (2020).