

Phosphodiesterase DosP Increases Persistence by Reducing cAMP which Reduces the Signal Indole[†]

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ABSTRACT

Persisters are bacteria that are highly tolerant to antibiotics due to their dormant state and are of clinical significance owing to their role in infections. Given that the population of persisters increases in biofilms and that cyclic diguanylate (c-di-GMP) is an intracellular signal that increases biofilm formation, we sought to determine whether c-di-GMP has a role in bacterial persistence. By examining the effect of 30 genes from *Escherichia coli*, including diguanylate cyclases that synthesize c-di-GMP and phosphodiesterases that breakdown c-di-GMP, we determined that DosP (direct oxygen sensing phosphodiesterase) increases persistence by over a thousand fold. Using both transcriptomic and proteomic approaches, we determined that DosP increases persistence by decreasing tryptophanase activity and thus indole. Corroborating this effect, addition of indole reduced persistence. Despite the role of DosP as a c-di-GMP phosphodiesterase, the decrease in tryptophanase activity was found to be a result of cyclic adenosine monophosphate (cAMP) phosphodiesterase activity. Corroborating this result, the reduction of cAMP via CpdA, a cAMP-specific phosphodiesterase, increased persistence and reduced indole levels similarly to DosP. Therefore, phosphodiesterase DosP increases persistence by reducing the interkingdom signal indole via reduction of the global regulator cAMP.

INTRODUCTION

It has long been established that lethal antibiotic treatments are unable to kill a small fraction of persistent bacteria (Hobby et al. 1942). This insensitivity to antibiotic treatment is not due to any inherent or developed resistance as cultures grown from these persister cells show the same sensitivity to the antibiotic as the parent culture (Bigger 1944; Lewis 2010). The persister phenotype has been exhibited in all bacteria tested (Lewis 2008), but the mechanisms underlying persistence have yet to be fully elucidated. However, it is clear that persisters are metabolically dormant (Kwan et al. 2013; Wood et al. 2013), and that toxins of toxin/antitoxin pairs increase persistence by inhibiting metabolic activity (Dörr et al. 2010; Kim and Wood 2010).

Biofilm formation provides protection to bacteria against environmental stress, and greater numbers of persister cells are found in biofilms and stationary-phase cultures in comparison to exponential-phase cultures (Lewis 2008). Though the majority of biofilm cells are sensitive to antibiotics, persisters account for the resilience of biofilms, as the high proportion of persister cells in a biofilm allows survival of the population (Lewis 2010).

Environmental signaling plays a role in persistence as demonstrated by *Pseudomonas aeruginosa*, in which the quorum sensing-linked molecules *N*-(3-oxo-dodecanoyl)-*L*-homoserine lactone and pyocyanin increase persistence in exponential-phase cultures (Möker et al. 2010). Since quorum sensing and biofilm formation invoke high levels of persistence, we reasoned that cyclic diguanylate (*c*-di-GMP), which increases biofilm formation (Römling et al. 2013), may play a role in modulating persistence. Two molecules of GTP are converted to *c*-di-GMP by diguanylate cyclases (DGCs), which contain GGDEF domains, and *c*-di-GMP is degraded into linear di-GMP (pGpG) by phosphodiesterases (PDEs), which contain EAL or HD-GYP domains. The importance of *c*-di-GMP in bacterial physiology and its tight regulation is evident by the presence of numerous DGCs and PDEs in a given strain; for example, in *E. coli* K-12 there are 12 proteins with a GGDEF domain, 10 proteins with an EAL domain, and 7 proteins with both EAL and GGDEF domains in a single polypeptide (Sommerfeldt et al. 2009; Weber et al. 2006). Additionally, our lab recently discovered a biofilm dispersal protein (BdcA) that binds *c*-di-GMP but does not act as a phosphodiesterase (Ma et al. 2011).

DosP, named as the direct oxygen sensing phosphodiesterase (Tuckerman et al. 2009), is a 90 kDa protein with an NH₂-terminal heme sensor-PAS-PAS-containing domain and a COOH-terminal GGDEF-EAL catalytic phosphodiesterase domain in which the GGDEF domain is inactive (Méndez-Ortiz et al. 2006). DosP cleaves both *c*-di-GMP (Schmidt et al. 2005) and

cAMP (Sasakura et al. 2002), although c-di-GMP is proposed as the physiological substrate due to higher activity towards c-di-GMP than cAMP (Schmidt et al. 2005). Nevertheless, DosP is important for maintaining cAMP levels (Yoshimura-Suzuki et al. 2005), which are tightly regulated at low intracellular concentrations (Hantke et al. 2011). DosP is active as a tetramer and is strongly inhibited by CO, NO, and etazolate, a selective cAMP PDE inhibitor (Sasakura et al. 2002). The heme binding domain of DosP is 60% homologous to the PAS oxygen sensing domain of FixL, an oxygen responsive biological sensor in rhizobia (Delgado-Nixon et al. 2000). The catalytic activity of DosP is therefore oxygen dependent (Kobayashi et al. 2010) and is enhanced 17 fold when saturated with O₂ (Tuckerman et al. 2009). DosP is also activated during entry into stationary phase and is positively regulated by RpoS, the stationary phase sigma factor (Sommerfeldt et al. 2009).

Environmental signaling is also conveyed by the cell signal indole. In *E. coli*, indole regulates several cellular processes including conferring multi-drug resistance (Hirakawa et al. 2005), increasing plasmid stability (Chant and Summers 2007; Field and Summers 2012), decreasing motility (Bansal et al. 2007; Lee et al. 2007c), and decreasing biofilm formation (Domka et al. 2006; Lee et al. 2007a; Lee et al. 2007b; Lee et al. 2009b). Indole is also an interspecies signal for bacterial biofilm formation and virulence (Chu et al. 2012; Lee et al. 2009a; Lee et al. 2007a; Lee et al. 2007b) and an interkingdom signal between bacteria and epithelial cells in the gastrointestinal tract (Bansal et al. 2010). In *E. coli*, indole is produced by tryptophanase (TnaA), which degrades tryptophan into indole, pyruvate, and ammonia (Newton and Snell 1964). Indole is primarily imported by Mtr (Yanofsky et al. 1991) and exported by the AcrEF-TolC multi-drug efflux system (Kawamura-Sato et al. 1999), although it is possible for small amounts of indole to cross the *E. coli* membrane independently of both transporters (Kamaraju et al. 2011; Piñero-Fernandez et al. 2011).

One positive regulator of indole is cyclic adenosine monophosphate (cAMP), a regulatory molecule used by species across multiple kingdoms. In bacteria, cAMP controls protein synthesis primarily through catabolite mediated repression of transcription (Rickenberg 1974). In *E. coli*, cellular cAMP levels correspond inversely to the carbon source, resulting in low concentrations in the presence of a readily metabolizable carbon source (e.g., glucose) (Rickenberg 1974). cAMP binds to CRP, the catabolite receptor protein in *E. coli*, to form the cAMP-CRP transcriptional regulator (Rickenberg 1974), which controls a large regulon and acts as a positive regulator for the *tna* operon, responsible for indole synthesis (Stewart and Yanofsky 1985).

In this work, we found that the phosphodiesterase DosP increases persistence via decreased activity of tryptophanase which results in reduced levels of the signal indole. This regulation of tryptophanase is mediated by cleavage of the cellular

signal cAMP by DosP. The involvement of regulatory signals cAMP and indole lends credence to the involvement of numerous pathways in persister cell formation.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are described in **Table I**. Cultures were grown in lysogeny broth (LB) (Sambrook et al. 1989) at 37°C with shaking unless indicated otherwise. Kanamycin (50 µg/mL) and chloramphenicol (30 µg/mL) were utilized to maintain the pCA24N- and pBS(Kan)-based plasmids. The pCA24N-*dosP* plasmid was sequenced to confirm the presence of *dosP*⁺.

Persister cell formation assay. Persistence was determined by comparing cell viability before and after lethal antibiotic treatment. Overnight cultures were inoculated into LB medium with appropriate antibiotics to maintain plasmids and grown to a turbidity of 1.0 at 600 nm. For strains containing plasmids, gene expression was induced with IPTG (1 mM) for 2 h and cultures were adjusted to a turbidity of 1.0 at 600 nm in fresh LB medium. Cells were then exposed to 100 µg/mL ampicillin or 5 µg/mL ciprofloxacin for 2 to 4 h. The antibiotic concentrations used were at least 10-fold greater than the minimum inhibitory concentration for the wild-type host strain (Kwan et al. 2013) to ensure that any altered resistance of the strains used was not a contributing factor in the persister results. To measure cell viability, samples were taken before and after antibiotic treatment, washed and serially diluted in 0.85% (w/v) NaCl solution, plated on LB agar, and grown overnight at 37°C to determine CFU/mL (Donegan et al. 1991). Experiments were performed with at least two independent cultures.

DNA microarrays. Overnight cultures of BW25113/pCA24N-*dosP* and BW25113/pCA24N were inoculated into LB medium with appropriate antibiotics to maintain plasmids, grown to a turbidity of 1.0 at 600 nm, and induced with IPTG (1 mM) for 90 min to produce DosP. Cell pellets were isolated and flash-frozen in ethanol/dry ice. RNALater buffer (Applied Biosystems, Foster City, CA, USA) was added to stabilize RNA during preparation. Total RNA was isolated from cells as described previously (Ren et al. 2004a) using a bead beater (Biospec, Bartlesville, OK, USA). cDNA synthesis, fragmentation, and hybridization to *E. coli* GeneChip Genome 2.0 arrays (Affymetrix, Santa Clara, CA, USA; P/N 900551) were performed as described previously (González Barrios et al. 2006). Genes were identified as differentially expressed if the expression signal ratio was higher than the standard deviation (1.72) and the P-value for comparing two chips was less than 0.05 (Ren et al. 2004b). The whole-transcriptome dataset is available in the NCBI Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo/>) through accession number GSE47427.

Proteomics. Overnight cultures of BW25113/pCA24N-*dosP* and BW25113/pCA24N were inoculated into LB medium with

appropriate antibiotics to maintain plasmids, grown to a turbidity of 1.0 at 600 nm, and induced with IPTG (1 mM) for 2 h to produce DosP. Cell pellets were isolated and flash frozen in ethanol/dry ice. Total soluble protein was extracted using B-PER (Bacterial Protein Extraction Reagents; Thermo Fisher Scientific, Waltham, MA, USA). In summary, 1 g of cell pellet was lysed by suspending in 4 mL of B-PER containing 100 µg/mL lysozyme (Thermo Fisher Scientific), 5 Kunitz units/mL DNase I (Qiagen, Hilden, Germany), and 12.5% (v/v) EDTA-free protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). The lysate was centrifuged and the supernatant, containing the soluble fraction, was collected. Samples were normalized using IR spectroscopy and 100 µg of protein was labeled with a Tandem Mass Tag Kit (Thermo Fisher Scientific). Proteins were digested with 2 µg of sequencing grade trypsin (Promega, Madison, WI, USA) overnight at 37°C. The digestion mixtures were filtered with 50 kDa MWCO centrifugal filters (EMD Millipore, Billerica MA, USA). Samples were dried using a Speedvac. The digestion mixtures were reconstituted in 100 µL of 4% (v/v) aqueous acetonitrile with 0.1% (v/v) formic acid. The mixture (2 µL) was loaded onto an Acclaim PepMap100 trapping column (100 µm x 2 cm, C18, 5µm, 100 Å, Thermo Fisher Scientific) at a flow rate of 20 µL/min using 4% (v/v) aqueous acetonitrile as a mobile phase. Peptides were separated on an Acclaim PepMap RSLC column (75 µm x 15 cm, C18, 2 µm, 100 Å, Thermo Fisher Scientific) with a 90 minute 4%-50% (v/v) linear gradient of acetonitrile in water containing 0.1% (v/v) formic acid. A Dionex Ultimate 3000 nano-LC system (Thermo Fisher Scientific) was used to deliver the gradient solution at 300 nL/min. Data was acquired over a 40 to 2000 m/z range with an LTQ Orbitrap Velos mass spectrometer (Thermo Fisher Scientific) using the following data-dependent parameters: full FT MS scan at a resolution of 60,000 followed by 10x ion trap MS² scans on the most intense precursor ions with CID activation. Charge states of +2 or higher were used to select precursors for MS²; monoisotopic precursor selection was enabled, and the isolation window was 2 m/z.

Proteome Discoverer 1.3 (Thermo Fisher Scientific) was used to process the mass spectra using the following search parameters: precursor tolerance 10 ppm, fragment tolerance 0.8 Da (ion trap), dynamic modifications including oxidation (+15.995 Da, M) and deamidation (+0.984 Da, N, Q), and a static modification with carbamidomethyl (+57.021 Da, C). The absolute XCorr threshold and the peptide without protein threshold were set to zero in the peptide scoring option. Proteins were identified from the Uniprot *E. coli* K-12 database (March, 2013). The generated msf file was loaded into Scaffold 4.0 (Proteome Software, Inc., Portland, OR, USA) and searched with X!Tandem (Global Proteome Machine) against the *E. coli* K-12 database.

Indole assays. Overnight cultures were inoculated into LB medium with appropriate antibiotics to maintain plasmids, and

grown to a turbidity of 1.0 at 600 nm. Strains containing plasmids were induced with IPTG (1 mM) for 2 h. Cells were pelleted and the supernatant was collected (i.e., extracellular sample). Cells were resuspended in fresh LB medium and sonicated to lyse cells using a 60 Sonic Dismembrator (Fisher Scientific, Hampton, NH, USA). Samples were pelleted to remove debris and the supernatant was collected (i.e., intracellular sample). Extracellular and intracellular indole concentrations were measured spectrophotometrically based on absorbance at 540 nm using Kovac's reagent and HCl-amyl alcohol as described previously (Domka et al. 2006). Experiments were performed with at least two independent cultures.

Biofilm formation. Overnight cultures were diluted to a turbidity of 0.05 at 600 nm in LB medium with or without 1 mM IPTG and with appropriate antibiotics to maintain plasmids. Cultures were grown for 24 h at 37°C in 96-well plates (300 μ L/well). Biofilm formation was assayed using crystal violet staining as described previously (Lee et al. 2009b). Cell growth (turbidity at 620 nm) was used to normalize the total biofilm formation (absorbance at 540 nm). Data points were averaged from at least 12 replicate wells using at least two independent cultures.

Intracellular cAMP levels. Overnight cultures were inoculated into LB medium with appropriate antibiotics to maintain plasmids, grown to a turbidity of 1.0 at 600 nm, and induced with IPTG (1 mM) for 2 h. Cells were pelleted, resuspended in fresh LB medium, sonicated to lyse cells using a 60 Sonic Dismembrator (Fisher Scientific), and centrifuged to remove debris. cAMP was acetylated and quantified using a competitive enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, MI, USA).

RESULTS

DosP increases persistence. To determine if proteins associated with c-di-GMP play a role in persistence, we quantified the number of persister cells surviving ampicillin treatment for 29 isogenic deletion mutants lacking genes encoding diguanylate cyclases and phosphodiesterases. We identified 12 proteins potentially related to persistence (DosC, YdeH, YeaP, YedQ, DosP, Gmr, CsrD, YhjK, BluF (YcgF), YdiV, YjcC, and YliE) based on changes in persistence of 3-fold or greater. We further tested these proteins by producing the 12 proteins via IPTG-inducible plasmids from the ASKA Collection and determining persistence upon production of the DGCs and PDEs. We found that producing DosP caused the most significant difference, increasing persistence to both ampicillin (4200 ± 400 fold) and ciprofloxacin (62 ± 3 fold) (**Fig. 1A**) without affecting overall cell growth (specific growth rate of BW25113/pCA24N-*dosP* of 0.66 ± 0.09 h⁻¹ vs. 0.74 ± 0.06 h⁻¹ for BW25113/pCA24N). The consistent trend across multiple antibiotic classes demonstrates the multidrug tolerance that is the trait of persister cells (Wiuff et al. 2005); hence, DosP increases persistence dramatically.

DosP reduces biofilm formation. DosP is an active phosphodiesterase which degrades c-di-GMP to linear pGpG (Schmidt et al. 2005; Tuckerman et al. 2009). We reasoned that if DosP is active *in vivo* as a phosphodiesterase, production of DosP should reduce biofilm formation since c-di-GMP increases biofilm formation in many bacteria (Hengge 2009). Corroborating this hypothesis, the production of DosP reduced mature biofilm formation by -25 ± 12 fold with low levels of DosP (from the uninduced leaky P_{T5-lac} promoter) and eradicated biofilm formation with high levels of DosP (1 mM IPTG induction) (**Fig. 1B**). This confirms that DosP is an active phosphodiesterase that reduces c-di-GMP *in vivo*.

BdcA also increases persistence. Since DosP reduces c-di-GMP and increases persistence, we tested persistence from production of BdcA, which sequesters c-di-GMP and causes biofilm dispersal (Ma et al. 2011). We found that producing BdcA from pCA24N-*bdcA* to reduce c-di-GMP increased persistence by 430 ± 80 fold relative to the empty plasmid. However, production of other c-di-GMP-related proteins did not affect persistence as expected based on DosP and BdcA; i.e., other PDEs did not necessarily increase persistence and DGCs did not necessarily reduce persistence. For example, GGDEF protein YeaP unexpectedly increased persistence by 9 ± 4 fold. Furthermore, unlike phosphodiesterase DosP, EAL protein YahA reduced persistence by -261 ± 30 fold. Therefore, these results suggested that c-di-GMP levels may not be the mechanism by which DosP increases persistence.

DosP inhibits tryptophanase. To investigate further the relationship between DosP and persistence, we performed a microarray study to determine the effect of DosP production on the transcriptome. We found differential expression (≥ 4 -fold) of 72 transcripts (**Table II**), including repression of genes involved in tryptophan synthesis (the precursor for indole (Newton and Snell 1964)), indole synthesis, and indole import: *trpE* (-8.0 fold), *trpL* (-7.5 fold), *trpD* (-6.5 fold), *tnaA* (-7.5 fold), *tnaL* (-6.1 fold), and *mtr* (-4.9 fold).

Additionally, we performed a proteomic study with DosP using the same conditions and found that one of the most significantly reduced proteins was tryptophanase (TnaA, -33.1 fold, **Table III**), the enzyme responsible for indole synthesis. Hence, both the microarray and proteomic studies indicated that DosP either directly or indirectly reduces cellular tryptophanase.

In addition to regulation of tryptophanase-related genes, the transcriptomic and proteomic results both suggest down-regulation of genes encoding several major components of or related to the tricarboxylic acid cycle and glyoxylate cycle: *aceE*, *acs*, *yjcH*, *actP*, *sdhC*, *sdhD*, *sdhA*, *sucB*, *sucC*, *sucD*, *aceA*, and *aceB*. The results also indicate down-regulation of genes encoding several stress-related proteins involved in starvation (*dps*, *sspA*, and *ndk*), oxidative (*katE* and *katG*), acid

(*hdeA* and *hdeB*), and UV (*uspE*) stress responses. Therefore, DosP causes a general reduction in cellular metabolism and stress responses, which may contribute to persistence through dormancy.

DosP and BdcA decrease indole. Based on the inhibition of tryptophanase by DosP, we measured indole concentrations and found that producing DosP reduced extracellular indole by -3.9 ± 1.4 fold and intracellular indole by -2.9 ± 0.8 fold (**Fig. 1C**). Corroborating this result, inactivating DosP increased extracellular indole by 2.0 ± 0.3 fold and intracellular indole by 2.0 ± 0.02 fold. We also tested whether the decreased tryptophanase activity observed with DosP was unique by producing other c-di-GMP-related proteins and found that there was no significant difference in extracellular indole concentrations for DGCs AdrA (1.3 ± 0.2 fold) and DosC (1.1 ± 0.2 fold) or for PDEs Gmr (-1.3 ± 0.04 fold) and YjcC (1.4 ± 0.2 fold). The DGC AdrA substantially increases c-di-GMP levels (Antoniani et al. 2010; Tagliabue et al. 2010), while DosC serves as the DGC component of the DosP-DosC c-di-GMP module and also increases c-di-GMP (Méndez-Ortiz et al. 2006; Tagliabue et al. 2010). Conversely, both PDEs Gmr (YciR) (Weber et al. 2006) and YjcC reduce c-di-GMP via cleavage. Unlike the DGCs and PDEs, we found that c-di-GMP binding protein BdcA reduced extracellular indole (-2.0 ± 0.1 fold), although less significantly than DosP. The trends in both indole and persistence are not consistent among the DGCs and PDEs, again demonstrating that c-di-GMP is not correlated with persistence.

DosP increases persistence via reduced indole. Since DosP reduced indole, we sought to test whether the signal indole was mediating persistence. We recently found that addition of indole to a $\Delta tnaA$ strain decreased persister formation with multiple antibiotics in a dose-dependent manner by up to -52 ± 1 fold with 2 mM indole (**Fig. 2A and B**) (Hu et al. 2014). The indole concentrations tested were consistent with previous studies regarding the role of indole signaling in biofilm formation (Lee et al. 2007b), multidrug export (Hirakawa et al. 2005), and stress responses (Hirakawa et al. 2010) (concentrations of 0.5-2.0 mM). Furthermore, transient intracellular concentrations are reported to reach as high as 60 mM (Gaimster et al. 2014). Additionally supporting the importance of indole signaling in persistence, absence of the indole importer Mtr increased persistence 28 ± 11 fold (**Fig. 2C**). Corroborating the role of indole in DosP-mediated persistence, when TnaA and DosP were produced concurrently from two IPTG inducible plasmids (i.e., pBS(Kan)-*dosP* and pCA24N-*tnaA*) in the same host, DosP no longer induced persistence (**Fig. 2D**). Together, these three sets of experiments show conclusively that indole inversely regulates persistence and that DosP increases persistence via regulation of TnaA.

cAMP is cleaved by DosP and modulates persistence. Since there was no consistent relationship between c-di-GMP and persistence and since DosP was originally characterized as a cAMP phosphodiesterase (Sasakura et al. 2002), we investigated

the possibility that DosP may modulate tryptophanase activity and persistence through cAMP cleavage. If this is the case, it would suggest that BdcA has the same effects through an uncharacterized binding affinity for cAMP. Based on the microarray conducted for production of DosP (**Table II**), 37 of the 72 significantly affected transcripts showed differential expression consistent with a reduction in the cAMP-CRP transcriptional regulator. Critically, cAMP-CRP is a positive regulator for transcription of the *tna* operon (Stewart and Yanofsky 1985), responsible for indole synthesis.

Initially, we tested the impact of cAMP on persistence through CpdA, a cAMP-specific phosphodiesterase (Imamura et al. 1996). We found that production of CpdA (i.e., reduced cAMP) increased persistence 235 ± 15 fold (**Fig. 3A**). Furthermore, absence of the single adenylate cyclase (Δ *cyoA*) producing cAMP in *E. coli* (Tuckerman et al. 2009) increased persistence 19 ± 4 fold (**Fig. 3B**). Similar to results with DosP and BdcA, production of CpdA reduced extracellular indole (-9 ± 1.1 fold) and intracellular indole (-8 ± 0.2 fold) (**Fig. 3C**), suggesting analogous cAMP reduction. Also, a cAMP enzyme immunoassay was performed to verify that DosP reduced intracellular cAMP (-2 ± 0.1 fold) whereas the positive control CpdA essentially eliminated all cAMP (-323 ± 81 fold) (**Fig. 3D**). Hence, cAMP inversely controls persistence through direct regulation of indole. CpdA eliminated nearly all cAMP yet did not increase persistence as significantly as DosP, suggesting that cAMP regulation may not be the only mechanism through which DosP increases persistence.

Since glucose inhibits intracellular cAMP accumulation (Buettner et al. 1973), we also tested the effect of glucose supplementation on persistence from DosP production to determine the extent to which DosP persistence is dependent on reducing cAMP. We found that glucose reduced DosP-mediated persistence by -25 ± 6 fold (**Fig. 3E**), a result which clearly demonstrates the cAMP dependence of DosP persistence while also suggesting the existence of additional DosP persistence mechanisms. In addition to cAMP cleavage, DosP may further contribute to persistence through the general down-regulation of metabolism noted in the transcriptomic and proteomic studies (**Tables II and III**). Nonetheless, the distinctly reduced effect of DosP on persistence with low intracellular cAMP (i.e., with glucose) and the consistent results for both CpdA and DosP suggest that DosP increases persistence by cleaving cAMP, to reduce *tna* operon transcription and indole synthesis.

DISCUSSION

Our results show that cAMP inversely regulates persistence by inducing the *tna* operon which increases indole. The phosphodiesterase DosP has activity against both c-di-GMP and cAMP (Sasakura et al. 2002), and here DosP degradation of cAMP leads to reduced indole concentrations and increased persistence. DosP activity is directly dependent on oxygen concentrations, which suggests that DosP mediates persister cell formation with changing oxygen availability. Our proposed

regulatory pathway (Fig. 4) implicates both the internal regulator cAMP and the external signal indole as mediators of persistence.

It was previously proposed that DosP may signal increased oxygen concentrations present when cells exhibit slow metabolism (Tuckerman et al. 2009). Since producing DosP leads to higher persistence, then perhaps DosP contributes to the increased persistence observed in slow growing cultures. Additionally, DosP activity may be regulated in cells located within different layers of biofilms, which contain a gradient of oxygen levels (Tuckerman et al. 2009). Oxygen content is higher in the top layer of biofilms due to air exposure (Rani et al. 2007), so DosP may act as a mechanism to maintain a persister sub-population among cells in the outer layer of biofilms.

cAMP concentrations fluctuate based on carbon metabolism (Rickenberg 1974) and exogenous addition of cAMP has been purported to increase persistence (Amato et al. 2013). Since cAMP uptake occurs via facilitated diffusion (i.e., energy-independent transport) (Goldenbaum and Hall 1979), exogenous cAMP is likely internalized. Therefore, cAMP was proposed to increase persistence by stimulating RelA expression to produce the stringent response alarmone ppGpp (Amato et al. 2013), which was previously implicated in multiple persister formation pathways (Wood et al. 2013). In contrast, our results show that decreasing cellular cAMP via phosphodiesterases increases persistence via diminished indole. Therefore, the specific role of cAMP in persistence may be complex, likely due to the vast size of the cAMP-CRP regulon (more than 180 genes) (Tan et al. 2001; Zheng et al. 2004). Versatile involvement of cAMP in persistence provides bacterial populations with mechanisms for maintaining persister subpopulations across a number of different growth conditions. Therefore, cAMP adds to the redundancy of persister mechanisms, similarly characterized for toxin/antitoxin systems (Wood et al. 2013), and ensures the presence of a persistent population to prevent sterilization of a bacterial population.

Our results indicating that indole reduces persistence are in contrast to those reported previously (Vega et al. 2012) but are consistent with our recent results involving endoribonuclease toxin YafQ of the *E. coli* YafQ/DinJ toxin/antitoxin system; we found that YafQ increases persistence by decreasing indole concentrations, resulting from a reduction in TnaA (Hu et al. 2014). Since *tnaA* is repressed in *E. coli* biofilm cultures (Ren et al. 2004a), our findings suggest that the total cell population contributes to high persister levels in biofilms by reducing intercellular signaling through indole. This is also reasonable since indole reduces biofilm formation (Domka et al. 2006; Lee et al. 2007a; Lee et al. 2007b; Lee et al. 2009b).

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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Table I. Bacterial strains and plasmids used in this study.

Strain	Genotype	Source
BW25113	<i>rrnB3</i> Δ <i>lacZ4787</i> <i>hsdR514</i> Δ (<i>araBAD</i>)567 Δ (<i>rhaBAD</i>)568 <i>rph-1</i>	(Baba et al. 2006)
BW25113 Δ <i>adrA</i>	BW25113 Δ <i>adrA</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>bdcA</i>	BW25113 Δ <i>bdcA</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>dosC</i>	BW25113 Δ <i>dosC</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>dosP</i>	BW25113 Δ <i>dosP</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>gmr</i>	BW25113 Δ <i>gmr</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>rtn</i>	BW25113 Δ <i>rtn</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>tnaA</i>	BW25113 Δ <i>tnaA</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yahA</i>	BW25113 Δ <i>yahA</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>ycdT</i>	BW25113 Δ <i>ycdT</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>ycgF</i>	BW25113 Δ <i>ycgF</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>ydaM</i>	BW25113 Δ <i>ydaM</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>ydeH</i>	BW25113 Δ <i>ydeH</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>ydiV</i>	BW25113 Δ <i>ydiV</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yeaI</i>	BW25113 Δ <i>yeaI</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yeaJ</i>	BW25113 Δ <i>yeaJ</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yeaP</i>	BW25113 Δ <i>yeaP</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yedQ</i>	BW25113 Δ <i>yedQ</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yegE</i>	BW25113 Δ <i>yegE</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yfeA</i>	BW25113 Δ <i>yfeA</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yfgF</i>	BW25113 Δ <i>yfgF</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yfiN</i>	BW25113 Δ <i>yfiN</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yhdA</i>	BW25113 Δ <i>yhdA</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yhjK</i>	BW25113 Δ <i>yhjK</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yhjH</i>	BW25113 Δ <i>yhjH</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yjcC</i>	BW25113 Δ <i>yjcC</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>ylaB</i>	BW25113 Δ <i>ylaB</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yliE</i>	BW25113 Δ <i>yliE</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yliF</i>	BW25113 Δ <i>yliF</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yneF</i>	BW25113 Δ <i>yneF</i> Ω Km ^R	(Baba et al. 2006)
BW25113 Δ <i>yoaD</i>	BW25113 Δ <i>yoaD</i> Ω Km ^R	(Baba et al. 2006)

Plasmid

pCA24N	Cm ^R ; <i>lacI</i> ^q , pCA24N	(Kitagawa et al. 2005)
pCA24N- <i>adra</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>adra</i>	(Kitagawa et al. 2005)
pCA24N- <i>bdcA</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>bdcA</i>	(Kitagawa et al. 2005)
pCA24N- <i>dosC</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>dosC</i>	(Kitagawa et al. 2005)
pCA24N- <i>dosP</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>dosP</i>	(Kitagawa et al. 2005)
pCA24N- <i>gmr</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>gmr</i>	(Kitagawa et al. 2005)
pCA24N- <i>yahA</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yahA</i>	(Kitagawa et al. 2005)
pCA24N- <i>yaiC</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yaiC</i>	(Kitagawa et al. 2005)
pCA24N- <i>ycgF</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>ycgF</i>	(Kitagawa et al. 2005)
pCA24N- <i>ydaM</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>ydaM</i>	(Kitagawa et al. 2005)
pCA24N- <i>ydeH</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>ydeH</i>	(Kitagawa et al. 2005)
pCA24N- <i>ydiV</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>ydiV</i>	(Kitagawa et al. 2005)
pCA24N- <i>yeaI</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yeaI</i>	(Kitagawa et al. 2005)
pCA24N- <i>yeaJ</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yeaJ</i>	(Kitagawa et al. 2005)
pCA24N- <i>yeaP</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yeaP</i>	(Kitagawa et al. 2005)
pCA24N- <i>yedQ</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yedQ</i>	(Kitagawa et al. 2005)
pCA24N- <i>yegE</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yegE</i>	(Kitagawa et al. 2005)
pCA24N- <i>yfeA</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yfeA</i>	(Kitagawa et al. 2005)
pCA24N- <i>yfgF</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yfgF</i>	(Kitagawa et al. 2005)
pCA24N- <i>yfiN</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yfiN</i>	(Kitagawa et al. 2005)
pCA24N- <i>yhdA</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yhdA</i>	(Kitagawa et al. 2005)
pCA24N- <i>yhjH</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yhjH</i>	(Kitagawa et al. 2005)
pCA24N- <i>yjcC</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yjcC</i>	(Kitagawa et al. 2005)
pCA24N- <i>ylaB</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>ylaB</i>	(Kitagawa et al. 2005)
pCA24N- <i>yliE</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yliE</i>	(Kitagawa et al. 2005)
pCA24N- <i>yliF</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yliF</i>	(Kitagawa et al. 2005)
pCA24N- <i>yneF</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yneF</i>	(Kitagawa et al. 2005)
pCA24N- <i>yoaD</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>yoaD</i>	(Kitagawa et al. 2005)
pCA24N- <i>cpdA</i>	Cm ^R ; <i>lacI</i> ^q , pCA24N P _{T5-lac} :: <i>cpdA</i>	(Kitagawa et al. 2005)
pBS(Kan)	Km ^R ; pBS(Kan)	(Canada et al. 2002)
pBS(Kan)- <i>dosP</i>	Km ^R ; pBS(Kan) P _{lac} :: <i>dosP</i>	This study

Table II. Summary of the largest changes in gene expression as a result of producing DosP (i.e., BW25113/pCA24N-*dosP* vs. BW25113/pCA24N). Differentially-expressed genes regulated by reduced cAMP-CRP are in bold.

Gene	b number	Fold Change	Description
<u>Amino Acid Biosynthesis, Catabolism, and Transport</u>			
<i>trpE</i>	b1264	-8.0	Anthranilate synthase, <i>trp</i> operon
<i>trpL</i>	b1265	-7.5	<i>trp</i> operon leader peptide
<i>trpD</i>	b1263	-6.5	Anthranilate phosphoribosyl transferase, <i>trp</i> operon
<i>tnaA</i>	b3708	-7.5	Tryptophanase, makes indole, <i>tna</i> operon
<i>tnaL</i>	b3707	-6.1	Tryptophanase leader peptide, <i>tna</i> operon
<i>mtr</i>	b3161	-4.9	Tryptophan and indole permease (import)
<i>astC</i>	b1748	-4.9	Arginine catabolic pathway
<i>dppA</i>	b3544	-4.9	Binding component of dipeptide ABC transporter (import)
<i>dppD</i>	b3541	-4.6	ATP binding component of dipeptide ABC transporter (import)
<i>glnH</i>	b0811	-4.6	Component of glutamine ABC transporter (import)
<i>sdaC</i>	b2796	-4.6	Serine proton-symporter (import)
<i>gcvH</i>	b2904	-4.9	H-protein of glycine cleavage system
<i>gcvT</i>	b2905	-4.9	T-protein of glycine cleavage system
<i>gcvP</i>	b2903	-4.0	P-protein of glycine cleavage system
<i>cstA</i>	b0598	-4.0	Peptide transporter (import), induced by CsrA during carbon starvation
<i>aspA</i>	b4139	-4.0	Aspartate-ammonia lyase
<u>Carbohydrate Catabolism and Transport</u>			
<i>gatC</i>	b2092	-7.5	Galactitol-specific enzyme IIC of phosphotransferase system
<i>gatB</i>	b2093	-7.5	Galactitol-specific enzyme IIB of phosphotransferase system
<i>gatD</i>	b2091	-6.5	Galactitol-1-phosphate dehydrogenase
<i>gatZ</i>	b2095	-6.5	GatYZ subunit
<i>gatA</i>	b2094	-5.7	Galactitol-specific enzyme IIA of phosphotransferase system
<i>gatY</i>	b2096	-4.6	GatYZ subunit
<i>acs</i>	b4069	-8.0	Acetyl-CoA synthetase
<i>yjcH</i>	b4068	-6.1	Cotranscribed with <i>acs</i>
<i>actP</i>	b4067	-5.7	Cotranscribed with <i>acs</i>, acetate/glycolate permease, <i>acs</i> pathway
<i>sdhC</i>	b0721	-5.7	Cytochrome b556 with SdhD, succinate dehydrogenase
<i>sdhD</i>	b0722	-5.7	Cytochrome b556 with SdhC, succinate dehydrogenase
<i>sdhA</i>	b0723	-4.0	Succinate dehydrogenase
<i>sucC</i>	b0728	-5.7	Succinyl CoA synthase subunit, CoA binding site
<i>sucB</i>	b0727	-4.9	Forms succinyl-CoA, <i>suc</i> operon
<i>gabD</i>	b2661	-5.3	Succinate semialdehyde dehydrogenase, utilizes NADP+
<i>gabT</i>	b2662	-5.3	4-aminobutyrate aminotransferase, GABA degradation pathway
<i>fbxB</i>	b2097	-5.3	Fructose biphosphate aldolase
<i>aldA</i>	b1415	-5.3	Aldehyde dehydrogenase A, repressed anaerobically by ArcA
<i>mglB</i>	b2150	-4.9	Binding component of galactose ABC transporter (import)
<i>rbsB</i>	b3751	-4.9	Component of ribose ABC transporter (import)
<i>csrB</i>	b4408	-4.6	Regulator of CsrA (carbohydrate metabolism and biofilm regulator)
<i>malE</i>	b4034	-4.6	Substrate binding component of maltose ABC transporter (import)

<i>pckA</i>	b3403	-4.3	PEP carboxykinase, gluconeogenesis
<i>rbsD</i>	b3748	-4.3	Ribose pyranase, required for efficient utilization of ribose
<i>ptsH</i>	b2415	-4.0	Sugar-non-specific component of PEP phosphotransferase system
<i>maeB</i>	b2463	-4.0	Component of malate dehydrogenase, citric acid cycle/gluconeogenesis
<i>eno</i>	b2779	-4.0	Phosphopyruvate hydratase (enolase), glycolysis

Stationary Phase Ribosome Interaction

<i>yfiA</i>	b2597	-7.0	Involved in ribosome modulation during stationary phase
<i>yqjD</i>	b3098	-4.0	Associates with 30S subunit of 70S and 100S ribosome during stationary phase

Membrane Transport Proteins

<i>ompC</i>	b2215	-7.0	Outer membrane protein
<i>lamB</i>	b4036	-6.1	Maltose sugar porin
<i>nmpC</i>	b0553	-5.3	General bacterial porin, silent gene in <i>E. coli</i> K-12

Stress Response

<i>katE</i>	b1732	-4.6	Catalase hydroperoxidase II, oxidative stress response
<i>ndk</i>	b2518	-4.6	Nucleoside diphosphate kinase, suggested involvement in stringent response
<i>ynaF</i>	b1376	-4.3	Stress induced protein, promotes adhesion over motility
<i>cspC</i>	b1823	-4.3	Cold-shock protein linked to <i>rpoS</i> mRNA stability
<i>hdeB</i>	b3509	-4.0	Acid stress response chaperone
<i>hdeA</i>	b3510	-4.0	Acid stress response

Miscellaneous Genes Induced by Stationary Phase or Starvation

<i>cspD</i>	b0880	-5.7	Toxin related to persister cells, increased in stationary phase
<i>csiD</i>	b2659	-5.7	Starvation induced gene, unknown function
<i>wrbA</i>	b1004	-5.7	Flavodoxin protein family, increased in stationary phase
<i>ftn</i>	b1905	-5.3	Iron storage protein ferritin, increased in stationary phase
<i>ygaF</i>	b2660	-5.3	L-2 hydroxyglutarate oxidase, increased in stationary phase
<i>rpsV</i>	b1480	-5.7	30S ribosomal subunit, increased in stationary phase
<i>yeaG</i>	b1783	-4.0	Unknown function, increased in stationary phase or acid/salt stress

Miscellaneous Genes or Unknown Function

<i>ryeE</i>	b4438	-4.9	Small regulatory RNA, cAMP activated
<i>msyB</i>	b1051	-4.9	Restores growth and protein export of <i>secY</i> and <i>secA</i> mutants
<i>malM</i>	b4037	-4.3	Unknown function, maltose regulon
<i>mipA</i>	b1782	-4.3	Scaffolding protein for murein synthesis
<i>fabA</i>	b0954	-4.3	Subunit of 3R-3-hydroxydecanoyl-ACP dehydratase
<i>yccJ</i>	b1003	-4.3	Predicted protein, unknown function
IS092	b4434	16.0	Small RNA (<i>isrB</i>), unknown function
<i>ibpB</i>	b3686	7.5	Small heat-shock protein
<i>spf</i>	b3864	6.1	non-translated protein, affects DNA polymerase I activity, inhibits <i>galK</i> (galactose metabolism)
<i>ybbD</i>	b0500	4.6	Conserved hypothetical protein, unknown function
<i>ydcL</i>	b1431	4.6	Lipoprotein, unknown function

Table III. Summary of the largest changes in protein levels as a result of producing DosP (i.e., BW25113/pCA24N-*dosP* vs. BW25113/pCA24N).

Protein	Accession	Fold Change	Description
TnaA	Q5UES8	-33.1	Tryptophanase
Replication			
YjiU	C3SIZ2	-57.2	Cell division, coordinate FtsZ for division
Phosphate Allocating Proteins			
Upp	E2QPW1	-14.3	Uracil phosphoribosyltransferase
Ndk	E2QPY4	-4.6	Nucleoside diphosphate kinase, involved in stringent response
Stress Response Proteins			
SspA	C3SRY7	-20.2	Induced by stringent starvation, inhibits accumulation of H-NS
Dps	E2QII0	-9.9	Starvation stress response
KatG	E2QIX4	-7.7	Catalase, oxidative stress response
UspE	C3TBD7	-5.3	Resistance to UV irradiation, stress inducible
Global Regulatory Proteins			
Hns	C3TCN2	-5.4	Global transcriptional regulator
IhfA	Q14F23	-3.2	Global transcriptional regulator
Chaperone/Translation/Housekeeping Proteins			
YhbC	C3SSN2	-14.9	Assists in maturation of 30S ribosomal subunit
FusA	C3SQS7	-14.1	Elongation factor G, facilitates ribosomal translocation
ClpB	E2QQ51	-5.9	ATP dependent protease
GroL	Q548M1	-4.3	Hsp60 chaperone for protein folding
RhlB	E2QHV7	-3.5	RNA helicase of degradosome
TufB	E2QFJ4	-3.4	Elongation factor Tu, coordinates charged tRNA
HslO	E2QFQ1	3.6	Hsp33, expressed under heat shock and activated by oxidative stress
Tig	E2QGI0	2.3	Chaperone involved in folding nascent cytosolic proteins
Metabolic Proteins			
AceE	E2QF44	-53.6	Component of pyruvate dehydrogenase complex
AceA	E2QJ37	-37.8	Isocitrate lyase, glyoxylate cycle
AceB	E2QJ36	-13.4	Malate synthase, glyoxylate cycle
FabB	E2QPI4	-9.8	Fatty acid biosynthesis
GapA	C3T6W2	-8.5	G3P dehydrogenase, required for glycolysis
SucD	C3TIK7	-5.4	Succinyl-CoA synthase
Pta	E2QPF8	-4.9	Acetate metabolism
AtpH	C3SL92	-3.3	Component of ATP synthase
DapD	E2QF93	-2.6	Lysine biosynthetic pathway
Ribosomal Proteins			
RpsA	C3TGB2	-7.9	S1 component of 30S ribosomal subunit
RpsF	C3SFQ7	-5.4	S6 component of 30S ribosomal subunit
RplD	C3SQU7	-4.8	L4 component of 50S ribosomal subunit
RpsG	C3SQS2	-3.5	S7 component of 30S ribosomal subunit
RplA	C3SIC2	2.6	L1 component of 50S ribosomal subunit
RplF	C3SR17	2.0	L6 component of 50S ribosomal subunit
Miscellaneous and Uncharacterized Proteins			
TorC	E2QJN9	-8.5	Inner membrane cytochrome
ElaB	C3T2J7	-6.3	Unknown function
YbfN	E2QI69	-3.5	Unknown function

FIGURE LEGENDS

Fig. 1. DosP is an active phosphodiesterase *in vivo* that reduces indole. (A) Persister cell formation for BW25113/pCA24N-*dosP* and BW25113/pCA24N grown to a turbidity of 1.0 in LB medium, induced with 1 mM IPTG for 2 h, adjusted to a turbidity of 1.0, and exposed to 100 μ g/mL ampicillin or 5 μ g/mL ciprofloxacin for 3 h. (B) Biofilm formation for BW25113/pCA24N-*dosP* and BW25113/pCA24N after 24 h of static growth at 37°C with and without 1 mM IPTG induction in LB medium. (C) Extracellular and intracellular indole concentrations for BW25113/pCA24N-*dosP* and BW25113/pCA24N after 2 h of DosP production via 1 mM IPTG. All data are averaged from two independent cultures and one standard deviation is shown.

Fig. 2. DosP induces persistence via reduced indole. Persister cell formation for the following strains and conditions: (A and B) BW25113 Δ *tnaA* grown to a turbidity of 2.0 in LB medium, exposed to 0, 0.5, 1, and 2 mM indole for 2 h, adjusted to a turbidity of 1.0, and exposed to (A) 100 μ g/mL ampicillin or (B) 5 μ g/mL ciprofloxacin for 2 h, (C) BW25113 Δ *mtr* and wild-type grown to a turbidity of 1.0 in LB medium and exposed to 100 μ g/mL ampicillin for 3 h, and (D) BW25113/pCA24N/pBS(Kan), BW25113/pCA24N/pBS(Kan)-*dosP*, and BW25113/pCA24N-*tnaA*/pBS(Kan)-*dosP* grown to a turbidity of 1.0 in LB medium supplemented with 2 mM *L*-tryptophan, induced with 1 mM IPTG for 2 h, adjusted to a turbidity of 1.0, and exposed to 100 μ g/mL ampicillin for 3 h. All data are averaged from at least two independent cultures and one standard deviation is shown.

Fig. 3. cAMP is cleaved by DosP and regulates indole and persistence. Persister cell formation for the following strains and conditions: (A) BW25113/pCA24N-*cpdA* and BW25113/pCA24N grown to a turbidity of 1.0 in LB medium, induced with 1 mM IPTG for 2 h, adjusted to a turbidity of 1.0, and exposed to 100 μ g/mL ampicillin for 3 h and (B) BW25113 Δ *cyaA* and wild-type grown to a turbidity of 1.0 in LB medium and exposed to 100 μ g/mL ampicillin for 3 h. (C) Extracellular and intracellular indole concentrations for BW25113/pCA24N-*cpdA* and BW25113/pCA24N after 2 h of CpdA production via 1 mM IPTG. (D) Cellular cAMP concentrations for BW25113/pCA24N-*dosP*, BW25113/pCA24N-*cpdA*, and BW25113/pCA24N after 2 h of induction via 1 mM IPTG. (E) Persister cell formation for BW25113/pCA24N-*dosP* and BW25113/pCA24N grown to a turbidity of 1.0 in LB medium with and without 1% (w/v) glucose, induced with 1 mM IPTG for 2 h, adjusted to a turbidity of 1.0, and exposed to 100 μ g/mL ampicillin for 3 h. All data are averaged from at least two independent cultures and one

standard deviation is shown.

Fig. 4. Schematic for persistence induced by DosP production. DosP is a phosphodiesterase that is activated by oxygen and cleaves both c-di-GMP and cAMP. cAMP and CRP form a transcriptional regulation complex which induces expression of the *tna* operon. TnaA synthesizes indole from *L*-tryptophan, and indole reduces persistence. Curved arrows indicate enzymatic reactions, \rightarrow indicates induction, and \perp indicates inhibition.

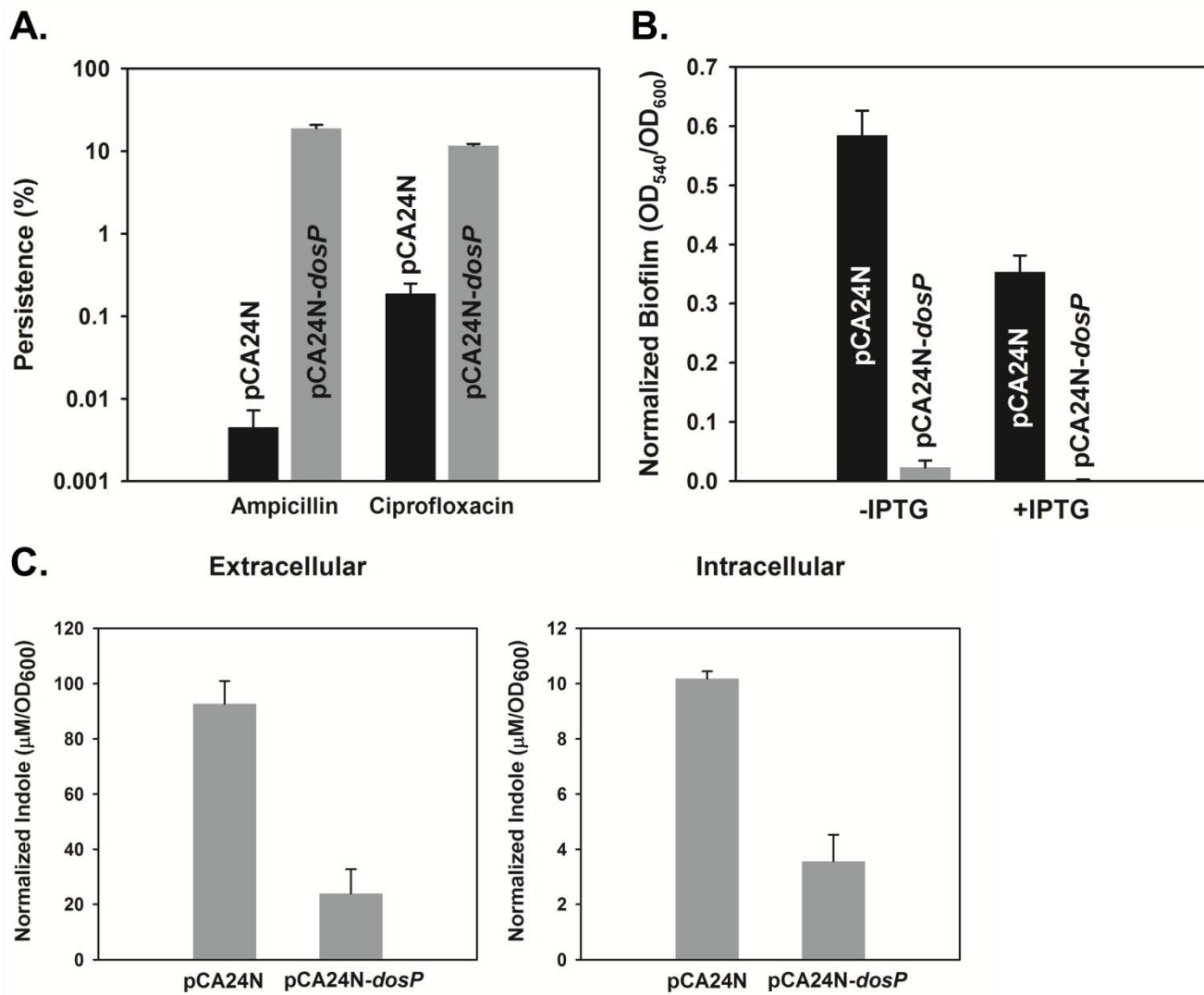


Figure 1

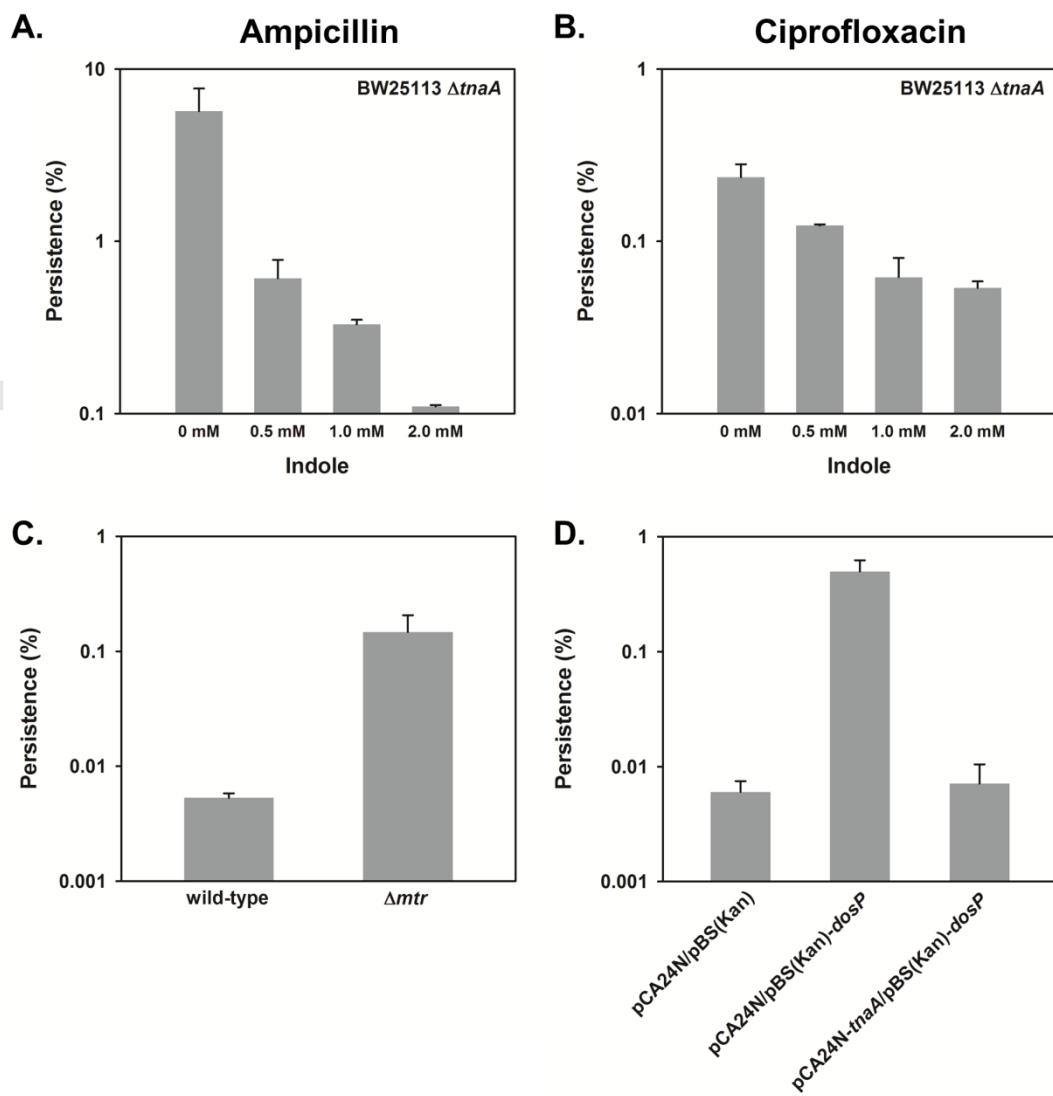


Figure 2

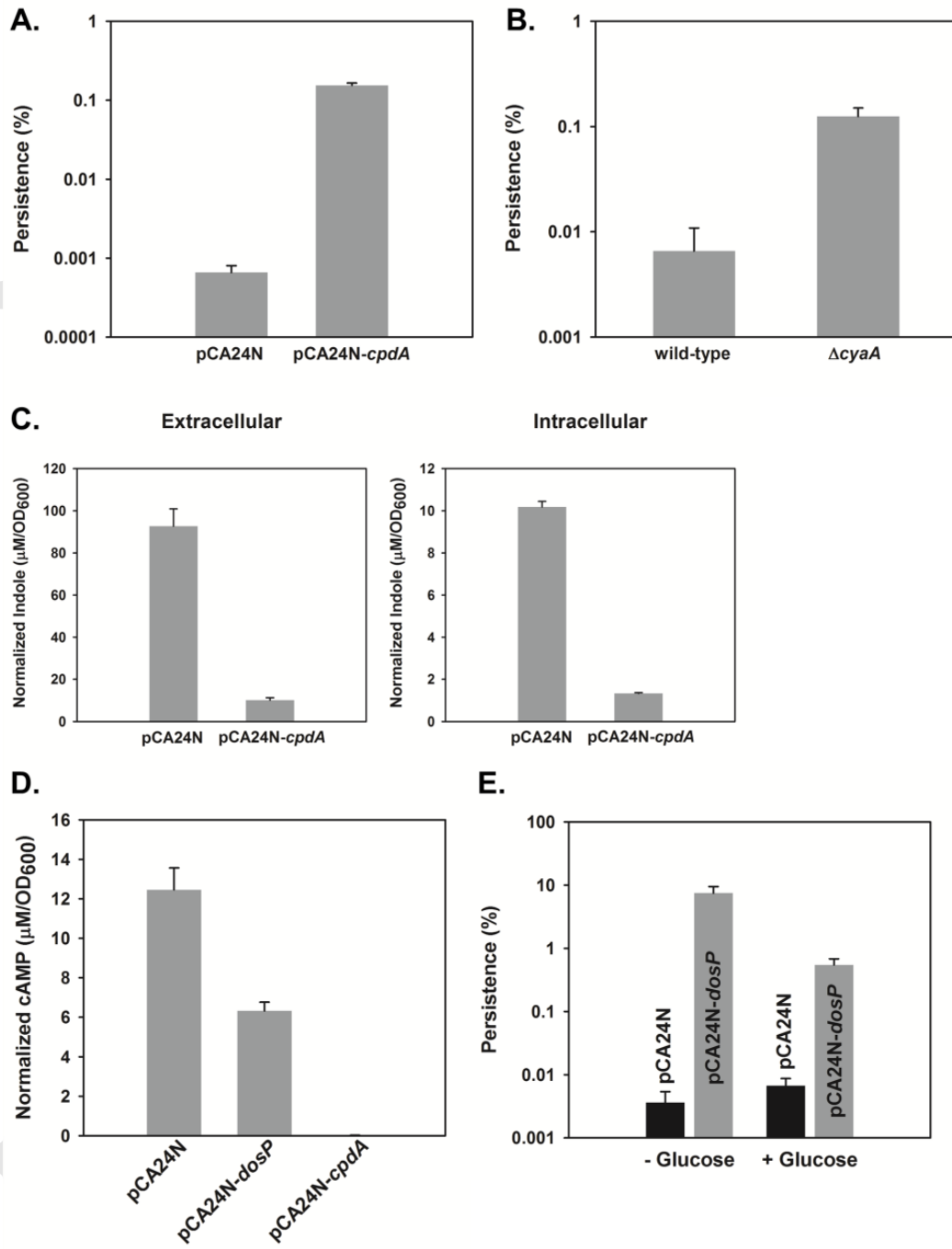


Figure 3

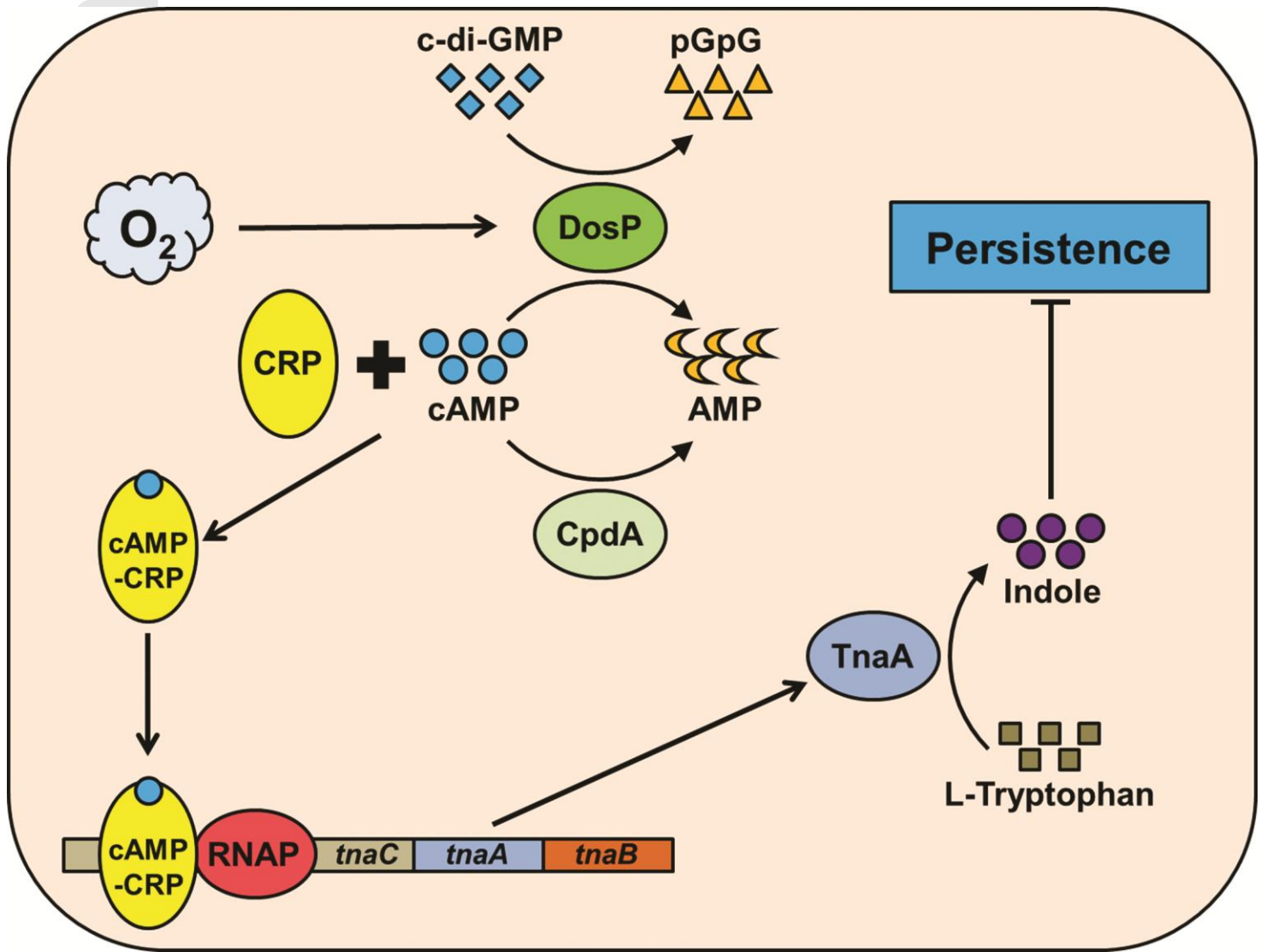


Figure 4