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Table S2: Strains, plasmids and primers used in this study

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Figure S2, *related to Figure 3:* Mass spectrometry confirmation of EF-P lysylation in vitro by PoxA at conserved lysyl residue 34.

Figure S3, *related to Figure 3:* PoxA requires ATP to lysylate EF-P and AMP formation by PoxA in vitro.

Figure S4, *related to Figure 4:* Structure of PoxA in complex with AMP

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Supplementary Methods

Table S1, related to Figure 1A: Insertion sites of Tn*10d*(*del25*) transposons leading to GSNO resistance.

	Strain ID	Genome position	9 bp repeat	orient.
yjeK				
1	924B1	4,578,642	GTT CGC GCA	(-)
***poxA* (*yjeA*)**				
2	30J4	4,586,109	AAC CTG GCA	(-)
3	29D1	4,586,213	CGC CCT GCA	(+)
4	30Y4	4,586,613	GGC AAA GCT	(+)
5	29C4, 30G2, 30W1	4,586,691	GGT TGA GCC	(+)
6.	30T5	4,586,691	GGT TGA GCC	(-)

Table S2: Strains, plasmids and primers used in this study.

Strain	**Genotype or relevant characteristics**	**Reference**
14028s	"wild type" *Salmonella enterica* serovar Typhimurium	ATCC
WN353	14028s *poxA::cm*	This work
WN354	14028s *yjeK::cm*	This work
WN356	14028s *poxA::km yjeK::cm*	This work
WN409	14028s *poxA::cm* pWN403	This work
WN412	14028s *yjeK::cm* pWN404	This work
TT18796	*proAB47*/*F'*128 (*pro lac*) *zzf-3832::Tn10Tet[del25]*	(Rappleye and Roth, 1997)
Plasmids		
pBAD18	An expression vector that places cloned genes under the control of the arabinose-inducible P_{BAD} promoter	(Guzman et al., 1995)
pWN403	Has a 1112 nt fragment encoding the *poxA* open reading frame cloned into the *KpnI* and *SphI* sites of pBAD18	This work
pWN404	A 1238 nt fragment containing the *yjeK* open reading frame cloned into the *EcoRI* and *XbaI* sites of pBAD18	This work
pKD3	A pir-deficient plasmid harboring the chloramphenicol resistance cassette used for gene deletion.	(Datsenko and Wanner, 2000)
pKD4	A pir-deficient plasmid harboring the kanamycin resistance cassette used for gene deletion.	(Datsenko and Wanner, 2000)

Primers	
WNp6	5' CTTCGTGACCTTGTCTTAAACTGGAGAAAGAATCATGAGCGTGTAGGCTGGAGCTGCTTC
WNp7	5' CACCAGACGATCAACACCCAGCGCCACGCCGGAACAATCCCATATGAATATCCTCCTTAG
WNp245	5' ACAGGACGCCAGGCGCTTGTTTGCCCTCCGCGTGCCGCGCGTGTAGGCTGGAGCTGCTTC
WNp246	5' TAAATCCAGCGGTGTTTTGCTCGGTTCGCCGCCGATTTCACATATGAATATCCTCCTTAG
WNp336	5' TTTGAATTCGCATAAGGTTCGCCATCCAAC
WNp337	5' TTTTCTAGAGTTAGTTTATCCTCGCCAAAGGG
WNp348	5' TTTGGTACCCTTCGTGACCTTGTCTTAAACTGGA
WNp349	5' TTTGCATGCTCCAGCCTTATGTTGTGGCAAA
PoxA-fwd	5' TTGTATTTCCAGGGCAGCGAAACGGCAACCTGGCAGCCGA
PoxA-rev	5' CAAGCTTCGTCATCATTACGCCCGATCGACCGTAAAAGCA
EFPS-fwd	5' GACGACGACAAGATGGCGACTTACTATAGCAACGATTTTC
EFPS-rev	5' GAGGAGAAGCCCGGTCTGCACCATTTTTCCCGATAACG
Arb1	5' GGCCAGCGAGCTAACGAGAC
Arb1a	5' GGCCAGCGAGCTAACGAGACNNNNGTTGC
Arb1b	5' GGCCAGCGAGCTAACGAGACNNNNGATAT
Arb1c	5' GGCCAGCGAGCTAACGAGACNNNNAGTAC
Tn10-3L	5' TCCATTGCTGTTGACAAAGGGAAT
Tn10-2R	5' ACCTTTGGTCACCAACGCTTTTCC
IS10-L	5' CAAGATGTGTATCTACCTTAAC
IS10-R	5' CAAGATGTGTATCCACCTTAACTTAATG

Table S3 (*related to Figures 2 and S1*)

Summary of Biolog Phenotype Microarray results of strains WN150 vs. WN353 (*poxA*) and WN354 (*yjeK*)

GROWTH CONDITIONS UNDER WHICH THE *POXA* AND *YJEK* MUTANTS RESPIRE MORE THAN WILD-TYPE

CARBON SOURCES
L-Arabinose, N-Acetyl-D-Glucosamine, D-Fructose

NITROGEN SOURCES
Ammonia, L-Aspartic Acid, L-Leucine, L-Methionine, L-Phenylalanine, D-Asparagine, D-Aspartic Acid, D-Valine, Glucuronamide, Cytosine, Guanine, Xanthine, D,L-α-Amino-Caprylic Acid
dipeptides: Ile-Ile, Ile-Met, Ile-Phe, Ile-Trp, Ile-Val, Leu-Ile, Leu-Leu, Leu-Met, Leu-Phe, Leu-Trp, Leu-Val, Lys-Phe, Met-Ile, Met-Leu, Met-Met, Met-Phe, Met-Trp, Met-Val, Phe-Ile, Phe-Phe, Phe-Trp, Trp-Asp, Trp-Gly, Trp-Leu, Trp-Phe, Trp-Trp, Trp-Tyr, Tyr-Leu, Val-His, Val-Ile, Val-Leu, Gly-lle, Ile-Leu, Leu-Tyr, Met-Tyr, Phe-Met, Phe-Tyr, Phe-Val, Trp-Val, Tyr-Val, Val-Met, Val-Phe, b-Ala-Ala, Met-b-Ala, Leu-b-Ala
tripeptides: Gly-Gly-D-Leu, Gly-Gly-lle, Gly-Gly-Leu, Gly-Phe-Phe, Leu-Gly-Gly, Leu-Leu-Leu

PHOSPHATE SOURCES
Phosphate, Pyrophosphate, Trimetaphosphate, Tripolyphosphate, Adenosine- 2'-Monophosphate, Dithiophosphate, Guanosine- 2'-Monophosphate, Guanosine- 3'-Monophosphate, Guanosine- 2',3'-Cyclic Monophosphate, Phospho-Glycolic Acid, 2-Deoxy-D-Glucose 6-Phosphate, Cysteamine-S-Phosphate, O-Phospho-D-Serine, O-Phospho-L-Serine, O-Phospho-L-Threonine, Uridine- 2'- Monophosphate, Uridine- 5'- Monophosphate, O-Phospho-D-Tyrosine, O-Phospho-L-Tyrosine, Phosphocreatine, 2-Aminoethyl Phosphonic Acid, Thymidine- 5'-Monophosphate, Inositol Hexaphosphate

NUTRIENT LIMITATION
* The mutants can respire on media deficient in sulfur whereas the wild-type strain cannot.
* The mutants can respire on the nutrient poor media on plate 5, including in the negative control well, whereas the wild-type strain cannot.

GROWTH CONDITIONS UNDER WHICH THE *POXA* AND *YJEK* MUTANTS RESPIRE LESS THAN WILD-TYPE

CARBON SOURCES
* L-Glutamine cannot be used by *poxA* and *yjeK* mutants as a carbon source.

NITROGEN SOURCES
*γ-Glu-Gly cannot be used by *poxA* and *yjeK* mutants as a nitrogen source.

ALKALINE STRESS
Mutants display comparatively poor growth at pH 9.5 to 10
Mutants also display relatively poor growth at pH 9.5 with the following deaminase substrates: Agmatine, Putrescine, L-Norleucine, L-Arginine, L-Methionine, L-Glutamine, L-Threonine, L-Phenylalanine, L-Histidine, L-Aspartic Acid, L-Serine, L-Asparagine

ACID STRESS
Mutants display comparatively poor growth at pH 5.
Mutants also display relatively poor growth at pH 4.5 with the following decarboxylate substrates: L-Ornithine, L-Lysine, 5-Hydroxy-L-Lysine, g-Hydroxy Glutamic Acid, Urea, L-Norvaline

COMPOUNDS THAT INHIBIT THE GROWTH OF *POXA* AND *YJEK* MUTANTS (RELATIVE TO ISOGENIC WILD TYPE)

CELL WALL SYNTHESIS INHIBITORS
Vancomycin
Cephalasporins: Cefsulodin, Cephalothin, Ceftriaxone, Cefotaxime, Cefoxitin, Cefamandole, Cefazolin, Cetoperazone, Cefmetazole, Cefuroxime
Beta-lactams: Amoxicillin, Azlocillin, Phenethicillin, Ampicillin, Piperacillin, Cloxacillin, Nafcillin, Oxacillin, Penicillin G, Moxalactam

CHELATORS, WATER SOLUBLE
Pyrophosphate, 1,10-Phenanthroline, EDTA

CHELATORS, LIPOPHILLIC
5,7-Dichloro-8-hydroxyquinoline, 5,7-Dichloro-8-hydroxy-quinaldine, 8-Hydroxyquinoline, 5-Chloro-7-Iodo-8-Hydroxyquinoline, Fusaric Acid

DNA INTERCALATING AGENTS
2-Phenylphenol, 4-Hydroxycoumarin, Coumarin

DNA SYNTHESIS
5-Azacytidine, Bleomycin, Phleomycin, Hexaminecobalt (III) Chloride, Furaltadone, Nitrofurantoin, Nitrofurazone, Myricetin

RNA SYNTHESIS
Rifampicin, Proflavine

TOPOISOMERASE INHIBITORS
Nalidixic Acid, Norfloxacin, Ciprofloxacin, Enoxacin

FOLATE ANTAGONISTS
Sulfanilamide, Sulfachloropyridazine, Sulfamethazine

RESPIRATION INHIBITORS / UNCOUPLERS
Tetrazolium Violet, Thioridazine, Crystal Violet, 18-Crown-6-Ether, Cinnamic Acid, FCCP, 3,5-Dinitrobenzene, CCCP, 2,4-Dintrophenol, Sodium Caprylate, Sorbic Acid, Pentachlorophenol, Ruthenium red, Capreomycin, Menadione, Dequalinium, Nifedipine, 4-Aminopyridine, Protamine Sulfate, Alexidine, Chlorhexidine

MEMBRANE DAMAGING AGENTS
Guanidine hydrochloride
Antimicrobial peptides: Colistin, Polymyxin B
Detergents: Dodecyltrimethyl Ammonium Bromide, Benzethonium Chloride, Methyltrioctylammonium Chloride, Cetylpyridinium Chloride, Domiphen bromide, Lauryl sulfobetaine

OXIDIZERS
Diamide, 1-Chloro-2,4-Dinitrobenzene, Plumbagin, Methyl viologen, D,L-Thioctic Acid

PROTEIN SYNTHESIS INHIBITORS
Blasticidin S, Cinoxacin, Fusidic acid, Spectinomycin
Aminoglycosides: Kanamycin, Amikacin, Gentamicin, Geneticin (G418), Hygromycin B, Streptomycin, Apramycin
Macrolides: Josamycin, Spiramycin, Puromycin, Tylosin, Oleandomycin
Tetracylines: Minocycline, Tetracycline, Demeclocyline, Penimepicycline

TOXIC ANIONS
Sodium metasilicate, Potassium chromate, Sodium Selenite, Sodium Orthovanadate, Sodium Dichromate

TOXIC CATIONS
Chromium Chloride, Ferric Chloride, Lithium Chloride, Aluminum Sulfate, Cesium chloride, Cupric chloride, Thallium (I) acetate, Nickel chloride, Cobalt chloride, Manganese (II) chloride, Zinc chloride, Antimony (III) chloride.

tRNA SYNTHETASE INHIBITORS
Glycine Hydroxamate, D,L-Methionine Hydroxamate, L-Glutamic-γ-Hydroxamate

OTHER
Sodium Nitrate
Sodium Lactate
Nitrofurazone
Hydroxyurea
D-Serine

Fungicides: Chloroxylenol, Disulphiram, Captan, Oxycarboxin, Dodine, Dichlofluanid, Patulin, Nordihydroguaiaretic acid
Poorly characterized: Amitriptyline, Chlorpromazine, Compound 48/80, Chelerythrine, D,L-Propranolol, Pridinol/Orphenadrine, Caffeine, Promethazine, Aminotriazole, Harmane, 2,4-Diamino-6,7-Diisopropylpteridine, Ornidazole, Chlorambucil, Ketoprofen, Gallic Acid, Trifluoperazine, Colchicine, Tinidazole, Cytosine arabinoside, Atropine, β-Chloro-L-Alanine

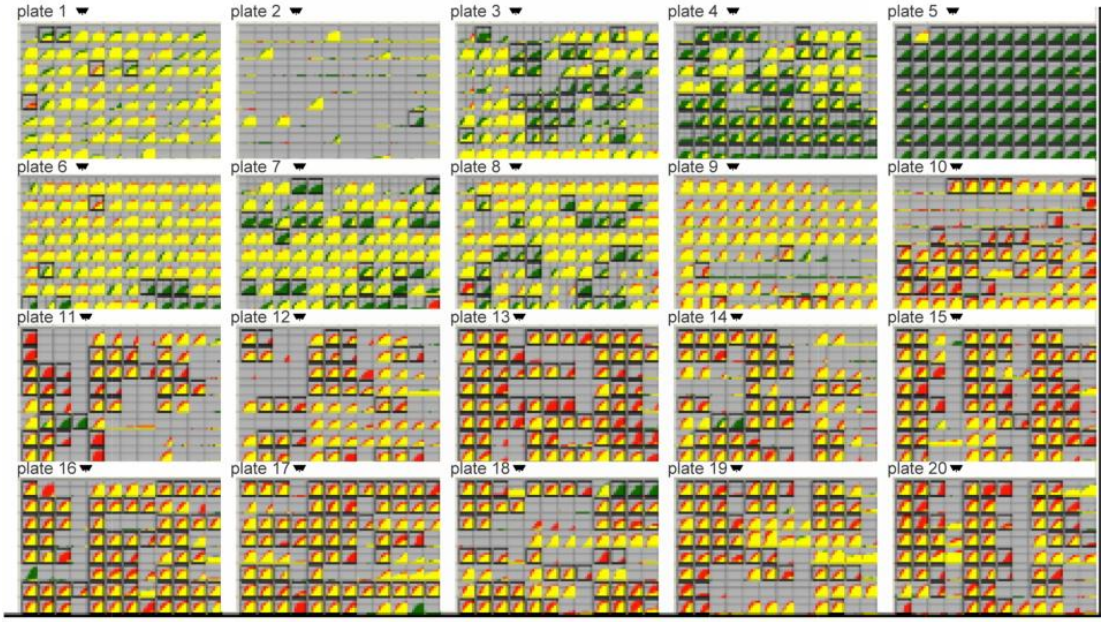
Table S4, *related to Figure 4* – Growth of PoxA alanine mutants on AB2 media or against GSNO. The greatly diminished growth of *poxA* mutant *Salmonella* on AB2 agar (Difco formulation) was previously reported and was measured here as a rapid indicator of PoxA activity. Growth inhibition by GSNO in a disk diffusion assay is reported as the diameter of the zone (including standard deviation) where no bacterial growth is observed (see Experimental Procedures).

strain	genotype	growth on AB2	GSNO (cm)
WN150	wild-type	+	5.6 ± 0.34
WN353	*poxA:cm*	-	3.6 ± 0.01
WN409	*poxA::cm* pPoxA	+	5.3 ± 0.01
LX029	*poxA* (S76A)	+	4.8 ± 0.03
LX046	*poxA* (E78A)	-	4.1 ± 0.03
LX047	*poxA* (R100A)	-	3.9 ± 0.01
LX039	*poxA* (E102A)	+	6.0 ± 0.08
LX040	*poxA* (H108A)	+	5.7 ± 0.18
LX031	*poxA* (F112A)	+	4.5 ± 0.03
LX041	*poxA* (E116A)	-	4.1 ± 0.01
LX042	*poxA* (Y118A)	+	5.2 ± 0.08
LX043	*poxA* (E244A)	-	3.9 ± 0.06
LX044	*poxA* (E251A)	-	3.8 ± 0.01
LX045	*poxA* (R303A)	-	3.8 ± 0.03

Figure S1, *related to Figure 2 and Table S3 – poxA* and *yjeK* mutant *S.* Typhimurium display numerous phenotypes with nearly complete overlap. Graphical depiction of the Biolog "phenotype microarray" performed on WN353 and WN354 compared to wild-type *Salmonella* 14028s. The chart represents twenty 96-well plates, with each small box represents a well on a 96-well assay plate that contains a different growth condition or growth inhibitor. Within each box the turnover of a tetrazolium dye (indicative of respiration) is shown for both the mutant and wild-type strain is measured by intensity (y-axis) versus time (x-axis). In cases in which the turnover of dye is similar between wild-type and mutant, the curve appears yellow. In conditions in which the mutant respires at a rate that is faster or slower than the wild-type strain, the area under the curve appears green or red, respectively. Plates along the top two rows (1 through 8) test the ability to respire on various carbon, nitrogen, sulfur or phosphate sources. Plates 9 through 20 test a variety of growth inhibitors and stress conditions. A summary of the conditions where tetrazolium turnover is affected in either mutant is provided in Table S3.

-figure on next page-

WN353 (*poxA::cm*) vs. 14028s wild-type



WN354 (*yjeK::cm*) vs. 14028s wild-type

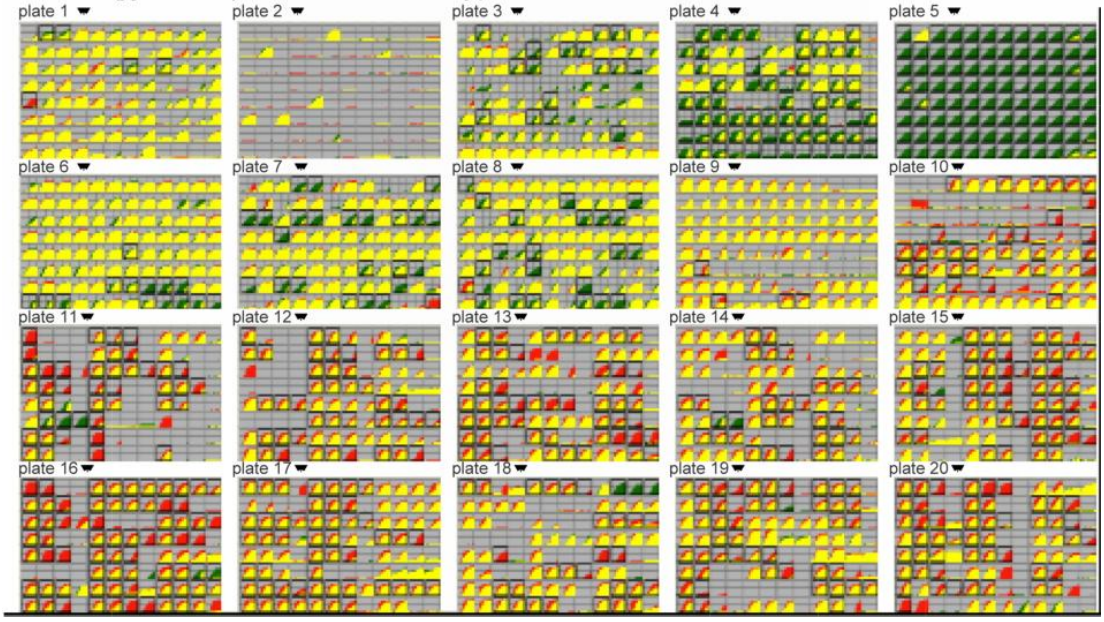


Figure S2, *related to Figure 3* – Mass spectrometry confirmation of EF-P lysylation *in vitro* by PoxA at conserved lysyl residue 34. Mass assignments for spectra generated by MALDI-TOF of tryptic peptides of modified EF-P are indicated on the predicted peptide PGK*GAFAR, where the modified lysyl residue is indicated with an asterisk.

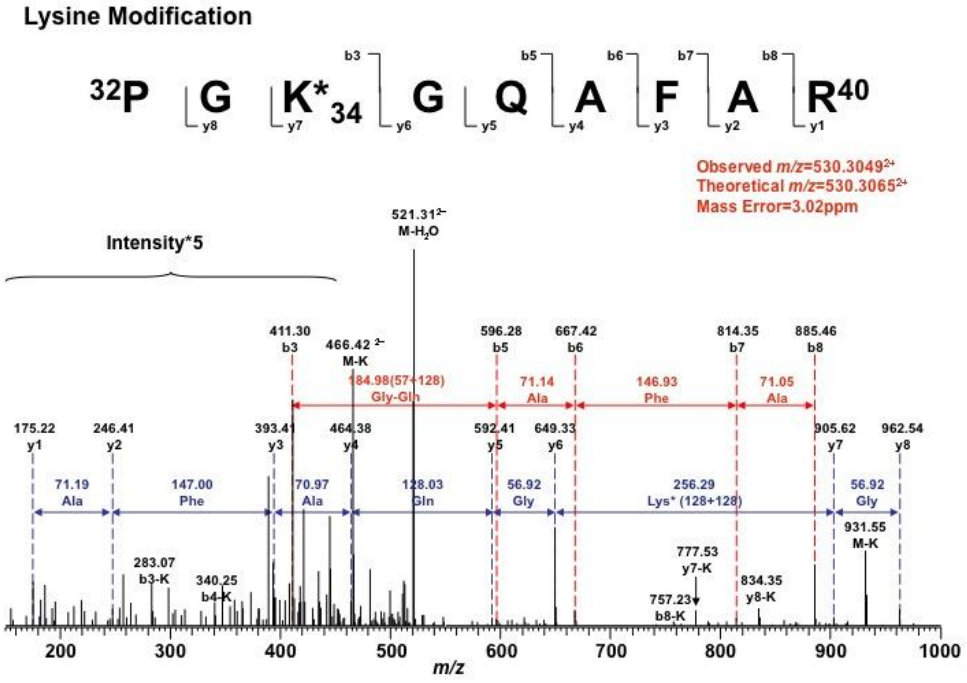


Figure S3, *related to Figure 3* – PoxA requires ATP to lysylate EF-P and AMP formation by PoxA *in vitro*. A [^{14}C]-Lys addition to EF-P catalyzed by PoxA was monitored by SDS-PAGE after various incubation intervals at 37°C either in the presence or absence of ATP using conditions identical to those in Figure 3. Top panel, coomassie stained PAGE, *bottom panel*, autoradiogram of lysylated EF-P. **B.** [α^{32}P**]-**AMP formation was monitored by thin layer chromatography and revealed by phosphorimaging. The presence (+) or absence (-) of PoxA, EF-P or Lys in reaction media is indicated. [α^{32}P**]-**AMP control (lane 6) was obtained with phenylalanyl-tRNA synthetase incubated in presence of Tyr and tRNA^{Phe}.

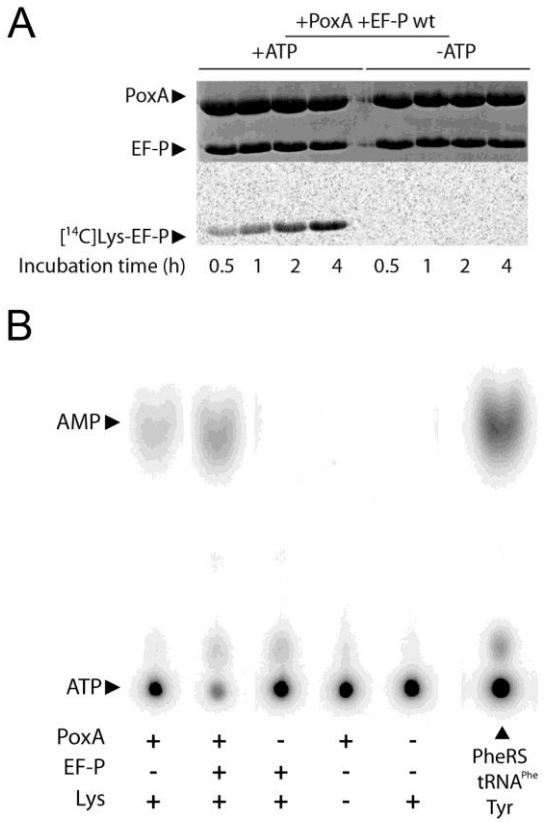
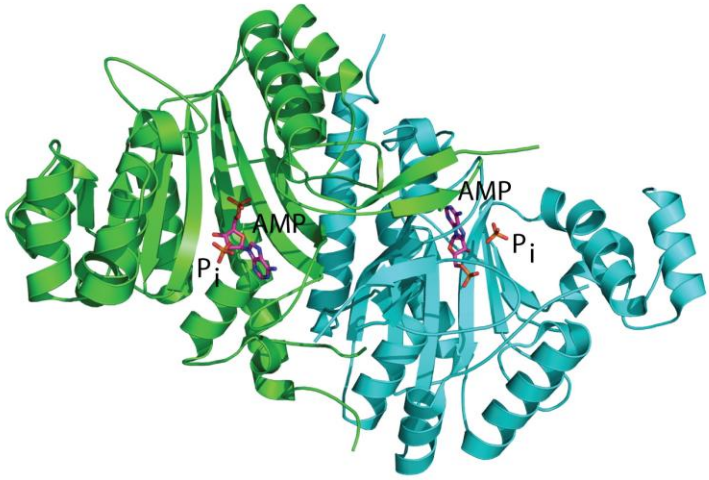


Figure S4, *related to Figure 4* - Structure of PoxA in complex with AMP (PDB 3G1Z). (A) Ribbon backbone of the PoxA dimer, with molecules A and B colored green and cyan, respectively, with the positions of the AMP and phosphate (Pi) molecules shown as a stick representation and labeled. (**B)** Overlay of PoxA (molecule A, green) with the *E. coli* lysyl t-RNA synthetase LysU (PDB code 1E22, blue). (**C)** Closeup of the nucleotide-binding site of PoxA. The AMP molecule, the phosphate ion representing the expected position of the gamma phosphate of ATP (from the overlay of PoxA with PDB 1E22), and residues of PoxA contacting these two moieties are shown in a stick representation and labeled.

-figure on next page-

A.



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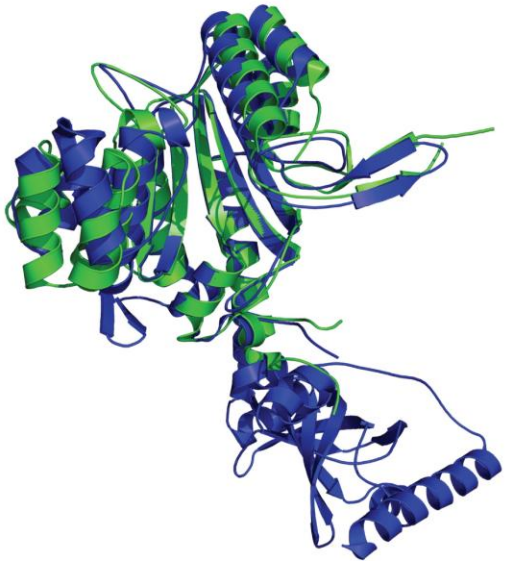


C.

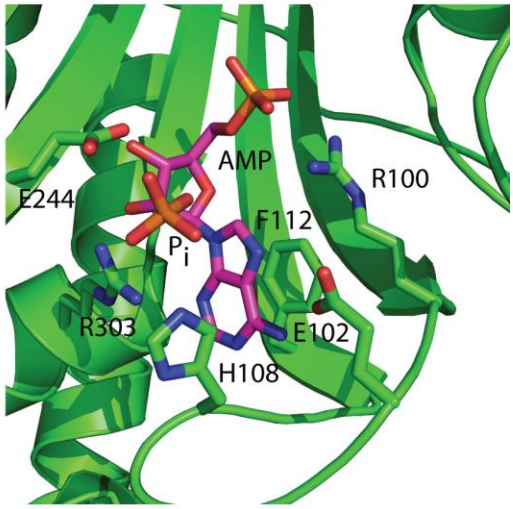
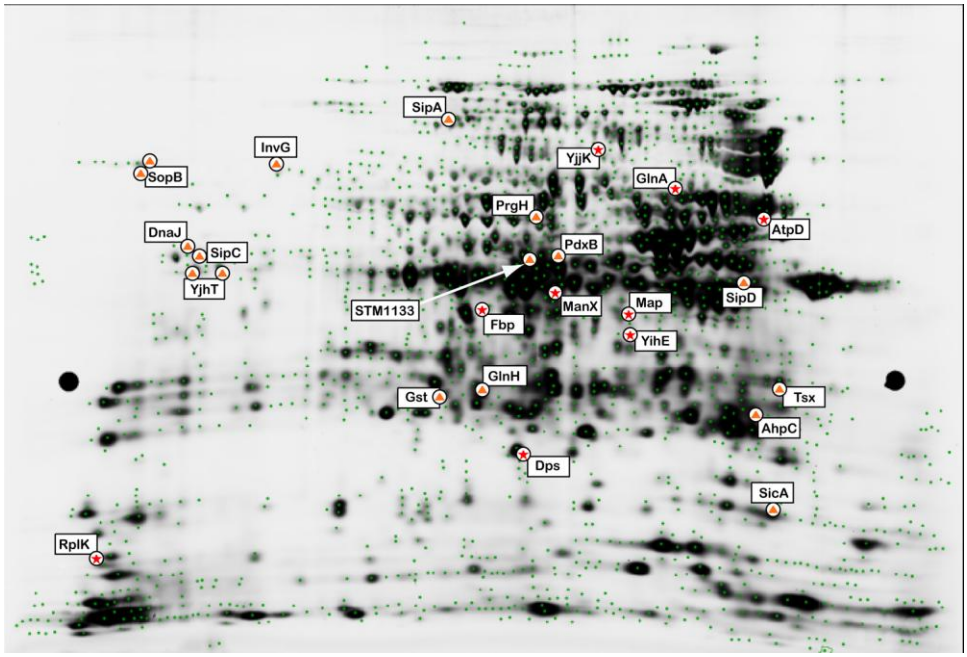


Figure S5, *related to Figure 5* – Image of *2D DIGE results*. Location of unambiguiously identified individual spots on 2D gels with red stars indicating proteins with higher levels in wild-type 14028s and orange triangles indicate proteins with higher levels in the isogenic *poxA* mutant. Each green dot is an individual protein as identified by DIGE.



Supplementary Methods

Bacterial strains and plasmids

The wild-type *Salmonella enterica* serovar Typhimurium 14028s was obtained from the laboratory of Samuel Miller at the University of Washington. Alleles were moved into the 14028s strain background by transduction using phage P22 HT105/1 *int-201* (Schmieger, 1971). The origins and properties of strains used in this study are outlined in Table S1.

Null mutations in *poxA* and *yjeK* were constructed by the protocol of Datsenko and Wanner using the *red-gam* recombinase as described (Datsenko and Wanner, 2000). Briefly, the *poxA* gene of S. Typhimurium strain LT2 was replaced by chloramphenicol or kanamycin resistance gene cassettes amplified from the plasmids pKD3 and pKD4 using primers WNp6 and WNp7. Similarly, the *yjeK* gene was replaced by a chloramphenicol resistance cassette amplified from pKD3 using primers WNp245 and WNp246.

Plasmid pWN403 (pBAD18-*poxA*) was constructed by PCR amplification of the *poxA* open reading frame from *S.* Typhimurium 14028s genomic DNA with primers WNp348 and WNp349. The resulting PCR product was digested with the restriction enzymes *KpnI* and *SphI* and cloned into the corresponding *KpnI* and *SphI* sites on the pBAD18 plasmid. pWN404 (pBAD18-*yjeK*) was constructed in a similar way using primers WNp336 and WNp337 to amplify a 1238 nt fragment containing the *yjeK* open reading frame for cloning into the *EcoRI* and *XbaI* sites in pBAD18.

Genetic Screen for GSNO Resistant Mutants

Salmonella was mutagenized with the T-POP *tetA* mini-transposon (Tn*10d*(*del25*)) (Rappleye and Roth, 1997) by phage P22-mediated transduction from a strain harboring the an F′-borne Tn*10d*(*del25*) insertion into a 14028s derivative carrying pNK2880, a plasmid that harbors a gene encoding the altered target specificity (ATS) Tn*10* transposase that decreases bias in the transponson site selection (Kleckner et al., 1991). Transductants were immediately plated on LB agar supplemented with 10 μg/ml tetracycline. Phage concentrations were adjusted such that each transduction would yield approximately 500 colonies, each colony representing an independent transposition event. This process was repeated several times to generate a large set of independently generated pools totaling approximately 40,000 transposition events. *Salmonella* pools harboring insertions were plated on M9 agar containing 500 μM GSNO at a cell density of 4 x 10^3 per plate.

M9 minimal agar supplemented with 0.2% glucose and GSNO was prepared as follows. One-hundred ml of sterile filtered 5X M9 minimal salts (Difco) was mixed into 380 ml of an autoclaved aqueous agar suspension in (7.5 grams in water) that had been cooled to 60°C. To this mixture, 5 ml of 20% glucose, 5 ml of 10 mM $CaCl_2$ and 1 ml of 1M $MgCl_2$ were added while stirring. The agar was allowed to cool to < 50° C before adding 500 μl of a 500 mM solution of GSNO for a final concentration of 500 μM. Twenty-five ml of agar were dispensed by pipette into 10 cm petri dishes.

Transposon insertions were rapidly mapped using a modification of a previous method by O'Toole and Kolter (O'Toole and Kolter, 1998). Two sequential PCR reactions were used to amplify the transposon-chromosome junctions. The first PCR (30 cycles: 95°C, 30 sec; 38°C, 30 sec; 72°C, 2 min) employed a primer complimentary to a

site within the transposon (Tn*10*-3L or Tn*10*-2R to sequence the left end or right end of the transposon, respectively) and an "arbitrary" primer (Arb1a, b, or c) containing random nucleotides near the 3' end. Amplifications that failed to produce a sequence with one arbitrary primer would often work with one of the other two primers. At low annealing temperatures the arbitrary oligonucleotides prime at random sites in the chromosome to yield fragments of mixed size adjacent to the transposon junction (as well as irrelevant products due to two arbitrary primers landing near one another). Fragments containing the transposon junction were amplified further with a second round of PCR (30 cycles: 95°C, 30 sec; 55°C, 30 sec; 72°C, 2 min) using a transposon-specific primer more proximal to the junction (IS10-L or –R) and a primer (Arb-1) complimentary to the non-random 5' end of the original Arb-1a, b, or c primers. The final PCR products were cleaned using a PCR clean-up kit (Qiagen) and submitted for sequencing using the relevant transposon specific primer (IS10-L or –R).

More than a third (65) of the highly GSNO-resistant mutants mapped to the dipeptide permease (*dpp*) operon. This locus had previously been demonstrated to be involved in importing GSNO into the bacterial cell and to be required for GSNO to display its cytostatic effects in laboratory culture (De Groote et al., 1995). Because the role of this locus in bacterial sensitivity to GSNO is relatively well understood and is not due to NO toxicity *per se*, these mutants were not examined further. A significant number (approximately 20) of transposon insertions initially identified in our screen failed to reproduce the GSNO-resistance phenotype when the genes were disrupted using other methods. These mutants were also discarded from our study.

Determination of the structure of PoxA

The structure of full-length PoxA (325 residues) in complex with ATP was determined by molecular replacement using a model generated automatically from the protein sequence by the Swiss-Model server (http://swissmodel.expasy.org/SWISS-MODEL.html). Molecular replacement determined that there were two molecules in the asymmetric unit, and after refinement of the model, a buried surface area of 7580 Å2 was calculated between the two molecules. The quaternary structure server PISA (http://www.ebi.ac.uk/msd-srv/prot_int/pistart.html) predicts that this protein is a dimer. The resulting model contains a nearly complete chain for residues 5-325 in both molecules, with 4 and 12 residues missing over that range in molecules A and B, respectively. The model shows good geometry, with 99.6% of residues found in the favored and additional allowed regions of the Ramachandran plot. The structure has been deposited into the Protein Data Bank (www.rcsb.org; PDB ID: 3G1Z).

Overall, the structure resembles the tRNA transferase domain of lysyl tRNA synthase and other aminoacyl tRNA synthetases, but without the anticodon binding domain at the N-terminus of the protein (see Figure S4B). Using the SSM server – full-length PoxA structure superimposes upon 1LYL, *E coli* lysyl tRNA synthetase from residue 165 with a Z-score of ~12 and an rmsd of 1.7 A. Figure S4 shows a closeup of the interactions with the nucleotide. ATP was added into the crystallization liquor, however AMP is observed in the crystal, probably due to slow hydrolysis of ATP by PoxA in the crystal. Addition phosphate ions from the crystallization liquor were also modeled, including a phosphate ion in the position of the γ-phosphate based on the

structure of other tRNA synthetases co-crystallized with non-hydrolysable ATP analogues (for example, see the structure of LysU with AMP-PCP and lysine (PDB 1E22)). In the PoxA-AMP complex, the adenine ring is held by ring stacking interactions between the aromatic ring of F112 and the guanidinium group of Arg303, with the N6 (NH2) and N1 of the adenine being in contact with the main chain carbonyl and amide of residue 101, respectively. The N6 also contacts a carboxyl oxygen of E102. The ribose ring is held by principally 3 hydrogen bonds, the O2 being assisted by the interaction of the main chain carbonyl oxygens of E244 and L245, while the O3 OH contacts one of the carboxyl oxygen atoms of E244. The alpha phosphate held by a strong interaction of the guanidinium group of Arg100, in which the two NH groups contact 2 phosphate oxygen atoms. The phosphate group nearest the AMP molecule forms a salt bridge with the guanidinium group of R303, with additional interactions involving the Nε of H108 and the carboxyl oxygen of E244.

PoxA was cloned into the expression plasmid p15TvLic, and the plasmid was transformed into *E. coli* BL21(DE3)-RIPL (Stratagene). These *E. coli* cells were then cultured in 1 L of Luria Broth (LB) at 37°C to an optical density (at 600 nm) of approximately 1.2, and 0.4 mM IPTG was added to induce protein expression. After induction, the cells were incubated overnight with shaking at 25°C. Cells were harvested by centrifugation, disrupted by sonication, and the insoluble material was removed by centrifugation. PoxA was purified using Ni-NTA affinity chromatography, cleaved with recombinant His-tagged TEV protease and dialyzed and stored in a buffer containing 10 mM HEPES (pH 7.5), 300 mM NaCl and 0.5 mM TCEP.

Crystallization was performed with protein concentrated to 25 mg/mL at room temperature (21 °C) using sitting-drop vapor diffusion with an optimized sparse matrix crystallization screen (Kimber et al., 2003). The crystal used for the data collection at (see Table S3) was obtained using crystallization liquor containing 1.6 M sodium/potassium dihydrogen phosphate, 0.1 M HEPES, pH 7.5, 0.5 mM ATP and 0.3 mM $MgCl_2$. Crystals were cryoprotected in N-Paratone oil and flash-frozen in liquid nitrogen prior to data collection.

The structure of PoxA was determined by molecular replacement using a model derived by inputting the protein sequence into the SWISS-MODEL server (http://swissmodel.expasy.org/swiss). Diffraction data collected at 100° K on a Rigaku Micromax-007 rotating anode generator equipped with Osmic mirrors, and diffraction data were recorded on an R-Axis IV++ detector and integrated and scaled using HKL2000 (Minor et al., 2006). The molecular replacement program PHASER (McCoy et al., 2005) as part of the CCP4 program suite (1994) was used to determine the initial positions of the individual monomers derived by SWISS-MODEL. The model was subsequently improved through alternate cycles of manual building and water-picking using COOT (Emsley and Cowtan, 2004) and restrained refinement against a maximum-likelihood target with 5% of the reflections randomly excluded as an R_{free} test set. All refinement steps were performed using REFMAC (Murshudov et al., 1997) in the CCP4 program suite, with final steps of refinement including TLS parameterization (Winn et al., 2001; Winn et al., 2003). In total, out of a possible 652 residues, 26 residues were omitted due to poor electron density (9 and 17 residues in molecules A and B, respectively), including the first 5 residues of both molecules as well as additional

residues in flexible loop regions. The final model contained two molecules of PoxA, 2 AMP molecules, 4 phosphate ions and solvent molecules refined to an R_{work} of 18.1% and R_{free} of 23.9%. Data collection, phasing and structure refinement statistics are summarized in Table S3. The Ramachandran plot generated by PROCHECK (Laskowski et al., 1993) showed excellent stereochemistry overall with 99.6% of the residues in the most favored and additional allowed regions.

Mutagenesis of the *Salmonella* chromosomal *poxA* locus

Plasmid pWN403 containing *poxA* was used as a template for site-directed mutagenesis using PCR amplification and DpnI digestion as described by Fisher and Pei (Fisher and Pei, 1997). Oligos used for various mutants are as follows: S76 (CAACCTCTATTTAATGACCGCGCCGGAATACCATATGAAACGCC and GGCGTTTCATATGGTATTCCGGCGCGGTCATTAAATAGAGGTTG); E78 (CTCTATTTAATGACCAGTCCGGCTTACCATATGAAACGCCTGC and GCAGGCGTTTCATATGGTAAGCCGGACTGGTCATTAAATAGAG); R100 (CCAGCTATGCCGCAGTTTCGCGAATGAAGAGATGGGACGAC and GTCGTCCCATCTCTTCATTCGCGAAACTGCGGCATAGCTGG); E102 (GCCGCAGTTTCCGTAATGCTGAGATGGGACGACATC and GATGTCGTCCCATCTCAGCATTACGGAAACTGCGGC); H108 (GAAGAGATGGGACGACATGCGAATCCGGAATTCACTATGCTGG and CCAGCATAGTGAATTCCGGATTCGCATGTCGTCCCATCTCTTC); F112 (GGACGACATCATAATCCGGAAGCTACTATGCTGGAGTGGTATCG and CGATACCACTCCAGCATAGTAGCTTCCGGATTATGATGTCGTCC); E116

(CCGGAATTCACTATGCTGGCATGGTATCGCCCGCATTAC and GTAATGCGGGCGATACCATGCCAGCATAGTGAATTCCGG); Y118 (CACTATGCTGGAGTGGGCCCGCCCGCATTACGATATG and CATATCGTAATGCGGGCGGGCCCACTCCAGCATAGTG); E244 (GGTGTACTACAAAGGTATTGCGCTGGCGAATGGTTTCCACG and CGTGGAAACCATTCGCCAGCGCAATACCTTTGTAGTACACC); E251 (GCGAATGGTTTCCAGGCTCTGACGGACGCACGTGAGCAAC and GTTGCTCACGTGCGTCCGTCAGAGCCTGGAAACCATTCGC); R303 (GTGGCGCTGGGTGTTGATGCACTGGTGATGCTGGCG and CGCCAGCATCACCAGTGCATCAACACCCAGCGCCAC). All oligo sequences are reported with 5' end on left.

After mutagenesis each *poxA* open reading frame harboring the mutation were PCR amplified from the plasmid using *Pfu* polymerase. Resulting products were electorporated into *Salmonella* strains 29D1 and 29C4 (see Table S1), each harboring Tn10d(del25) transposon within the *poxA* gene. These strains also harbored a pKD46 plasmid to facilitate recombination of the PCR amplified mutant *poxA* gene that was introduced via electroporation with the transposon-containing allele on the *Salmonella* chromosome. The transposon encodes a tetracycline-resistance cassette that can be counter-selected in the presence of fusaric acid as described (Bochner et al., 1980; Karlinsey, 2007; Maloy and Nunn, 1981). Fusaric acid resistant clones were verified to contain the intended mutation by sequencing the chromosomal *poxA* region.

Phenotype microarrays

Phenotype microarray testing was performed under contract by Biolog's PM Services group (Hayward, CA). The basic growth medium for the analysis has been described published previously. The array was conducted in duplicate after incubation of the strains at 37°C for 24 h. Reproducibility was measured using correlation plots of the independent runs. Reproducibility analysis indicates the number of wells where the difference of average height (area under the curve divided by number of reads) between duplicate runs is above an arbitrary threshold. Mutant strains WN353 and WN354 are to resistant to chloramphenicol due to the presence of the chloramphenicol acetyl transferase gene cassette used for gene replacement. Growth of these mutants on chloramphenicol and thiamphenicol served as an internal control for the success of these assays.

Susceptibility to gentamicin and sulfometuron methyl

The minimal inhibitory concentration of gentamicin for wild-type and mutant *Salmonella* strains was determined in 96-well assay format. Overnight cultures of *Salmonella* strains were diluted 100-fold into LB medium containing two-fold serial dilutions of gentamicin ranging from 0.8 to 25 μg/ml. Visible growth was measured by turbidity at each concentration.

Sensitivity to sulfometuron methyl was assessed by disk diffusion on M9 agar supplemented with 83 μg/ml valine using 0.25 in. disks soaked with 2 mg/ml sulfometuron methyl in acetone. Disks were allowed to dry briefly before being placed on the agar, and the diameter of the zone of growth inhibition was measured after 16 hrs at 37°C.

GSNO disk diffusion assay

Susceptibility to (GSNO) was determined by a disk diffusion method (De Groote et al., 1995). Briefly, 15 µl of 500 mM GSNO were added to a 0.25-in. paper disk placed over a lawn of 10^6 bacteria on M9 minimal agar with 0.2% glucose (25 ml of agar in a 10 cm petri dish). The zone of growth inhibition after a 16 h incubation at 37°C was used as a measure of susceptibility.

Mouse virulence assay

Female 6–8-week-old C56BL/6 (The Jackson Laboratory, Bar Harbor, ME) mice were used for the determination of *Salmonella* virulence. *Salmonella* were grown overnight in LB medium and diluted in phosphate-buffered saline (PBS; Difco). Approximately 1200 cfu were administered intraperitoneally and mice monitored twice daily for signs of disease. Moribund mice were euthanized according to the animal care and use regulations of the University of Washington.

Lysylation of EF-P by PoxA in vitro

Salmonella EF-P was amplified by PCR (primers EFPS-fwd and EFPS-rev) and ligated in the vector pET-51b Ek/LIC (Novagen), that harbors the Strep-Tag II coding sequence at the 5' end of the inserted fragments. *Salmonella* PoxA was cloned in the vector p15TV-L (GenBank accession EF456736) using primers PoxAS-fwd and PoxAS-rev yielding a protein bearing a 6-His tag at its N-terminus. Both proteins were expressed in BL21 (DE3) in 0.5 L of autoinduction medium (Roy and Ibba, 2008) containing 200

mg/L of ampicillin (EF-P) or 50 mg/mL of kanamycin (PoxA) by incubation overnight at 37°C with agitation. Cell free extracts were prepared by sonication in buffer A (100 mM Tris-HCl pH 8.0, 150 mM NaCl, 2 mM β-mercaptoethanol) containing 0.5 mM phenylmethanesulphonylfluoride and diisopropylfluorophosphate. S100 supernatant containing EF-P was applied on 2 ml of Strep-Tactin Superflow Agarose (Novagen). The resin was washed with 50 ml of buffer A and EF-P was eluted with buffer A containing 2 mM of desthiobiotin. S100 supernatant containing PoxA was applied on 2 ml of TALON matrix (Clontech). The resin was washed with 200 mL of buffer A and the protein was eluted with buffer A containing 200 mM imidazole. Both proteins were dialyzed against a buffer containing 50 mM Tris-HCl pH 8.0, 150 mM NaCl, and 2 mM β-mercaptoethanol. Proteins were dialyzed a second time against the latter buffer containing 50% glycerol. 4.5 mg of EF-P and 48 mg of PoxA were recovered and stored at -20°C.

EF-P lysylation was performed at 37°C in a mixture containing 100 mM HEPES –NaOH, pH 7.2, 20 mM $MgCl_2$, 30 mM KCL, 10 mM ATP, 30 μM [^{14}C]-Lys (215 cpm/pmol), 20 μM EF-P, and the reaction was initiated by addition of 20 μM PoxA. At various time intervals, 10 μl aliquots were added to 3 μl of protein loading dye and analyzed by SDS-PAGE. After migration, the gel was stained with Coomassie dye and lysylated EF-P with [^{14}C]-Lys was revealed by phosphorimaging. For MS analysis, EF-P was modified by PoxA in the reaction medium described above containing 15 μM PoxA, 40 μM EF-P and 40 mM Lys. A negative control was simultaneously carried out without addition of Lys. After 4 hours of incubation at 37°C, 10 μl of the reaction mix were analyzed on SDS-PAGE and the proteins were revealed by Coomassie staining. The gel slices containing EF-P were cut and trypsic digest of the protein was analyzed by MS-

MS. Structural Docking was performed with Autodock 3.0 (Morris et al., 2008) and active site cavities displayed with PocketPicker (Weisel et al., 2007).

Two-dimensional gel electrophoresis and DIGE

For each strain, cultures were grown to early stationary phase (OD600 = 1.5) in LB medium at 37°C. Cells were pelleted by centrifugation and total protein was extracted by sonication. Prior to labeling, proteins were precipitated with the Ettan 2-D Clean-up Kit (GE Healthcare, Piscataway, NJ) according to the manufacturer's instructions. Cytoplasmic proteins from three biological replicates each of both wildtype and *poxA* mutant strains were labeled with Cy5 minimal dye (GE Healthcare). Equal amounts of protein from all six samples were pooled to form an internal standard (IS) that was labeled with Cy2 minimal dye (GE Healthcare). Briefly, 50 μg of protein were incubated with 400 pmol of CyDye for 1 h at room temperature in the dark. The reaction was stopped by the addition of 10mM lysine. Fifty μg each of the labeled internal standard and protein sample (100 μg total protein) were pooled, mixed with an equal volume of 2x sample buffer (7 M urea, 2 M thiourea, 0.065 M CHAPS (Sigma, St. Louis, MO), 0.13 M dithiothreitol (DTT), and 0.02% IPG buffer, pH 3-11 NL (GE Healthcare)), and incubated on ice for 10 min. Samples were brought to a final volume of 0.45 ml with rehydration solution (7 M urea, 2 M thiourea, 0.065 M CHAPS, 0.018 M DTT, 0.02% IPG buffer, pH 3-11 NL, and 0.001% bromophenol blue) and used to passively rehydrate Immobiline Drystrips (pH 3-11 NL, 24 cm, GE Healthcare) for 14 h at room temperature. Rehydrated strips were equilibrated and focused under the following conditions: 300 V for 2 h, 1000 V for 2 h, a gradient of 5000 V/h to 20,000 V·h, and a gradient of 8000 V/h

to 60,000 V·h. Second dimension SDS-PAGE was carried out on 8-16% tris-glycine gels (Jule Biotechnologies, Mildford, CT). Gels were run at 80 V, 10 mA/gel, and 1 W/gel for 1 h, and then at 500 V, 38 mA/gel, and 13 W/gel until the dye front was approximately 1 cm from the bottom of the gel. Gels were scanned using a Typhoon scanner (GE Healthcare) with subsequent analysis carried out with the DeCyder 6.5 software suite (GE Healthcare). The normalized spot volume ratios from Cy5-labeled spots were quantified relative to the Cy2-labeled spots on the same gel. Normalized volume ratios of Cy5-labeled proteins were standardized to the Cy2-labeled IS and compared between gels. Six gels, three biological replicates each of wildtype and *poxA*, were analyzed using Student's t-test and ANOVA to identify changes in protein abundance.

To identify the proteins, 20 µg of labeled IS were added to 400 µg of unlabelled protein and focused as described above. Gels were imaged using the Typhoon scanner and matched to the previous set of analytical gels. Spots of interest were excised using the Ettan Spot Picker (GE Healthcare) and subjected to an overnight trypsin digest. Following peptide extraction, samples were analyzed by mass spectrometry at the Advanced Protein Technology Centre (Hospital for Sick Children, Toronto, ON, Canada). Briefly, tandem mass spectra were extracted using the LTQ Ion Trap (Thermo Scientific). Charge state deconvolution and deisotoping were not performed. All MS/MS samples were analyzed using Mascot (Matrix Science, London, UK; version Mascot). Mascot was set up to search the NCBInr_20090719 database (selected for *Salmonella*, unknown version, 100246 entries) assuming the digestion enzyme trypsin. Mascot was searched with a fragment ion mass tolerance of 0.50 Da and a parent ion

tolerance of 3.0 Da. Iodoacetamide derivatives of cysteine were specified in Mascot as a fixed modification. Deamidations (of unknown or asparagine) and oxidation of methionine were specified in Mascot as variable modifications.

Scaffold (version Scaffold_2_06_00, Proteome Software Inc., Portland, OR) was used to validate MS/MS based peptide and protein identifications. Peptide identifications were accepted if they could be established at greater than 50.0% probability as specified by the Peptide Prophet algorithm (Keller et al., 2002). Protein identifications were accepted if they could be established at greater than 95.0% probability and contained at least one identified peptide. Protein probabilities were assigned by the Protein Prophet algorithm (Nesvizhskii et al., 2003). Proteins that contained similar peptides and could not be differentiated based on MS/MS analysis alone were grouped to satisfy the principles of parsimony.

AMP formation assay

Formation of AMP by PoxA was monitored in a reaction mix containing 100 mM Hepes-NaOH pH 7.2, 30 mM KCl, 10 mM $MgCl_2$, 100 µM ATP, 0.5 µCi of [α ^{32}P]-ATP and 10 mM Lys. The reaction was conducted at 37°C in presence or absence of 28 µM PoxA or EF-P from *Salmonella*. After 30 min of incubation, the reaction was quenched by mixing equal volumes of reaction mix and acetic acid. Remaining [α^{32}P]-ATP and [α^{32}P]-AMP within 0.4 µl of quench mix were separated by thin layer chromatography on PEI cellulose plates (Sigma) developed in Acetic Acid:1 M NH_4Cl:water (2.5:5:42.5). Radiolabeled products were visualized and quantified by phosphorimaging. Synthesis of [α^{32}P]-AMP, for migration control, was obtained by incubating 1 µM phenylalanyl-tRNA

synthetase in the reaction mix described above in which Lys was substituted by 2 mM Tyr and in presence of transcript $tRNA^{Phe}$ (Roy et al., 2004).

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