The Evolution of SlyA/RovA Transcription Factors

From Repressors to Counter-Silencers in *Enterobacteriaceae*

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Abstract

Gene duplication and subsequent evolutionary divergence have allowed conserved proteins to develop unique roles. The MarR family of transcription factors (TFs) has undergone extensive duplication and diversification in bacteria, where they act as environmentally-responsive repressors of genes encoding efflux pumps that confer resistance to xenobiotics, including many antimicrobial agents. We have performed structural, functional, and genetic analyses of representative members of the SlyA/RovA lineage of MarR TFs, which retain their ancestral functions, including repression of their own expression and that of divergently-transcribed multi-drug efflux pumps, as well as allosteric inhibition by aromatic carboxylate compounds. In contrast to other MarR family members, SlyA and RovA have acquired the ability to counter-silence horizontally-acquired genes, which has greatly facilitated the evolution of *Enterobacteriaceae* by horizontal gene transfer. SlyA/RovA TFs in different species have independently evolved novel regulatory circuits to provide the enhanced levels of expression required for their new role. This demonstrates the ability of TFs to acquire new functions as a result of evolutionary divergence of *cis*-regulatory sequences.

Introduction

As organisms adapt to new or changing environments, their regulatory networks must evolve to ensure that individual genes are appropriately expressed in response to environmental signals (1). An important mechanism for the evolution of conserved essential proteins, including transcription factors (TFs), is gene duplication, which allows the subsequent diversification of gene and protein families and the development of new functions (2). More than 50% of bacterial genes are believed to have descended from original duplication events (3-5), providing a broad foundation from which bacteria can evolve complex and adaptive traits.

The MarR family is an ancient family of TFs, predating the divergence of archaea and bacteria (6). It has undergone extensive gene duplication events, with recent estimates suggesting that bacteria encode an average of seven MarR TFs per genome (7). MarR TFs typically function as environmentally-responsive repressors of genes encoding efflux pumps that export xenobiotics, including many antimicrobial agents. The prototypical MarR protein of *Escherichia coli* represses a single operon, *marRAB*, which encodes a transcriptional activator (MarA) required for the expression of the AcrAB efflux pump, which in turn confers resistance to β-lactams, quinolones, and tetracyclines (8-10). MarR is allosterically regulated by many small molecules, in particular small aromatic carboxylate compounds such as salicylate, which induce a structural change that reduces the affinity of MarR for DNA (9, 11-13) and derepresses the expression of its cognate promoters. MarR TFs are defined by the presence of a winged helix-turn-helix (wHTH) DNA-binding domain (14). Dimerization allows MarR TFs to recognize palindromic sequences via the α4 recognition helix, which makes sequence-specific contacts with the major groove, while the wing makes sequence-independent contacts via the minor groove (15).

The SlyA lineage of MarR TFs originally resulted from a MarR family gene duplication. SlyA has been best characterized in *Salmonella enterica* serovar Typhimurium, where it serves primarily to upregulate virulence genes (16-18). Although this contrasts with the classical repressive role of MarR TFs, work in our and other laboratories has demonstrated that SlyA positively regulates genes by a counter-silencing mechanism, in which repression of AT-rich promoters by the histone-like nucleoid-associated protein H-NS is relieved by SlyA (16, 19). SlyA cooperatively remodels the H-NS-DNA complex in concert with the response regulator PhoP (19, 20), which is activated by conditions found within phagosomal compartments, including low Mg^{2+}(21), acidic pH (22), and cationic antimicrobial peptides (23). SlyA orthologs, represented by Hor, Rap, and RovA in *Pectobacterium* (*Erwinia*), *Serratia*, and *Yersinia*, respectively (24), are conserved in every species of *Enterobacteriaceae*, even including endosymbionts such as *Sodalis glossinidius* (25), which have undergone extensive gene loss and degenerative evolution (26). This high degree of conservation suggests that the SlyA lineage occupies an essential role in the regulatory network organization of *Enterobacteriaceae*. Although conclusive mechanistic evidence to demonstrate that other SlyA orthologs function as counter-silencers has not yet been obtained, existing evidence is strongly suggestive of counter-silencing, as several are known to up-regulate horizontally-acquired traits, which are generally repressed by H-NS, in a number of species, including *Yersinia* spp. (27-29), *Dickeya dadantii* (30), *Pectobacterium carotovorum* (24) and *Shigella flexneri* (31).

We sought to understand the evolutionary transition of the SlyA/RovA TF lineage from the ancestral function of MarR family TFs as environmentally-responsive and dedicated repressors of small regulons to counter-silencers of extensive networks of horizontally-acquired genes. To do this, we performed structural and comparative analyses of representative members of the SlyA

lineage to identify the evolutionary changes that allowed SlyA to adopt its new role. Here we show that SlyA has retained the ability to undergo conformational changes in response to aromatic carboxylates, regulate gene expression in an environmentally-responsive manner, and repress the expression of a linked drug efflux system. We further show that SlyA/RovA promoters have evolved to support the higher levels of expression that are required for counter-silencing. Finally, we show that linked efflux pumps are not conserved in some *Enterobacteriaceae*, even though SlyA/RovA TFs have been evolutionarily retained, suggesting that these regulators have been conserved not due to their primordial role in regulating antimicrobial resistance but rather as a consequence of their counter-silencing function, which is essential to maintain the regulated expression of horizontally-acquired genes in *Enterobacteriaceae*.

Results

Salicylate-mediated inhibition of SlyA activity. As environmentally-responsive repressors whose conformation and regulatory actions are modulated by small aromatic carboxylates (11, 13), MarR family TFs are inhibited by salicylate *in vitro* (11). To determine whether the SlyA lineage has retained ancestral MarR characteristics, we performed *in vitro* transcription assays (IVTs) of *slyA* and the divergently transcribed *ydhIJK* efflux pump operon. Supercoiled plasmid DNA containing the *slyA-ydhIJK* region was incubated with RNA polymerase (RNAP) and increasing SlyA concentrations in the presence or absence of salicylate. SlyA repressed *slyA* transcription approximately 5.3-fold, while *ydhI* transcription was inhibited ~19-fold (Fig. 1A, B). The addition of 2mM sodium salicylate reduced SlyA-mediated repression to 2.8-fold and 3.2-fold, respectively, indicating that the sensitivity to aromatic carboxylates observed in classical MarR TFs has been retained by SlyA.

We then confirmed that the *ydhIJK* operon encodes a functional antimicrobial efflux pump by growing wildtype, *slyA*, *ydhIJK*, and *slyA ydhIJK* mutant strains in the presence of an aromatic carboxylate with antimicrobial activity, fusaric acid (Fig. 1C). Both the *ydhIJK* and *slyA ydhIJK* mutant strains exhibited delayed growth in the presence of fusaric acid relative to the wildtype strain, suggesting that *ydhIJK* confers fusaric acid resistance in wildtype *S*. Typhimurium. Conversely, *slyA* mutants exhibit improved growth in the presence of fusaric acid, as predicted when *ydhIJK* is derepressed. Collectively, these observations indicate that the *S.* Typhimurium SlyA TF has retained ancestral functions characteristic of the MarR family.

To determine whether salicylate inhibits SlyA-mediated counter-silencing as well as repression, expression of the counter-silenced *pagC* gene was measured by qRT-PCR in the presence or absence of 2mM sodium salicylate (Fig. 1D). Wildtype cultures grown in salicylate phenocopied a *slyA* mutant strain, with a >14-fold reduction in *pagC* expression, indicating that salicylate is a general allosteric inhibitor of SlyA function, most likely inducing a structural change that reduces affinity for DNA as described in other MarR TFs (9, 14). Unexpectedly, SlyA retained an ability to interact with DNA upstream of the *ydhI* promoter even in the presence of salicylate (Fig. S3), but this interaction may be non-specific, as salicylate inhibited SlyA interaction with a 20 bp DNA segment centered near the -35 position of the *ydhI* TSS, which exhibited the strongest binding by SlyA.

To determine whether salicylate-mediated inhibition correlates with structural changes in SlyA, ^{1}H,^{15}N-TROSY NMR spectra of *S.* Typhimurium SlyA in the presence or absence of salicylate were collected. The apo-SlyA spectrum is well dispersed (Fig. 2), with ~85% of the expected resonances observed. However, wide variation among individual resonances with respect to peak width and intensity may signify [1] weak non-specific interactions between SlyA dimers,

[2] varying rates of exchange of amide protons with solvent, or [3] conformational exchange. Previous studies of MarR found that apo-MarR is also highly disordered (14), suggesting that the observed spectral characteristics of apo-SlyA can be ascribed to the presence of multiple conformational states in solution. Addition of salicylate to SlyA induces chemical shift perturbations throughout the spectrum and nearly 90% of expected resonances are now observed. The large-scale changes in chemical shifts show that backbone amides throughout the protein are stabilized in a different environment relative to apo-SlyA. These observations are consistent with ligand binding to SlyA dimers inducing global structural changes, likely stabilizing a single protein conformation in solution, a conformation that is no longer able to interact with specific high-affinity DNA binding sites.

Crystal structure of salicylate-SlyA. To further analyze the mechanism of allosteric inhibition of SlyA, the structure of the salicylate-SlyA co-crystal was determined. Studies by other groups have previously determined the structure of apo-SlyA and the SlyA bound to DNA (32), demonstrating that SlyA is similar in overall structure to other MarR proteins, consisting of six alpha helices (Fig. 3). Helices α1, α5, and α6 make up the dimerization domain, while α3 and α4, along with the wing region between α4 and α5 comprise the wHTH DNA-binding domain. These two domains are separated by α2. Dolan *et al.* (32) previously observed that the two recognition helices of the apo-SlyA dimer are only ~15Å apart, in a closed conformation. During interaction with a high-affinity binding site, the helices move a significant distance to accommodate the 32Å distance between major grooves.

SlyA formed large crystals in the presence of 75mM sodium salicylate (Fig. S4). However, we were unable to obtain usable crystals of apo-SlyA. Diffraction data set and refinement statistics

are summarized in Tables S1 and S2, respectively. The two SlyA molecules in the asymmetric unit form two different SlyA dimers in this crystal form. Space group symmetry operations generate the other subunit in each dimer. The dimers are very similar in structure, and further discussion will focus on the dimer formed by polypeptide chain A. We were unable to observe electron density for the tips of the wings, so these regions are absent from our structural model. Difference electron density maps (|Fo|-|Fc|) identified two salicylate molecules bound per SlyA monomer at sites referred to as Site I and Site II (Fig. 3A-C). Salicylate molecules interact with these sites via hydrophobic interactions with their aromatic rings, while the carboxylate and hydroxyl groups are positioned to interact with the solvent. Site I is composed of residues from α2, α3, and α4, as well as I58 in the loop between α3 and α4, and is well positioned to sterically inhibit DNA binding. It should be noted that the residue numbers in the deposited PDB file are not in register with the residue numbers in this text which are based on alignment of SlyA orthologs. Comparison with apo-SlyA and SlyA-DNA structures (32) indicates that this salicylate molecule causes the α4 recognition helix to rotate by ~35° around its axis, disrupting specific contacts with the DNA major groove. Site II is formed by residues from both dimer subunits, almost completely sequestering the salicylate molecule from the solvent. The buried polar groups of salicylate interact with S7' and R14' in α1 of one subunit and R17 in α1 and H38 in α2 from the other subunit. A third salicylate binding site was observed on the surface of each subunit of the dimer. However, this site is adjacent to SlyA residues involved in crystal packing contacts and may not be biologically relevant.

Evolutionary analysis of the SlyA TF lineage. To identify other variables that might have contributed to the evolution of the SlyA lineage, we performed an evolutionary analysis of

representative SlyA proteins throughout the *Enterobacteriaceae*. Phylogenetic analysis reveals four clusters of SlyA orthologs (Fig. 4A). SlyA orthologs in enteric pathogens including *E. coli*, *S.* Typhimurium, and *S. flexneri* form cluster I. Orthologs in more distantly related pathogens such as *Yersinia* spp. and *Proteus mirabilis* make up cluster II, while orthologs in insect endosymbionts such as *S. glossinidius* and plant pathogens such as *D. dadantii* make up clusters III and IV, respectively. Despite having significantly diverged from cluster I, orthologs in both clusters II and IV are known to function as pleiotropic regulators (27, 30). This suggests that the SlyA lineage began to diverge from the ancestral function of MarR TFs prior to the divergence of *Enterobacteriaceae* from other bacteria. To determine if allosteric inhibition is likely to be conserved throughout the SlyA lineage, we aligned the sequences of SlyA orthologs from different bacterial species to analyze the conservation of residues involved in salicylate binding (Fig. S5, Table S3). Cluster I exhibited complete conservation of the salicylate-binding residues. Cluster II exhibited the greatest variation among the free-living species, with both the *Proteus mirabilis* and *Y. pseudotuberculosis* proteins exhibiting an H38Y mutation, and the *P. mirabilis* protein encoding an additional S7A mutation, either of which would alter the second salicylate binding site. Some members of cluster IV (*P. carotovorum*) also exhibited a mutation in site II (I20V). The orthologs from the endosymbiotic species of cluster III also exhibited a unique set of polymorphisms, with all examined species exhibiting a V35I mutation (sites I and II), and the *Wigglesworthia glossinidia* protein containing an A10S mutation (site II).

As the ancestral function of MarR TFs is the negative regulation of genes encoding drug efflux pumps, a function that is conserved in *S.* Typhimurium, we examined *slyA* orthologs throughout the *Enterobacteriaceae* for linkage to the *ydhIJK* efflux operon (Fig. 4B). Linkage is conserved among the enteric pathogens of cluster I, although some, such as *S. flexneri*, no longer

encode the efflux pump YdhK. However, linkage to the *ydhIJK* operon is no longer conserved in clusters II, III, or IV, as the entire operon has been lost in nearly all of the species examined. The *ydhIJK* locus does not appear to have been exchanged for another drug efflux system. Rather, the region upstream of *slyA* is highly variable, resulting in the replacement of *ydhIJK* by a wide variety of sequences (Fig. S6). Sequences homologous to the proximal portion of the *ydhI* gene are detectable even in species that have lost functional coding sequences (*e.g., Y. pseudotuberculosis, D. dadantii*). The retention of *slyA* orthologs outside of cluster I despite the loss of the linked efflux system suggests that proteins in the SlyA lineage have evolved to serve a function distinct from that of classical MarR family TFs. SlyA is convergently transcribed with *slyB*, encoding a putative lipoprotein. Although this linkage appears to be more strongly conserved, with *slyB* present in all examined species with the exception of *W. glossinidia*, the significance of this conservation is unclear, as the function of *slyB* is unknown.

Despite being closely related, *rovA* in *Y. pseudotuberculosis* and *Y. pestis* (data not shown) is not linked to a functional *ydhIJK* operon, whereas *ydhIJK* is retained in *Y. enterocolitica*. This indicates that *ydhIJK* was not lost by the former species until after the divergence of *Yersinia* from the other *Enterobacteriaceae*. Other species of *Enterobacteriaceae* are likely to have lost *ydhIJK* more recently, as each became adapted to its own specific niche. This appears to represent an example of parallel evolution, with multiple species independently losing *ydhIJK* as their own SlyA orthologs adapted to new roles.

Functional characterization of a distantly related SlyA ortholog. To understand how the SlyA lineage adapted to its emergent role as an important pleiotropic regulatory protein in the *Enterobacteriaceae*, we compared the functions of SlyA from *S.* Typhimurium ($SlyA_{STM}$) and

RovA of *Y. pseudotuberculosis* and *Y. enterocolitica*, a relatively divergent ortholog that also functions as a counter-silencer (27-29, 33). RovA of *Y. pseudotuberculosis* exhibits 76% identity with $SlyA_{STM}$ (Fig. 4A, Fig. S5). RovA is essential for virulence in *Y. enterocolitica*, and *Y. pestis* (27, 34) and has been suggested to function both as an activator, interacting with RNAP (35), and as a counter-silencer, alleviating H-NS-mediated repression (28, 29, 33). Notably, direct activation has not been demonstrated for any other member of the SlyA lineage, and the most direct evidence to suggest that RovA functions as an activator is derived from IVT studies using small linear fragments of DNA as a template (35). We have previously shown that small linear fragments are not necessarily representative of physiological regulatory events in intact cells and can generate spurious results in IVT assays (16). Genetic analysis of the *inv* gene, which is positively regulated by RovA, in an *hns* mutant strain of *E. coli* also suggested that RovA functions as both a counter-silencer and an activator. However, these experiments failed to consider the potential contribution of the H-NS paralog, StpA, which is up-regulated in *hns* mutants and can provide partial complementation (36, 37), which complicates the interpretation of regulatory studies in an *hns* mutant strain.

We attempted to corroborate the published findings by performing IVT analysis of *inv* expression in the presence of RovA. However, expression of *inv* exhibited only a very modest (~1.5-fold) increase following the addition of RovA (Fig. 5A), which became saturated at a 20 nM concentration; these results are not supportive of direct *inv* activation by RovA. In contrast, RovA was confirmed to function as an auto-repressor, like other MarR family TFs, as IVT analysis demonstrated a 4-fold decrease in *rovA* expression following the addition of RovA protein, with the effect reaching saturation at a 500 nM concentration (Fig. 5B). RovA has also retained the ability to respond to salicylate (Fig. 5C), despite the presence of an H38Y mutation in the second

salicylate-binding pocket (Table S3), as 5mM salicylate completely inhibited RovA-mediated repression, similar to our observations with SlyA. It is also notable that RovA has retained salicylate sensitivity despite the loss of the linked YdhIJK efflux pump in *Y. pseudotuberculosis*. To prove that RovA functions as a counter-silencer in *Y. pseudotuberculosis*, we measured *inv* expression in wild-type and *rovA* mutant strains expressing H-NST from enteropathogenic *E. coli* (H-NST$_{EPEC}$). Mutations in *hns* cannot be generated in *Yersinia* as *Yersinia* spp. carry a single essential *hns* gene, unlike many other members of the *Enterobacteriaceae* which encode *hns*-like genes such as *stpA*, which are able to partially compensate for the loss of *hns*. H-NST$_{EPEC}$ is a truncated *hns* homolog that has been demonstrated to function as a dominant negative form of H-NS by binding and inhibiting the activity of wild-type H-NS protein (38). The expression of *inv* in *rovA* mutant bacteria was approximately four-fold lower than in wild-type cells (Fig. 5D). However, *inv* expression was fully restored upon inhibition of H-NS by *hnsT*$_{EPEC}$, demonstrating that RovA functions solely as a counter-silencer of the *inv* gene. The *rovA* and *slyA* genes are capable of complementing each other in both *S.* Typhimurium and *Y. pseudotuberculosis* when expressed *in trans* (Fig. 5E, F), up-regulating both *inv* and *pagC*, further indicating that RovA functions as a counter-silencer, like SlyA. Together, these observations demonstrate that RovA has retained the ancestral characteristic of environmentally-responsive repression exhibited by other members of the MarR TF family, despite being one of the most divergent members of the SlyA lineage. However, in contrast to MarR TFs outside of the SlyA lineage, it is also able to function as a counter-silencer of horizontally-acquired genes, as exemplified by *inv*.

SlyA counter-silencing requires high expression levels. It is interesting to note that SlyA does not play a major regulatory role in *Escherichia coli*, the most well-studied member of the

Enterobacteriaceae. SlyA is only known to regulate two *E. coli* genes, *hlyE* and *fimB* (39-41), despite exhibiting a high degree of homology (91% identity) to SlyA in *S.* Typhimurium. To better understand the different roles of SlyA in the regulatory hierarchy of *E. coli* and *S.* Typhimurium, we performed a comparative genetic analysis of the *S*. Typhimurium ($slyA_{STm}$) and *E. coli* ($slyA_{Eco}$) alleles. In allelic exchange experiments, $slyA_{STm}$ was swapped with $slyA_{Eco}$ to determine the effect on the expression of the counter-silenced *S.* Typhimurium *pagC* gene (Fig. 6A). We observed that $SlyA_{Eco}$ is able to counter-silence the expression of *pagC* similarly to $SlyA_{STm}$, suggesting that the diminished role of SlyA in *E. coli* is not attributable to differences in protein sequence. This suggested that the importance of different SlyA lineage proteins within their respective regulatory networks may be determined by differences in levels of expression. To test this hypothesis, *slyA* promoter-ORF chimeras were constructed and assessed for their ability to counter-silence *pagC*. Chimeric constructs in the chromosome were unstable, so counter-silencing was instead assessed in a *slyA* mutant carrying pKM05, a plasmid with the $slyA_{STM}$ ORF transcribed by the *E. coli* promoter ($PslyA_{Eco}$), or pKM07, a plasmid with the $slyA_{STM}$ ORF transcribed by its native promoter ($PslyA_{STM}$). Although *pagC* expression was similar with either construct under non-inducing conditions, expression was approximately 10-fold lower with *slyA* expressed from $PslyA_{Eco}$ under inducing conditions (Fig. 6B), suggesting that the diminished role of SlyA in *E. coli* is at least partially attributable to differences in *slyA* expression levels in *S*. Typhimurium and *E. coli*. Although *slyA* expression driven by $PslyA_{Eco}$ is only slightly lower under non-inducing conditions, it is approximately 3-fold greater when driven by $PslyA_{STM}$ under inducing conditions (Fig. 6C), suggesting that $PslyA_{Eco}$ is less responsive to environmental signals associated with virulence gene expression in *Salmonella*, and thus is unable to regulate *slyA* expression in a manner appropriate for counter-silencing.

Parallel experiments in *Y. pseudotuberculosis* revealed that *rovA* is also more strongly expressed from its native promoter (P*rovA*$_{Yps}$) than from a chimera expressing *rovA* from P*slyA*$_{Eco}$ (Fig. S7).

Discussion

The ancient MarR family of transcription factors is represented throughout the bacterial kingdom and in many archaeal species (42). This study sought to understand how the SlyA/RovA lineage of MarR TFs in the *Enterobacteriaceae* evolved to acquire the novel function of counter-silencing. Our observations demonstrate that SlyA proteins have retained vestiges of their ancestral functions, e.g., the environmentally-responsive repression of small molecule efflux systems, while acquiring an ability to act as pleiotropic counter-silencers of horizontally-acquired genes. The latter role has been facilitated by the parallel evolution of cis-regulatory elements that support higher levels of gene expression.

SlyA, like other MarR family TFs, is subject to allosteric inhibition by small aromatic carboxylate compounds such as salicylate, which bind and stabilize the SlyA dimer in a conformation unfavorable for DNA binding (Fig. 2, Fig. S3). However, the specific structure and arrangement of the effector binding sites varies significantly among the MarR family. Our structural data indicate that SlyA binds a total of six salicylate molecules per dimer, although two of these are not likely to be biologically relevant (Fig. 3). Although the general structure and architecture of the salicylate-SlyA complex is similar to complexes formed by other MarR TFs, including MarR (14) and MTH313 from *Methanobacterium thermautrophicum* (43), the various TFs differ significantly in their specific interactions with salicylate. MarR binds a total of four salicylate molecules per dimer, whereas MTH313 binds only two (Fig. 3D, E). All four salicylate binding sites in MarR flank the wHTH domain and are partially exposed to the solvent, whereas

none of these sites correspond to the salicylate binding sites of SlyA. MTH313 binds salicylate asymmetrically at sites similar to sites I and II of SlyA, binding only one salicylate molecule per monomer. It is possible that the structural variability in the MarR family with respect to salicylate binding reflects an inherent evolutionary flexibility and ligand promiscuity. The MarR family sensor region may have evolved to interact with a variety of small molecules, and salicylate may simply represent a promiscuous probe for potential interactions. Alternatively, recent studies indicate that the true ligand of MarR may be copper, which oxidizes a cysteine residue (C80) to promote disulfide bond formation between MarR dimers, resulting in subsequent tetramerization and inhibition of DNA binding (44). The copper atom is thought to be liberated from membrane-associated proteins upon oxidative stress induced by bactericidal xenobiotic agents. As almost all characterized MarR TFs have a reactive cysteine residue corresponding to a region near Site II of SlyA, cysteine oxidation, which has also been described in the MarR family TF OhrR (45), may be a common inhibitory mechanism. Indeed, SlyA contains a cysteine residue that is conserved throughout the *Enterobacteriaceae* at position 82 (Fig. S5).

A phylogenetic analysis of SlyA orthologs in *Enterobacteriaceae* demonstrates that the SlyA lineage is strongly conserved, even in endosymbiotic species exhibiting significant genome loss (Fig. 4, Fig. S5). This suggests that SlyA has a central and essential role in the transcriptional regulatory networks of these species. For example, a recent analysis of the endosymbiont *W. glossinidia* found that *slyA* is subject to significant evolutionary constraints (46). This conservation does not appear to be due to its role as a regulator of antimicrobial resistance, as *slyA* does not exhibit significant linkage to the efflux pump operon *ydhIJK*, outside of the enteric pathogens in cluster I. Rather, SlyA proteins appear to function predominantly as pleiotropic counter-silencers, facilitating the integration of horizontally-acquired genes, including virulence genes, into existing

regulatory networks. A general counter-silencing role has been suggested in multiple enterobacterial species including *E. coli* (18, 41), *Salmonella* (16, 19, 20) and *Shigella* (31) in cluster I, *Yersinia* in cluster II, and *Dickeya* (30, 47), *Serratia* and *Pectobacterium* (*Erwinia*) (24) in cluster IV. Although similar evidence does not currently exist for the endosymbionts in cluster III, this may simply reflect the limited genetic analyses that have been performed in these species.

To understand the evolutionary adaptations to accommodate counter-silencing by the SlyA/RovA lineage, we compared three orthologs, $slyA_{STM}$, $slyA_{Eco}$ and *rovA*. The $slyA_{STM}$ and *rovA* genes are essential for *Salmonella* and *Yersinia* virulence, whereas $slyA_{Eco}$ plays a negligible role in *E. coli*, despite exhibiting a much higher degree of similarity to $slyA_{STM}$ than does *rovA*. These differing roles are attributable to differences in expression, as $SlyA_{Eco}$ is able to function as a counter-silencer in *S.* Typhimurium when its expression is driven by the *S.* Typhimurium promoter (Fig. 6). We also observed that $slyA_{Eco}$ transcription is significantly reduced in medium with low Mg^{2+} concentrations, a condition associated with the *Salmonella*-containing vacuole (SCV) of macrophages, in which SlyA cooperates with the response regulator PhoP to counter-silence virulence genes necessary for intracellular survival (16). This suggests that the diminished role of $slyA_{Eco}$ in *E. coli* may result from its inability to respond to appropriate environmental cues. This is further reinforced by the observation that RovA of *Yersinia* spp. is able to function as a counter-silencer in *S.* Typhimurium when expressed *in trans* from an inducible promoter, and that the *rovA* promoter of *Y. pseudotuberculosis* drives *rovA* transcription more strongly than $PslyA_{Eco}$ in a chimeric strain (Fig. S7). We conclude that the ability of a given SlyA ortholog to serve as a counter-silencer is contingent on its level and pattern of expression. Notably, another group recently demonstrated a significant expansion of the SlyA regulon in *E. coli* when $slyA_{Eco}$ is overexpressed, with 30 operons exhibiting regulation by SlyA, 24 of which are also repressed by

H-NS (48), indicating that $SlyA_{Eco}$ is capable of functioning as a counter-silencer but is not expressed under the appropriate conditions. In *S*. Typhimurium, the appropriate conditions are those associated with the SCV. However, SlyA orthologs in other species such as the plant pathogens of cluster IV and the endosymbionts of cluster III are likely to require expression under vastly different conditions corresponding to their environmental niches. The loss of the divergently-transcribed *ydhIJK* operon in *Y. pseudotuberculosis* and other enteric species may be a consequence of genetic alterations to enhance *rovA* expression as well as the redundancy of drug efflux pumps. An additional possibility is that enhanced *slyA/rovA* expression might result in hyper-repression of *ydhIJK*, which would negate the usefulness of the pump to the cell. Even the plant pathogens of cluster IV, which are most likely to encounter small phenolic compounds in the plant environment (49), have failed to retain *ydhIJK*. It is also notable that the *slyA* orthologs in the four species (*E. coli*, *S.* Typhimurium, *Y. enterocolitica*, *Y. pseudotuberculosis*) in which the transcriptional start sites have been characterized initiate transcription at different positions (29, 50-52), indicating that each species has evolved its cis-regulatory circuit independently (Fig. S6).

Previous studies have demonstrated that regulatory evolution can promote adaptation to new niches (53). The SlyA/RovA TF lineage provides a unique example of parallel regulatory evolution to achieve a common functional objective. Throughout the *Enterobacteriaceae*, the associated *ydhIJK* pump genes have been repeatedly lost, yet their regulators have been retained, presumably to facilitate the evolution of an appropriately responsive regulatory circuit to enable counter-silencing. This suggests that SlyA/RovA proteins possess intrinsic features that predispose them for a counter-silencing role, perhaps the abilities to respond to environmental stimuli and to recognize a variety of AT-rich target DNA sequences. Studies are underway to characterize these features and to determine their contribution to the evolutionary capacity of *Enterobacteriaceae*.

Materials and methods.

Bacterial strains and general reagents. All oligonucleotides and plasmids used in this study are described in Table S4 and Table S5, respectively. Unless otherwise indicated, bacteria were grown in Luria Bertani (LB) broth with agitation. *Salmonella enterica* serovar Typhimurium strains were constructed in the ATCC 14028s background and grown at 37°C, unless otherwise indicated. The 14028s *slyA* mutant strain was described previously (16). *Yersinia pseudotuberculosis* YPIII (54) and YP107 (55) (a gift from P. Dersch, Helmholtz Centre for Infection Research) were used as the wildtype and *rovA* strains, respectively, and grown at 24°C. A *ydhIJK* deletion mutant was constructed using the λ-Red recombinase system (56) and oligonucleotides WNp318 and WNp319. A *slyA ydhIJK* strain was generated by introducing the *slyA::Cm* cassette from 14028s *slyA::Cm* (16) to 14028s *ydhIJK* via P22HTint-mediated transduction. To exchange the wild-type *S*. Typhimurium *slyA* coding sequence with the *E. coli* allele, *S.* Typhimurium *slyA* was replaced with a *thyA* cassette, via FRUIT (57), using the oligonucleotides STM-slyA-targ-F and STM-slyA-targ-R . The *slyA* coding sequence from *E. coli* K-12 was then amplified using the primers STM-Eco-slyA-F and STM-Eco-slyA-R, which include 40 and 41 bases, respectively, from the regions flanking the *S*. Typhimurium *slyA* coding sequence. This fragment was electroporated into the *slyA::thyA* mutant strain and the resulting transformants were plated on minimal media containing trimethoprim, as described for the FRUIT method (57), to select for replacement with the *E. coli slyA* allele.

Cloning. A 2249 bp region containing both *slyA* and *ydhI* was amplified by PCR using the primers SlyAreg-F and SlyAreg-R. The resulting fragment was digested with *Bam*HI and *Hin*dIII and ligated into the low-copy IVT scaffold vector pRW20 (16) to generate pRW39. An IVT target

containing the 2379 bp *rovA* region from *Y. pseudotuberculosis* was generated using the primers BamHI-rovA-F and EcoRI-rovA-R. The resulting fragment and pRW20 were both digested with *Bam*HI and *Eco*RI and ligated together to construct pRW54. An IVT target containing the 2708 bp *inv* region from *Y. pseudotuberculosis* was generated via PCR using the primers BamHI-inv-F and EcoRI-inv-R. The resulting fragment and pRW20 were both digested with *Bam*HI and *Eco*RI and ligated together to construct pRW55. To perform *rovA* and *slyA* complementation studies, both genes were cloned into the arabinose-inducible expression vector pBAD18 (58). The *rovA* gene was amplified using the oligonucleotides EcoRI-rovA-F and KpnI-rovA-R. The *slyA* gene was amplified using the oligonucleotides EcoRI-slyA-F and KpnI-slyA-R. Both fragments were digested with *Eco*RI and *Kpn*I, and ligated into pBAD18, generating pRW58 (*slyA*) and pRW59 (*rovA*). pRW60 was constructed by cloning an *N*-terminal 6×His-tagged copy of *rovA* into pTRC99A. The *rovA* gene was amplified from YPIII genomic DNA by PCR using the oligonucleotides 6HisRovA-F and 6HisRovA-R. The resulting fragment and pTRC99a were digested with *Nco*I and *Bam*HI, and ligated together. An *E. coli* P_{slyA}-S. Typhimurium *slyA* coding sequence chimera was constructed using overlapping PCR. The *slyA-ydhIJK* intergenic region was amplified from *E. coli* K12 genomic DNA using primers KMp178 and KMp206, and from *S.* Typhimurium 14028s using oligonucleotides KMp177 and KMp206. The corresponding *slyA* ORF from *S.* Typhimurium was amplified with primers KMp207 and KMp181. The ORF and promoter segments were amplified along with 40bp of complementary overlapping sequence. Products from the ORF and promoter reactions were mixed 1:1 and amplified using primers KMp178 (*E. coli*) or KMp177 (*S.* Typhimurium) and KMp181 to amplify the promoter/ORF chimeras. The resulting fragment and pTH19Kr (59) were digested with *Bam*HI and *Hin*dIII and ligated together, generating pKM05 (*E. coli* promoter) and pKM07 (*S.* Typhimurium promoter). Plasmids were

transformed into S. Typhimurium 14028s *slyA* for gene expression analysis. The *hnsT* coding sequence was amplified from E2348/69 genomic DNA using the oligonucleotides EcoRI-hnsT-F and HindIII-hnsT-R. Both pBAD18 and the resulting PCR fragment were digested with *Eco*RI and *Hin*dIII, agarose gel-purified, and ligated together to generate pRW57.

Fusaric acid resistance assays. Cultures were grown overnight in LB broth at 37°C, then diluted 1:100. Thirty microliters of this dilution were added to 270 µl of LB containing 30 µg/ml freshly prepared fusaric acid in 100-well BioScreen plates (GrowthCurves, Piscataway, NJ). Cultures were grown with continuous maximum shaking at 37°C and regular OD_{600} measurements were taken on a BioScreen C MBR. Fresh 20 mg/ml stock solutions of fusaric acid were prepared in DMF (dimethyl formamide).

NMR analysis of SlyA-salicylate. SlyA protein for NMR analysis was prepared as an *N*-terminal 6×His-tagged protein from cells grown in M9 minimal medium supplemented with ^{15}N-ammonium chloride (Cambridge Isotope Labs, Tewksbury MA). SlyA protein expression was induced by the addition of 2 mM IPTG (isopropyl-ß-D-thiogalactopyranoside) and the protein purified to homogeneity using Ni-affinity chromatography as previously described (20), except that samples were dialyzed in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1mM EDTA, and 3 mM DTT following purification. Protein and ligand solutions for NMR experiments were prepared in 25 mM sodium phosphate, 150 mM NaCl buffer at pH 7.0 containing 10% D_2O. NMR spectra were collected on a Bruker DMX 500hHz spectrometer (Bruker, Billerica MA) on samples equilibrated at 35°C and consisting of 250-350 µM ^{15}N-labeled His-SlyA in the absence or

presence of 2 mM sodium salicylate. Spectra were processed using NMR-Pipe (60) and analyzed using NMR-View (61).

SlyA-salicylate crystallization. SlyA was over-expressed and purified for crystallization as previously described (20), except that samples were dialyzed in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.1 mM EDTA, and 3 mM DTT following purification. Cryo I and II sparse matrix crystallization screens (Emerald Biosystems, Bainbridge Island, WA) were used to determine initial conditions for His-SlyA crystal formation by sitting-drop vapor diffusion in 24-well crystal trays. Equal volumes (4µL) of SlyA and crystallization solutions were mixed before plates were sealed and kept at room temperature. Crystals appeared within 4-7 days in 20% PEG 300, 10% glycerol, 0.1 M phosphate/citrate buffer, pH 4.2, 0.2M ammonium sulfate (condition #14). Subsequent crystal growth was performed using lab-made 20% PEG 400, 10% glycerol, 0.1 M phosphate/citrate buffer, pH 4.2, and 0.2 M ammonium sulfate. Crystallization of SlyA with sodium salicylate was achieved by adding dilutions of a 2M sodium salicylate aqueous stock solution to the protein-crystallization solution mixture. SlyA-salicylate crystals appeared within 7-10 days. A single crystal appeared at a concentration of 75 mM sodium salicylate and grew to a maximum size of 500µ x 300µ x 150µ (Fig. S4). This crystal was used for X-ray diffraction experiments.

The crystal was frozen at 100°K in its crystallization solution for diffraction data collection on GM/CA-CAT beamline 23-ID-D at the Advanced Photon Source. The space group for the crystals is $P2_12_12$ with two SlyA molecules in the asymmetric unit. The diffraction data were processed with HKL2000 (62). Dataset statistics are shown in Table S1. The crystal structure of the salicylate complex of SlyA was solved using a model of a previously investigated structure

of apo-SlyA (unpublished data). That apo-structure was solved using the molecular replacement program, MOLREP (63) with a search model generated by applying Swiss-Model (64) and the SlyA amino acid sequence to PDB entry 2FBH (65). The structural model for the salicylate complex was refined using REFMAC-5 (66) in the CCP4 suite (67). Rfree (68) was calculated using 5% of the data in the test set. A high-resolution limit of 2.3Å was applied for the refinement, consistent with standards appropriate when the structure was solved. This is the resolution at which Rmerge for the data set drops below 0.40. XtalView (69) and Coot (70) were used to examine sigma A weighted |Fo|-|Fc| and 2|Fo|-|Fc| electron density maps (71). MOLSCRIPT(72), and Raster3D (73) were used to produce structural figures for this paper. Table S2 contains refinement statistics for the structure. Coordinates and structure factors have been deposited in the Protein Data Bank with identifier 3DEU.

6×His-RovA purification. RovA was purified using the same protocol as described previously for SlyA (16). However, overexpression cultures were grown at 24°C to OD_{600}=0.5. IPTG was added to a final concentration of 1mM, and cultures were incubated at 24°C for an additional four h before the cells were harvested by centrifugation and cell pellets stored at -80°C for the subsequent purification of RovA.

***In vitro* transcription.** IVT assays were performed essentially as previously described (16) with the following modifications. All SlyA IVT assays were performed at 37°C. All RovA IVT assays were performed at 24°C. Where indicated, sodium salicylate was added to IVT reactions prior to the addition of SlyA or RovA. All oligonucleotides and templates used in IVT reactions are indicated in Table S6.

RNA isolation and qRT-PCR. RNA was purified using Trizol (Life Technologies, Carlsbad CA) according to the manufacturer's protocols. cDNA was generated using the QuantiTect Reverse Transcription Kit (Qiagen, Hilden, Germany), and quantified in a BioRad CFX96 (BioRad, Hercules CA), using SYBR Green Master Mix (74).

For the analysis of *pagC* expression under inducing conditions, *S.* Typhimurium cultures were grown to early stationary phase (OD_{600}≈2.0) at 37°C in LB broth, then washed three times in N-minimal medium containing either 10 µM (inducing) or 10 mM $MgCl_2$ (non-inducing). Cultures were re-suspended in the appropriate N-minimal medium and incubated for an additional 30 min at 37°C before cells were harvested for RNA purification studies. For the complementation of *slyA* with *slyA* or *rovA in trans*, overnight cultures were diluted to 0.05 OD_{600} and grown for two h at 24°C before adding arabinose to a final concentration of 0.02% w/v. Cultures were grown for an additional six h before harvesting RNA.

Y. pseudotuberculosis strains YPIII and YP107 were diluted from overnight cultures to 0.05 OD_{600} in LB and grown with shaking at 24°C. For the inhibition of H-NS via H-NST_{EPEC} over-expression, and complementation of *rovA* with *rovA* or *slyA in trans*, YPIII and YP107 cultures carrying either pBAD18 or pRW57 were grown for two hrs before arabinose was added to a final concentration of 0.02% w/v. Cultures were grown for an additional six hrs before harvesting RNA. For *slyA*/*rovA* complementation studies, rpoAYS primers targeting *rpoA* were used as loading controls, as *rpoA* is sufficiently conserved between the two species as to allow use of the same primers.

Acknowledgments

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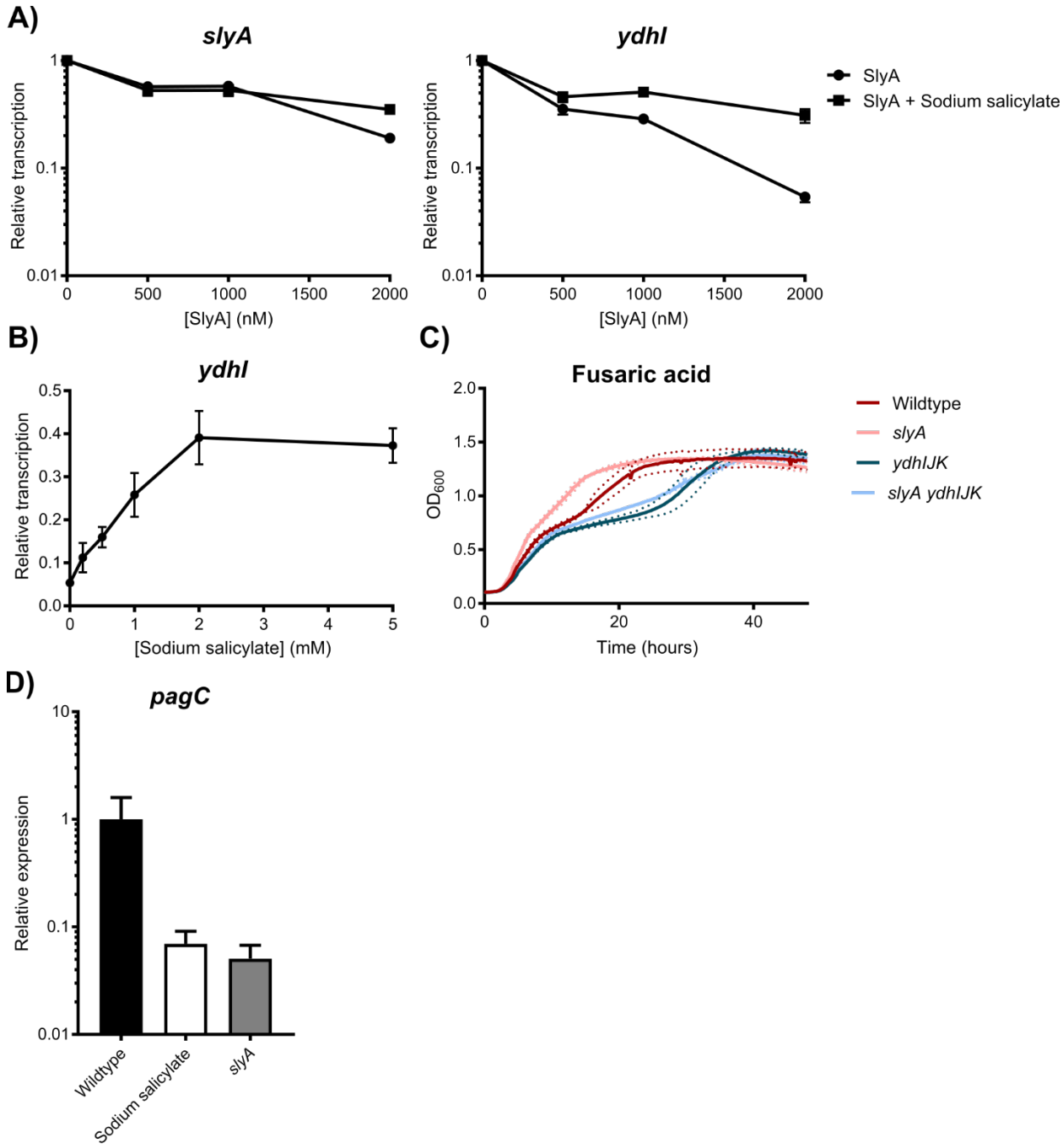


Figure 1. SlyA retains the ancestral functions of MarR family TFs. To determine whether SlyA is an autorepressor like other members of the MarR family, *in vitro* transcription (IVT) assays were performed on supercoiled template DNA containing *slyA* and the divergently expressed *ydhI* gene in the presence of increasing SlyA concentrations (A). Reactions were performed with or without 2mM sodium salicylate to determine whether SlyA is inhibited by

small aromatic carboxylate compounds like other MarR TFs. (IVT analysis of SlyA-mediated regulation of *pagC* is shown in Fig. S1.) To determine whether SlyA exhibits a dose-dependent response to salicylate, IVT assays were performed on *ydhI* in the presence of 2µM SlyA and increasing concentrations of sodium salicylate (B). Wildtype, *slyA*, *ydhIJK*, and *slyA ydhIJK* cultures were grown in the presence of 30 µg/ml fusaric acid and cell density (OD_{600}) measured over time to determine if *ydhIJK* encodes a functional anti-microbial efflux pump (C). Data represent the mean (solid line) of three independent experiments, each consisting of three replicates. Dashed lines represent the SD. (Growth curves in the absence of fusaric acid is shown in Fig. S2.) To determine whether salicylate also inhibits SlyA counter-silencing activity, *pagC* transcripts were quantified by qRT-PCR from early stationary phase ($OD_{600}\approx 2.0$) cultures in minimal N-medium containing 10 µM $MgCl_2$ in the presence or absence of 2 mM sodium salicylate (D). Transcript levels are normalized to *rpoD*, and data represent the mean ± SD; $n\geq 3$.

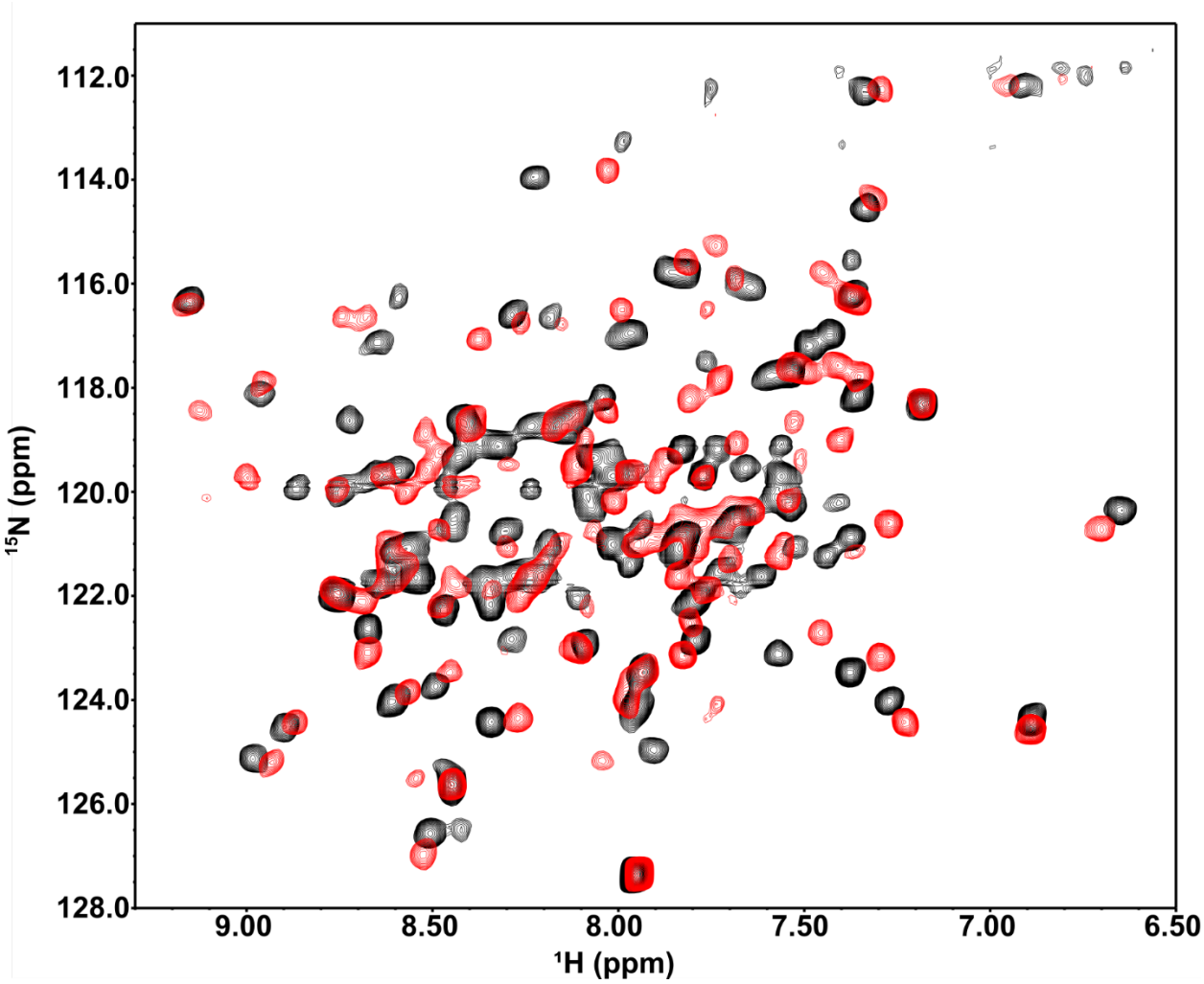
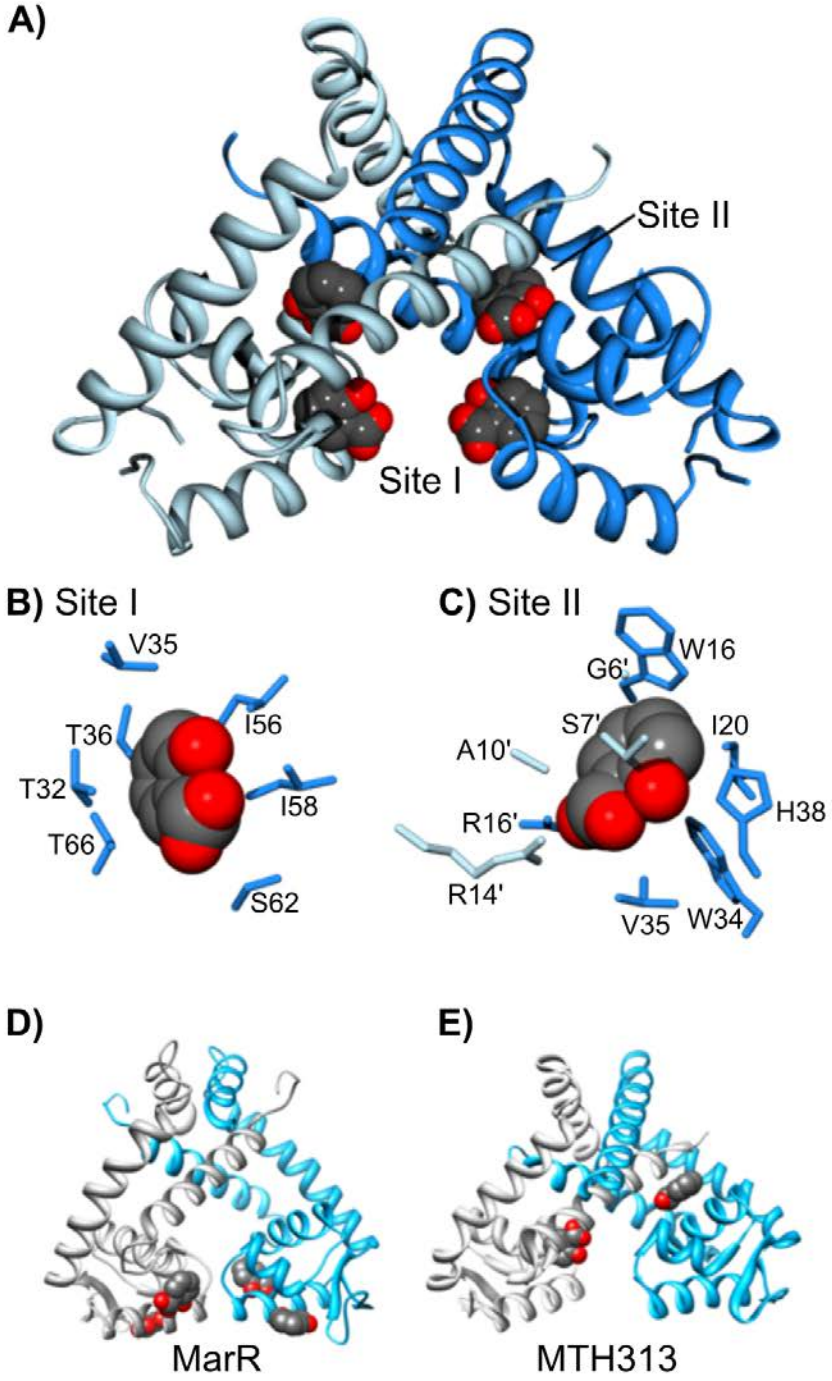


Figure 2. SlyA undergoes significant structural alterations upon salicylate binding. To assess salicylate-induced SlyA structural changes, ^{1}H,^{15}N-TROSY NMR spectroscopy was performed on 0.3mM uniformly-labelled ^{15}N-SlyA in the absence (black) or presence (red) of 2mM sodium salicylate. (Differential DNA Footprinting Analysis, or DDFA, of the SlyA-DNA interaction in the presence or absence of salicylate is shown in Fig. S3.)


A)
Site II
Site I
B) Site I
V35
T36
I56
T32
I58
T66
S62
C) Site II
W16
G6'
S7'
I20
A10'
H38
R16'
R14'
V35
W34
D)
MarR
E)
MTH313

562

Figure 3. Structure of the SlyA dimer bound to salicylate. The crystal structure of the SlyA dimer with salicylate bound at sites I and II was determined at a resolution of 2.3Å (A). Residues responsible for coordinating bound salicylates are shown for sites I (B) and II (C). The structures of salicylate-bound MarR (D; PDB entry 1JGS) and MTH313 (E; PDB entry 3BPX) dimers are shown for comparison. (An image of the SlyA-salicylate crystal is shown in Fig. S4.)

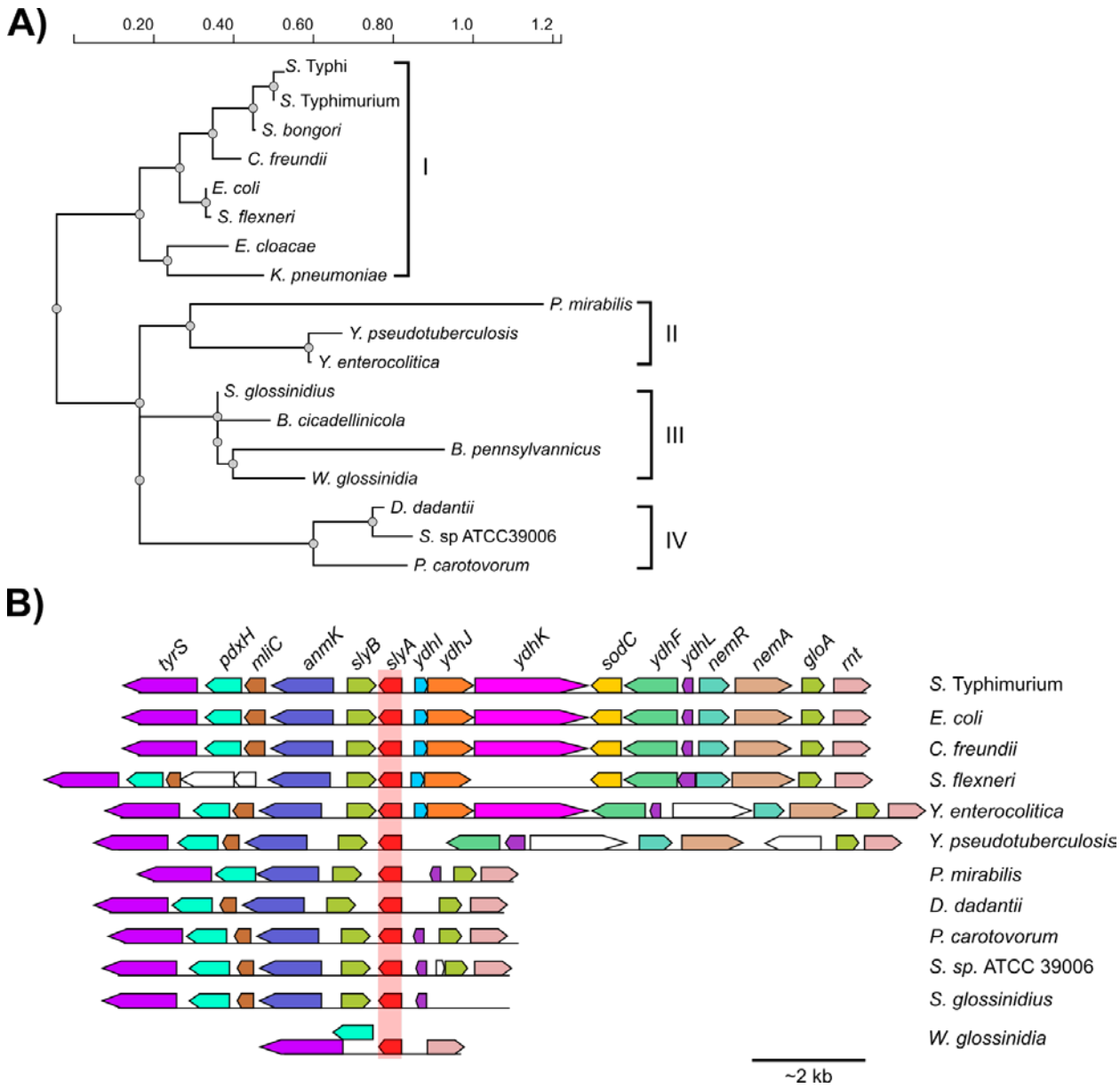


Figure 4. The SlyA TF lineage in *Enterobacteriaceae*. A phylogram of the SlyA TF lineage was generated using PhyML (75), and *slyA* lineage genes from representative members of *Enterobacteriaceae* were aligned (A). The *slyA* genes fall into four clusters: cluster I consists of most enteric species, including *S*. Typhimurium and *E. coli*; cluster II contains more distantly related pathogenic species from *Proteus* and *Yersinia*; cluster III includes endosymbiotic species

such as *S. glossinidius* and *W. glossinidia*; cluster IV contains plant pathogens such as *Pectobacterium (Erwinia)* and *Dickeya*. (A multiple sequence alignment of these SlyA orthologs can be found in Fig. S5.) Regions flanking *slyA* were examined for genetic linkage to the *ydhIJK* efflux locus (B). The *slyA* gene is highlighted in red. The efflux pump encoded by *ydhK* is only found in a small subset of species, suggesting that SlyA has evolved to serve a role independent of *ydhIJK* repression in *Enterobacteriaceae*. (A multiple sequence alignment of the region upstream of the *slyA* coding sequence is shown in Fig. S6.)

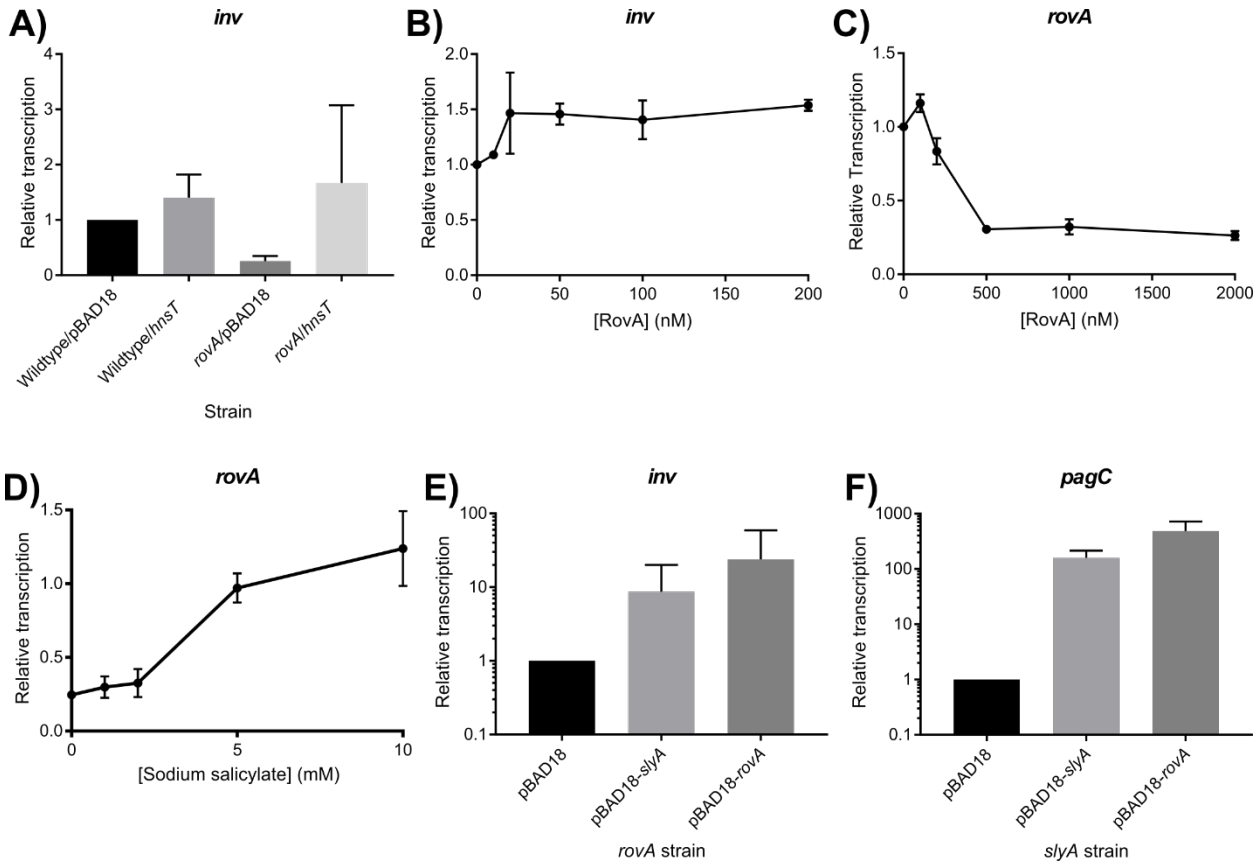


Figure 5. Environmentally-responsive repressing and counter-silencing functions are conserved in the SlyA TF lineage. To determine whether RovA functions as a counter-silencer in *Y. pseudotuberculosis*, transcription of the Rov-regulated *inv* gene was quantified by qRT-PCR in wildtype or *rovA* mutant strains expressing $hnsT_{EPEC}$, which inhibits H-NS (A). Although *inv* expression is decreased in a *rovA* mutant strain, expression is fully restored by the inhibition of H-NS, indicating that RovA functions as a counter-silencer. IVT assays of the *inv* regulatory region in the presence of increasing RovA concentrations detected only a minimal impact on transcription levels, suggesting that RovA is unlikely to function as a classical activator (B). IVT assays of *rovA* in the presence of increasing RovA concentrations of RovA demonstrate that RovA functions as an autorepressor (C). RovA remains sensitive to inhibition by small aromatic

carboxylate molecules, as increasing concentrations of sodium salicylate inhibited RovA-mediated *rovA* repression in IVT assays. Reciprocal complementation studies were performed by providing both *rovA* and *slyA in trans* expressed on the arabinose-inducible pBAD18 vector in *rovA* or *slyA* mutant *Y. pseudotuberculosis* and *S.* Typhimurium strains, respectively, and measuring transcription of *inv* and *pagC* via qRT-PCR. Transcript levels are normalized to *rpoA* in both species, and data represent the mean ± SD; n=3.

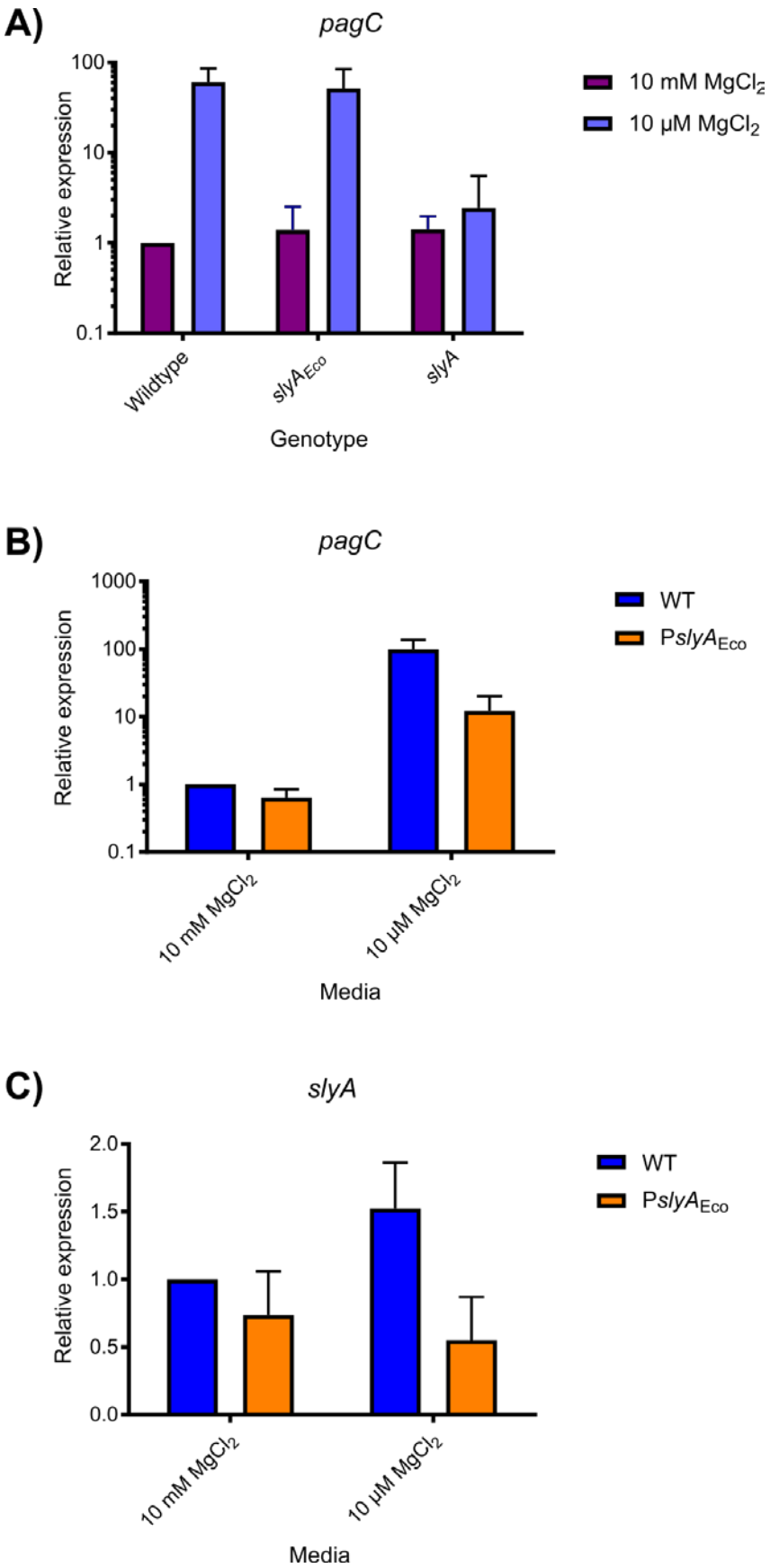

A)
pagC
Relative expression
100
10
1
0.1
Wildtype
slyAEco
slyA
Genotype
10 mM MgCl2
10 μM MgCl2
B)
pagC
Relative expression
1000
100
10
1
0.1
10 mM MgCl2
10 μM MgCl2
Media
WT
PslyAEco
C)
slyA
Relative expression
2.0
1.5
1.0
0.5
0.0
10 mM MgCl2
10 μM MgCl2
Media
WT
PslyAEco

Figure 6. *S*. Typhimurium SlyA is a more effective counter-silencer than *E. coli* SlyA due to higher expression levels. Expression of the SlyA counter-silenced *pagC* gene was measured by qRT-PCR in *S.* Typhimurium strains encoding wildtype *slyA*, $slyA_{Eco}$, or mutant *slyA* under either inducing (10 µM $MgCl_2$) or non-inducing (10mM $MgCl_2$) conditions (A). Expression of *pagC* (B) and *slyA* (C) was compared in *S*. Typhimurium strains in which *slyA* transcription was driven by either the wildtype (WT) or *E. coli* (P$slyA_{Eco}$) *slyA* promoter. Transcript levels are normalized to *rpoD* and data represent the mean ± SD; n=3. (A comparative analysis of a P$slyA_{Eco}$-*rovA* chimera is shown in Fig. S7.)

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