Structural basis for the regulation of β-glucuronidase expression by human gut Enterobacteriaceae


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The gut microbiota harbor diverse β-glucuronidase (GUS) enzymes that liberate glucuronic acid (GlcA) sugars from small-molecule conjugates and complex carbohydrates. However, only the Enterobacteriaceae family of human gut-associated Proteobacteria maintain a GUS operon under the transcriptional control of a glucuronide repressor, GusR. Despite its potential importance in Escherichia coli, Salmonella, Klebsiella, Shigella, and Yersinia opportunistic pathogens, the structure of GusR has not been examined. Here, we explore the molecular basis for GusR-mediated regulation of GUS expression in response to small-molecule glucuronides. Presented are 2.1-Å-resolution crystal structures of GusRs from Escherichia coli and Salmonella enterica in complexes with a glucuronide ligand. The GusR-specific DNA operator site in the regulatory region of the E. coli GUS operon is identified, and structure-guided GusR variants pinpoint the residues essential for DNA binding and glucuronide recognition. Interestingly, the endobiotic estradiol-17-glucuronide and the xenobiotic indomethacin-acyl-glucuronide are found to exhibit markedly differential binding to these GusR orthologs. Using structure-guided mutations, we are able to transfer E. coli GusR’s preferential DNA and glucuronide binding affinity to S. enterica GusR. Structures of putative GusR orthologs from GUS-encoding Firmicutes species also reveal functionally unique features of the Enterobacteriaceae GusRs. Finally, dominant-negative GusR variants are validated in cell-based studies. These data provide a molecular framework toward understanding the control of glucuronide utilization by opportunistic pathogens in the human gut.

Microorganisms compete in the gastrointestinal (GI) tract for sources of carbon in the form of simple and complex carbohydrates and have been shown to work in synergy to process dietary fiber that cannot be degraded by the host (1, 2). Microbial carbohydrate utilization plays a critical role in the diversity, abundance, and metabolic activity of both commensal and potentially pathogenic bacteria in the mammalian intestine (3, 4). As researchers have shown, the ability to access the energy present in oligosaccharides provides a competitive advantage to the members of the Bacteroidetes that harbor polysaccharide-utilization loci (PULs), which encode enzymatic and membrane-spanning machinery that catabolizes a range of complex carbohydrates (5–10). This leads to the question of how other members of the microbiota that lack PULs are able to compete for energy within the GI tract.

The GUS operon, which was first described more than 30 y ago in Escherichia coli, provides a potential answer to this question (11–15). This operon encodes proteins involved in processing glucuronidated ligands, including β-glucuronidase (GUS). GUS enzymes are glycosyl hydrolases that remove glucuronic acid (GlcA) sugars linked to endobiologic and xenobiotic compounds by phase II drug-metabolizing UDP-glucuronosyltransferase (UGT) enzymes in protective host tissues (e.g., liver and intestines) (16–21). A wide range of chemicals are conjugated to GlcA, including hormones, neurotransmitters, environmental pollutants, and drugs like cancer chemotherapeutics, immunosuppressants, and nonsteroidal antiinflammatory drugs (NSAIDs) (22–25). The microbial GUS-mediated reactivation of these compounds in the gut may play a role in their serum exposure via enterohepatic recirculation (26–29). This pathway also causes the intestinal damage and dose-limiting toxicities of the anticancer drug irinotecan and several NSAIDs (30–32). Microbe-selective GUS inhibitors have been shown to alleviate these toxicities in mice, providing an early demonstration of nontoxic drugs specific to the microbiome (30–34).

GUS is the product of the gusA gene, which in the GUS operon is followed by the inner-membrane GlcA-specific transporter gusB and nonspecific outer-membrane channel gusC genes (Fig. 1) (12, 15). As we show below, similar operons are found only in other Enterobacteriaceae, including Salmonella, Klebsiella, Yersinia, and Shigella taxa, all of which are potential intestinal and systemic pathogens. Importantly, the Enterobacteriaceae lack PULs, suggesting that they might rely on systems like the GUS operon to harness available forms of carbon (35, 36). The GUS operons in the Enterobacteriaceae are under the control of the transcriptional repressor GusR, which is expected to respond to the presence of glucuronidated ligands by dissociating from the regulatory region of the operon and thus allowing operon transcription. Similar to the lac and other E. coli operons, the GUS operon is also sensitive to catabolite repression by glucose, the global metabolic regulator (13, 37, 38). In E. coli, GusR and a related repressor, UxuR, were previously found to bind to two operator elements, termed sites 1 and 2, in the regulatory region of the GUS operon (14–16, 39, 40). To date, however, the structural and biochemical understanding of the control of glucuronide utilization by opportunistic pathogens in the human gut is limited. The authors declare no conflict of interest.

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Data deposition: The atomic coordinates and structure factors reported in this paper have been deposited in the Protein Data Bank, www.pdb.org (PDB IDs codes 6AYH, 6AYI, 6AZ6, and 6AZH).

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Significance

Commensal microbiota establish nutrient-utilization niches in the gastrointestinal tract. While the large intestine is dominated by the Bacteroidetes that degrade complex carbohydrates, the small intestine contains Proteobacteria and Firmicutes that compete with host tissues for small-molecule sources of carbon. Here, we show that the Enterobacteriaceae family of Proteobacteria, including Escherichia coli, Salmonella, Klebsiella, Shigella, and Yersinia pathobionts, maintains DNA operator- and glucuronidated ligand-specific glucuronide repressor (GusR) transcription factors that uniquely respond to glucuronidated ligands.

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bases of the interaction between GusR and the operator DNA, as well as the binding of glucuronidases to GusR, have remained undefined.

Here, we describe the crystal structures of two Enterobacteriaceae GusR proteins, from *E. coli* and from *S. enterica*, and outline the DNA binding characteristics of *E. coli* GusR within the regulatory region of its GUS operon. We further pinpoint the molecular determinants of GusR recognition of glucuronidated ligands both in vitro and in cell-based studies. Together, these data advance our understanding of how GI microbiota that lack the capacity to process energy-dense oligosaccharides up-regulate a system to scavenge GlcA from available glucuronidases.

**Results**

**GusR Crystal Structures.** We determined the 2.1-Å-resolution crystal structures of the GusR proteins from the gut microbial Enterobacteriaceae species *E. coli* (EcGusR) and *S. enterica* (SeGusR) in complexes with *p*-nitrophenyl-*β*-D-glucuronide (PNPG) (Fig. S1). PNPG is a standard in vitro GUS assay substrate, that provided cocrystal crystals with the two GusRs examined. Both EcGusR and SeGusR are α-helical homodimers, with each monomer composed of DNA- (α1 to α3) and effector-binding domains (α4 to α10) (Fig. 2). While the DNA-binding domain (DBD) of one monomer is disordered in the EcGusR structure (Fig. 2A), both DBDs are fully ordered and visualized in the SeGusR homodimer (Fig. 2B). EcGusR and SeGusR share 59% sequence identity and 0.85-Å root-mean-square deviation (rmsd) across 176 equivalent Ca positions. EcGusR and SeGusR also share 3.4- and 3.5-Å rmsd and 17 and 16% sequence identity, respectively, with the *E. coli* TetR protein that defines this family of ligand-regulated transcriptional repressors. Thus, GusR appears to use a TetR-like fold to create separate DBDs and effector-binding domains (EBDs) to recognize its DNA binding site in a manner controlled by glucuronidase effector ligands.

**DNA-Binding Domain.** The GusR DBDs exhibit a helix-turn-helix (HTH) DNA-binding motif composed of α2 and α3 (Fig. 3A). The HTH is the most common DNA binding fold in bacterial transcriptional factors, and is highly conserved within the TetR family of ligand-controlled regulators (41). Previous crystal structures of *E. coli* TetR [Protein Data Bank (PDB) ID code 1QPI] and *Staphylococcus aureus* QacR (SaQacR; PDB ID code 1JT0), which both share a 3.4-Å rmsd and 17% sequence identity with EcGusR, reveal that these repressor proteins utilize their HTH motifs to bind as homodimers to their palindromic operator sites (42, 43). In 1987, Blanco reported that the GUS operon in *E. coli* was under the control of GusR and UxuR, a second TetR-like repressor that shares 14% sequence identity with EcGusR, and that both repressors appeared to bind to two operator elements, sites 1 and 2 (44). Site 1 is 30 base pairs (bp) in length and is located 200 bp upstream from the GUS operon’s ribosome binding site (rbs), while site 2 (40 bp) is only 50 bp from the same rbs (Fig. S1). The 1987 report employed cell-based lac gene fusion experiments to examine operator site interactions (44). Here, we studied the specificity of *E. coli* GusR and UxuR interactions with predicted operator sites in vitro using isothermal titration calorimetry (ITC). We cloned the gene for *E. coli* UxuR (EcUxuR), recombiantly overexpressed the protein in *E. coli*, and purified it to homogeneity. We then compared the abilities of EcGusR and EcUxuR to bind to operator sites 1 and 2 using ITC. We found that EcGusR bound the 30 bp distal site 1 with 0.2 μM affinity but failed to bind to the 40 bp site 2 located in closer proximity to the start of the GUS operon (Fig. 3B and C and Fig. S1). Furthermore, we found that EcUxuR failed to bind to either site in the conditions tested (Fig. 3B). Thus, for *E. coli*, while EcUxuR showed no affinity for these DNA duplexes, site 1 was validated as an element capable of binding EcGusR in vitro.

Next, we sought to understand the effect that a specific duplex DNA element might have on EcGusR’s ability to bind a glucuronide ligand. We incubated EcGusR with excess operator site 1 or site 2 and then utilized increasing concentrations of the glucuronide ligand PNPG as the titrant for ITC. Without DNA, EcGusR bound to PNPG with a Kd of 0.2 μM (Fig. 4B and Fig. S2). However, in the presence of 18-fold molar excess site 1, EcGusR’s affinity for PNPG is reduced more than 20-fold, to 4.1 μM. By contrast, 18-fold molar excess site 2 did not change EcGusR’s affinity for PNPG, which remained at 0.2 μM. Therefore, *E. coli* GusR’s affinity for the effector ligand decreases when its cognate DNA element, site 1, is present. The inverse experiment was also performed, in which saturating levels of PNPG were added to EcGusR, and operator site 1 or site 2 DNA duplexes were then titrated in ITC studies. These
the equivalent residue in EcGusR is Y49 (Fig. 3A). Backbone contacts in the SaQacR side chain was found to form both base-specific and phosphate interactions with the glucuronide as well as additional hydrogen bonds with the glucuronide carboxylate (Fig. 4B). Interestingly, the GlcA enzyme also uses a lysine and tyrosine to recognize the same GlcA carboxylate within the enzyme’s active site (33). In the GusR effector-binding pocket, each hydroxyl of the glucuronide sugar forms a hydrogen bond with three polar GusR residues that are highly conserved (Fig. 4A). Thus, the sugar moiety of the bound glucuronide makes six contacts with a total of five GusR residues. The p-nitrophenol group, by contrast, interacts with only three protein side chains, two of which utilize relatively less specific van der Waals contacts. Because this portion of the ligand varies depending on the specific glucuronide bound, it is perhaps not surprising that fewer side chains and less specific contacts are formed with this group (Fig. 4A). Taken together, these structural data reveal that GusR uses intimate polar contacts in recognizing the GlcA moiety of a bound effector ligand (Fig. 4A).

Next, we compared the ability of EcGusR, SeGusR, and various mutant proteins to bind to glucuronide effector ligand (PNPG) in vitro by ITC. We found that EcGusR and SeGusR bound PNPG with a $K_d$ of $0.2 \pm 0.07\, \mu M$ and $2.7 \pm 0.4\, \mu M$, respectively (Fig. 4B and Table S2). The ligand-binding pockets of the two receptors are identical except at three positions—R73/H72, M87/L86, and H126/Y125 for EcGusR/SeGusR, respectively (Fig. 4A). An M87L mutation in EcGusR reduced PNPG binding by 8-fold (Table S2), and an EcGusR M87A mutation led to a greater than 100-fold reduction in PNPG affinity compared with wild type (Fig. 4B).

We find that mutations of the carboxylate-contacting residues in EcGusR or SeGusR (K125/124; Y164/163) eliminate or significantly reduce PNPG binding (Fig. 4B). Replacement of lysine with alanine in both EcGusR and SeGusR produces GusR variants with no binding to PNPG, while mutation of the tyrosine to alanine decreases ligand binding by 70- or 200-fold for SeGusR and EcGusR, respectively (Fig. 4B). Eliminating only the hydroxyl group of the tyrosine side chain via phenylalanine mutations still reduces binding by 6- to 50-fold for the Se and Ec receptors, respectively, highlighting the importance of this polar interaction in the GUS enzyme’s effector-binding pocket, each hydroxyl of the glucuronide sugar forms a hydrogen bond with three polar GusR residues that are conserved in sequences of GusR proteins (Fig. 4A and Fig. 4B). The GusR effector-binding pocket, each hydroxyl of the glucuronide sugar forms a hydrogen bond with three polar GusR residues that are highly conserved (Fig. 4A). Thus, the sugar moiety of the bound glucuronide makes six contacts with a total of five GusR residues. The p-nitrophenol group, by contrast, interacts with only three protein side chains, two of which utilize relatively less specific van der Waals contacts. Because this portion of the ligand varies depending on the specific glucuronide bound, it is perhaps not surprising that fewer side chains and less specific contacts are formed with this group (Fig. 4A). Taken together, these structural data reveal that GusR uses intimate polar contacts in recognizing the GlcA moiety of a bound effector ligand (Fig. 4A).
GusR DNA-binding domain structure and operator site binding.

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Fig. 3. GusR DNA-binding domain structure and operator site binding. (A) Ribbon representation of the EcGusR DBD (green) highlighting DNA-binding residues, and an alignment of GusR sequencing in this region with other TetR family members. Residues important for DNA binding are highlighted in black and gray. (B) DNA binding affinities measured by isothermal titration calorimetry for wild-type and variant factors and using two predicted operator sites, 1 and 2 (Fig. S1). Error values are reported in parentheses and italicized, and represent SDs. NB, no binding. (C) An ITC heat-map trace of EcGusR binding to DNA operator site 1. (D) An ITC heat-map trace of EcGusR preincubated with PNPG and DNA operator site 1 as the ligand titrant.

Potential Firmicutes GusR Functional Orthologs. We next sought to determine whether GUS expression is controlled by a potential GusR ortholog in gut microbes from the Firmicutes phylum. In Bacteroidetes, the other dominant phylum in the human gut, GUS enzymes and other glycosyl hydrolases are part of well-characterized PULs identifiable by proximal starch-utilization system factors (36). Analogous gram-positive PULs have been described, specific to butyrate-producing bacteria of the Firmicutes phyla (7). Previously, we reported the crystal structures of the GUS proteins from the non-butyrate-producing Firmicutes taxa Clostridium perfringens and Streptococcus agalactiae; thus, we focused on these two Firmicutes species (33). We found through sequence analysis that Firmicutes do not maintain a GUS operon akin to that observed within the Enterobacteriaceae family of the Proteobacteria phylum. We hypothesized, however, that a glucuronide-responsive transcriptional regulator similar to GusR may be encoded either near the Firmicutes gusA genes or elsewhere on the chromosomes of these two species. Thus, we searched locally around the gusA gene in C. perfringens and S. agalactiae to identify a putative GusR ortholog, and searched each organism’s reference genome globally to find the closest homolog by sequence identity to the confirmed GusRs outlined above (Fig. S3).

In the global search of the C. perfringens reference genome (1152363755), we identified a predicted FadR protein (CpFadR) that shares 20 and 21% sequence homology with EcGusR and SeGusR, respectively (Fig. 6A and Fig. S4). Thus, we synthesized the gene for CpFadR, overexpressed, purified, and crystallized the CpFadR protein, and determined its structure to 1.9-Å resolution (Fig. 6A, Fig. S5, and Table S1). The structure reveals that, like EcGusR and SeGusR, CpFadR is a homodimer that folds into a distinct DDB and EBD, and exhibits a similar overall structure to the Enterobacteriaceae GusRs, proteins, with 3.4-Å rmsd over 168 and 176 equivalent Cα positions with EcGusR and SeGusR, respectively (Fig. 6A). However, despite their fold similarities, the effector-binding pockets of CpFadR and the GusRs are distinct. CpFadR places four amino acid residues into the pocket the GusRs employ to accommodate the ligand in the PNPG–GusR structures; the corresponding CpFadR residues would appear to sterically block glucuronide binding (Fig. 6A). Because CpFadR is an apo (ligand-free) state in the crystal structure we resolved, the protein could potentially reposition these residues upon binding to ligand. Therefore, we tested the ability of CpFadR to bind

the NSAID metabolite indomethacin-acyl-glucuronide (Indo-glucuronide) binds only to SeGusR (Kd 15 μM) (Fig. 5). However, a single ligand-binding pocket L86M mutation in SeGusR confers 15 μM affinity to E17-glucuronide, and improves SeGusR’s affinity for Indo-glucuronide to 3.0 μM (Fig. 5). The corresponding mutation in EcGusR (M87L) reduces E17-glucuronide affinity to 19 μM but confers indomethacin-glucuronide binding capabilities to this variant protein (Fig. 5B). An alanine mutation in this position (MST4) eliminates EcGusR’s affinity for E17-glucuronide and Indo-glucuronide (Fig. 5). Taken together, these data reveal that GusRs bind to glucuronide conjugates primarily via contacts with the sugar moiety but also appear to exhibit species-specific preferences for distinct non-sugar groups, perhaps reflecting differences in glucuronide utilization within the human gut. Additionally, we show that the affinity for a particular ligand can be adjusted by single-residue changes within the GusR effector-binding pocket. Future studies will address GusR binding by other glucuronidated ligands.

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In both *C. perfringens* and *S. agalactiae*, a putative GntR family transcriptional regulator was identified locally in the region adjacent to the *gusA* genes (reference genomes 1152363755 and 674113006, respectively). This was also the most significant hit in the global search of the *S. agalactiae* genome. The GntRs identified share less than 13% sequence identity with EcGusR and SeGusR. CpGntR retains none of the six residues identified in the GusRs to be important for ligand binding, while two residues are conserved in SaGntR (Fig. S4). Despite these differences, we hypothesized, given their proximity to *gusA* genes, that CpGntR and SaGntR may be structural or functional orthologs of the Enterobacteriaceae GusRs. Thus, we synthesized both GntR genes, overexpressed both proteins recombinantly in *E. coli*, and purified them to homogeneity. While we failed to obtain crystals of CpGntR, the apo structure of SaGntR was determined to 1.9-Å resolution (Fig. 6B and Table S1). We found that SaGntR is structurally distinct from GusR, forming a homodimer but exhibiting little structural similarity with the glucuronide repressor proteins. SaGntR utilizes a winged helix-turn-helix DBD motif, and its EBD is composed of six α-helices packed to create a barrel-like structure with no evident ligand-binding pocket (Fig. 6B). Furthermore, neither CpGntR nor SaGntR exhibited binding to PNPG, PNP-Gluco, or free GlaC in ITC studies (Fig. S2). Taken together, these structure–function data from the Firmicutes taxa *C. perfringens* and *S. agalactiae* indicate that their putative GusR orthologs are not glucuronide-responsive factors. Thus, these species appear to regulate *gusA* gene expression in a manner distinct from the Enterobacteriaceae.

**Molecular Determinants of GusR-Mediated Regulation in *E. coli*.** We sought to validate the molecular contacts necessary for GusR function in living *E. coli* cells. Because the *gusA* gene is under the control of GusR in this member of the Enterobacteriaceae family, we started by examining changes in GUS activity in cultured *E. coli* BL21 cells in the presence of different potential effector ligands (Fig. 7A). We treated cells with potential effectors for 2 h, and then effectors were removed by three rounds of cell pelleting and washing before cell lysis and the subsequent assays of GUS activity (13). We found that free GlaC did not increase GUS activity at concentrations up to 10 mM (Fig. 7A). However, increasing concentrations of the GusR ligand PNPG led to a dose-dependent increase in GUS activity, starting with

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**Fig. 4.** GusR effector-binding domain structure and function. (A) Superposition of PNPG (yellow)-bound EcGusR and SeGusR monomers (green and blue, respectively), with a close-up highlighting side chains that contact PNPG. Residues examined by mutagenesis are indicated (bold). (B) PNPG binding affinities measured by ITC for GusR wild-type (WT) and mutant GusR proteins. Error values are reported in parentheses and italicized, and represent SDs. NB, no binding.
basal activity at 1.95 μM PNPG and rising to robust activity at 1 mM PNPG (Fig. 7A). By contrast, PNP-Gluco, the near-isostructural ligand to PNPG (Fig. S2), did not increase GUS expression even at 10 mM (Fig. 7A). These cell-based results support the in vitro ITC data above indicating that EcGusR binds to PNPG but not to GlcA or PNP-Gluco. Thus, we conclude that EcGusR responds to the presence of PNPG to de-repress the GUS operon, allowing for gusA expression and an increase of GUS enzyme activity in cultured E. coli cells.

Last, we examined how specific GusR residues affect the production of GUS activity in living E. coli cells. We introduced plasmids into E. coli that contained the gene for wild-type GusR or the K125A GusR variant that failed to bind ligand in vitro (Fig. 4B). E. coli with an empty plasmid [BL21(DE3)] showed robust GUS activity in the presence of PNPG, likely resulting from endogenous GusR (Fig. 7B). Cells containing an expression plasmid for wild-type GusR failed to show GUS activity when PNPG was withheld but exhibited GUS activity when induced with 1 mM PNPG (Fig. 7B). By contrast, cells containing an expression plasmid for the K125A GusR variant exhibited no GUS activity even when induced with 1 mM PNPG, indicating that this form of GusR acted as a dominant negative in E. coli cells that still retained their endogenous GusR protein (Fig. 7B). Additionally, the expression plasmid for E. coli UxuR yielded moderate GUS activity with PNPG, similar to levels seen when expression plasmids for wild-type and key mutants of S. enterica α1α2α3α4α5α6α7α8α9α10Y164R69K125E97R73M87L159T163H126F74W62Y122I126

Fig. 6. Putative GusR orthologs from C. perfringes and S. agalactiae. (A) Superposition of EcGusR and C. perfringes FadR monomers (green and red, respectively), with a close-up of residues of the putative effector-binding pockets. CpFadR side chains that sterically clash with PNPG are in bold and boxed. (B) Ribbon representation of the 1.9-Å crystal structure of the S. agalactiae GntR (orange; PDB ID code 6AZ6) homodimer.
GusR were added to *E. coli* cells (Fig. 7B). These results indicate that EcUxuR is unable to repress GUS expression in *E. coli*. However, we tested a form of SeGusR in which we combined both the ligand-insensitive mutant K124A and the *E. coli* DNA operator targeting SCAI mutations into a single SeGusR (SCAI + K124A) variant protein. This variant exhibited robust repression of GUS activity in *E. coli* cells (Fig. 7B). Taken together, these cell-based studies define the molecular determinants important for glucuronide recognition by the GusR transcriptional repressor that controls GUS activity levels in potential gut pathogens like *E. coli* and *S. enterica* (Fig. 8). Furthermore, they show that DNA-binding site-specific but ligand-insensitive receptors can act in a dominant-negative fashion in living *E. coli* cells.

**Discussion**

The structural basis of GusR-mediated glucuronide recognition by members of the human gut Enterobacteriaceae pathobionts *E. coli* and *S. enterica* is described. By sequence analysis, we find that additional human GI Enterobacteriaceae pathobionts *Shigella*, *Klebsiella*, and *Yersinia* appear to encode GusR orthologs adjacent to GUS operons (Fig. 8). Each of these GusR proteins maintains the conserved residues established here to be functionally important for glucuronide recognition in vitro and in vivo (Fig. 8). In the National Center for Biotechnology Information (NCBI) database, we also found GusR orthologs in *Buttiauxella*, *Erwinia*, and *Raoultella* taxa—Proteobacteria, typically associated with aquatic and soil environments but known to be opportunistic human pathogens. The GusRs from these taxa also maintain the residues shown here to be necessary for glucuronide binding (Fig. 8). The Proteobacteria, and particularly Enterobacteriaceae, can act as condition-specific opportunistic pathogens (45–47). Indeed, a dysbiotic state of microbial imbalance has been associated with the increase in abundance of the Enterobacteriaceae in patients with chronic inflammation and colorectal cancer (45, 48, 49). We speculate that GusR operons provide some Enterobacteriaceae with the ability to utilize intestinal endobiotic and xenobiotic glucuronides as nutrients. UGT enzymes expressed throughout the GI tract have been shown to be efficient producers of a range of phenolic glucuronides (50, 51), some of which we show here to be GusR ligands (Fig. 5 and Fig. S2). Thus, in the presence of suitable glucuronides, GusR operon-containing Enterobacteriaceae may be poised to use this unique source of carbon for colonization and potential expansion in opportunistic conditions.

We find that all microbes encoding a bona fide GusR also maintain elements of a GUS operon, but not all retain a complete GUS operon composed of gusA, gusB, and gusC genes. The NCBI database contains whole-genome sequences for six strains of *E. coli*, and the genomes of five of these six strains encode full GUS operons; by contrast, the pathogenic O157:H7 strain lacks the gusA gene that encodes the GUS enzyme. Commensal *E. coli* has been shown to limit the colonization of pathogenic O157:H7 (52, 53), suggesting that an intact GUS operon may provide a competitive advantage within the GI tract. All sequenced strains of GusR-encoding Enterobacteriaceae taxa maintain a complete operon, with the exception of one *Klebsiella* and two *Shigella* strains, which harbor truncated GUS operon genes (Table S3). Taken together, these observations support the conclusion that

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**Fig. 8.** Sequence alignment of GusRs identified from the NCBI database, with the secondary structure of EcGusR shown above the alignment. Residues important for glucuronide and nonglucuronide recognition are highlighted in red and brown, respectively. Residues important for DNA binding are highlighted in gray.
GUS operons are largely, but not universally, retained in human gut-associated Enterobacteriaceae. The Proteobacteria are at low abundance in the large intestine, where the primary nutrient source is complex carbohydrates (9, 45, 54, 55). The dominant phyla of the large intestine is Bacteroidetes, which utilize their PUL-encoded machinery to degrade complex carbohydrates (9, 36, 56). The Proteobacteria are associated with the small intestine in the healthy mammalian gut, and appear to compete with host tissues for sugars and other simple carbohydrates in this region of the GI tract (54, 55, 57). Intact GUS operons within the Enterobacteriaceae pathobionts would appear to give them a competitive advantage for nutrient consumption over other Proteobacteria and Firmicutes microbes in the small intestine, particularly downstream from the bile duct that delivers glucuronosides from the liver (19, 22, 23).

As we have shown with our estrogen- and indomethacin-glucuronides, different microbial species are equipped to respond to distinct endobiocid xenobiotic glucuronides, perhaps reflecting differences in glucuronide utilization in the gut (Fig. 5). The molecular basis for this distinction involves a key effector-binding pocket residue, M87 in EcGusR and L86 in SeGusR, and mutational swaps in this position alter ligand binding specificity (Fig. 5). We note that, of the residues that contact the p-nitrophenol group in our crystal structures (Fig. 4A), the only position that varies in the sequences of the Enterobacteriaceae GusRs (Fig. 5). It samples Leu, Met, and Thr amino acids, while the F74/73 (EcGusR/SeGusR), L160/159, and T163/162 residues near the nonglucuronide moiety are almost completely conserved; only Butiauxella GusR harbors a change in one of these positions, replacing T163/162 with an asparagine (Fig. 8). These observations support the conclusion that the M87/L86 position is an important site for ligand binding specificity in the GusR family of transcription factors.

Although we were able to express, purify, and study EcUxuR, we were unable to identify its DNA-binding element or determine its crystal structure. Furthermore, EcUxuR failed to repress GUS activity in our cell-based studies (Fig. 7B). Previous research has shown that this GusR homolog binds to a glucuronide metabolite, 1-fructuronate, which may stabilize the protein and perhaps make it amenable for future crystallographic analysis (58). Finally, because different ligands (e.g., indomethacin- and estrogen-glucurononides) share some residues essential for binding to distinct GusR proteins (e.g., E. coli and S. enterica), it is possible that distinct GUS enzymes will also show marked preferences for differing chemical classes of glucuronide substrates. Examining this possibility will be the subject of future GUS structure, function, and inhibition studies.

Methods
Cloning and Expression. E. coli GusR and UxuR were cloned from genomic DNA, all other genes were synthesized by GenScript. Genes were cloned into a pLIC vector with an N-terminal 6× Histagged DNA, all other genes were synthesized by GenScript. Genes were cloned into the correct phase of EcGusR, using the Phaser molecular replacement tool in BioSAXS.

Crystallization. Initial crystallization conditions were identified in 96-well sitting-drop trays using a Rigaku Phoenix Liquid Handler with 15 mM GUS and 10-fold molar excess PNPG (greater than 10-fold molar excess) could be reproduced in the laboratory in hanging-drop vapor-diffusion crystallization trays (Qiaogen). However, EcGusR crystals could be reproduced in 96-well sitting-drop trays, and could be looped and cryoprotected in 20% glycerol. Data from these crystals were collected to 2.1-Å resolution at the X24A Source (APS) using General Medical Sciences and National Cancer Institute Collaborative Access Team (GM/CA CAT) beamline 23ID-D at the APS. Selenium-substituted SeGusR was crystallized as described above and could be employed for single-wavelength anomalous dispersion phasing, as outlined below.

Selenomethionine-substituted SaGntR was screened in the same way as described above for EcGusR and SeGusR. Screening hits were refined in 15-well Qiaen vapor-diffusion hanging-drop trays. Final crystals were grown overnight at room temperature in 0.2 M triammonium citrate, 12% PEG 3350, and CpGntR at 15 mg/mL. Crystals were cryoprotected in the same condition as the crystallant with 20% glycerol.

Selenomethionine-substituted CpGntR was screened in the same way as described above for EcGusR and SeGusR. Screening hits were refined in 15-well Qiaen vapor-diffusion hanging-drop trays. Final crystals were grown overnight at 20 °C in 0.2 M sodium formate, 20%, PEG 3350, and CpGntR at 6.8 mg/mL. Crystals were cryoprotected with Fomblin (Sigma-Aldrich).

Structure Determination. An inverse-beam collection method at a wavelength of 0.97939 Å was applied to selenomethionine-substituted SeGusR crystals and 120° ECA resolution. Collection data were autoprocessed by X-ray Detector Software (XDS) (60, 61). The structure of SeGusR was determined using the AutoSol tool in PHENIX (62). After phasing, initial model building was performed in PHENIX via the AutoBuild function. Subsequent refinements and manual building were performed in PHENIX and Coot (63), respectively. The final model consisting of 192 amino acids was built with one molecule in the asymmetric unit (ASU). The final model was built and refined with an Rwork and Rfree of 0.1833 and 0.2220, respectively (Table S1). Atomic coordinates and structure factors have been deposited in the Protein Data Bank with ID code 6AYH.

EcGusR cryocrystallized with PNPG diffracted to 2.1-Å resolution. Collection data were autoprocessed by X-ray Detector Software (XDS) at a wavelength of 1.0332 Å. Collection data were autoprocessed by XDS. The structure of EcGusR was used to determine the correct phase of EcGusR, using the Phaser molecular replacement tool in PHENIX. After phasing, initial model building was performed in PHENIX via the AutoBuild function. Subsequent refinements and manual building were performed in PHENIX and Coot, respectively. The final model consisting of 648 amino acids was built with four molecules in the ASU. The final model was refined with an Rwork and Rfree of 0.1823 and 0.2280, respectively (Table S1).
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2385.
330:831
22.
427.
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μ
of 0.1940 and 0.2289, respectively
Structure
502:96
95.
1249.
1132.
25:967
217.
105.
337.
541:407
99.
Image 0x1 to 19x816
consisting of 404 amino acids was built with two molecules in the ASU. The
building were performed in PHENIX and Coot, respectively. The final model
in PHENIX via the AutoBuild function. Subsequent refinements and manual
were buffer-exchanged using Bio-Spin 6 columns (Bio-Rad) into GusR
and then gradually cooled back to room temperature. The resultant dsDNAs
in a 1:1 molar ratio (1 mM). The mix of ssDNAs was heated to 95 °C for 5 min
implements were ordered from Integrated DNA Technologies (IDT) of predicted
the Protein Data Bank with ID code 6AZH.
Preparation of dsDNA. Single-stranded DNA (ssDNA) sequences and com-
plements were ordered from Integrated DNA Technologies (IDT) of predicted
operator sites. ssDNAs were dissolved in annealing buffer (100 mM potassium
acetate, 30 mM Heps, pH 7.5). Complementary ssDNAs were mixed together in a 1:1 molar ratio (1 mM). The mix of ssDNAs was heated to 95 °C for 5 min and then gradually cooled back to room temperature. The resultant dsDNAs
were buffer-exchanged using Bio-Spin 6 columns (Bio-Rad) into GusR 5200 buffer (buffer C).

isothermal Titration Calorimetry Binding Studies. All ITC measurements were performed at 25 °C using an Auto-ITC200 microliterometer (MicroCal/GE Healthcare). The calorimetry cell (volume 200 μL) was loaded with GusR wild-type or mutant protein at a concentration of 50 μM or 100 μM for weak binding mutants. The syringe was loaded with a substrate (dsDNA or gluc-
uronide conjugate) concentration of 1 to 2 mM in a buffer identical to that employed for the protein. A typical injection protocol included a single 0.2-μL first injection followed by 26 1.5-μL injections of the substrate into the cal-
orimetry cell. The spacing between injections was kept at 180 s and the reference power at 8 μcal/s. A control experiment was performed by titrating ligand (dsDNA or glucuronide conjugate) into buffer under identical settings to determine the heat signals that arose from compound dilution; these were subtracted from the heat signals of protein–compound in-
teraction. The data were analyzed using Origin for ITC, version 7.0, software supplied by the manufacturer, and fit well to a one-site binding model.

In Vivo Induction Assay. A 5-ml overnight culture of BL21-Gold competent cells harboring pliC-His empty vector, UxuR, GusR, or a mutant thereof was grown in LB broth with 100 μg/mL ampicillin. Twenty microliters of the overnight culture was added to 2 ml of fresh LB broth and incubated at 37 °C for 1 h while shaking. PNPG or PNPG-Gluc at a final concentration of 1 mM was employed to induce the GUS operon of each culture, along with a no-inducer control. Additionally, cultures harboring protein expression plasmids were induced with 0.1 mM IPTG to induce protein expression. These cultures were incubated for another 3 h at 37 °C while shaking. One-
milliliter samples of each culture were transferred to a microcentrifuge tube and spun down at 13,000 × g for 10 min in a Sorvall (model Legend Micro 17). The supernatant was decanted and the cells were suspended in 1 ml of fresh LB broth with 100 μg/mL chloramphenicol (Cam), and then centrifuged again for 10 min and resuspended in another 1 ml LB-Cam mix. These twice-
washed cell samples were optimized by adding a single drop of 0.1% SDS and two drops of chloroform and vortexing for 30 s. Twenty microliters of the aforementioned washed cell samples was added to each well in a 96-well Corning flat clear-bottom black polystyrene microplate. Finally, 80 μL of buffer (1.25 mM PNPG, 150 mM NaCl, 20 mM Heps, pH 7.5) preincubated at 37 °C was added before starting the assay in the plate reader to monitor absorbance at 410 nm (PERStar® A13).