Molecular dissection of the glutamine synthetase-GlnR nitrogen regulatory circuitry in Gram-positive bacteria

Brady A. Travis¹, Jared V. Peck², Raul Salinas¹, Brandon Dopkins¹, Nicholas Lent¹, Viet D. Nguyen¹, Mario J. Borgnia³, Richard G. Brennan¹ & Maria A. Schumacher¹✉

How bacteria sense and respond to nitrogen levels are central questions in microbial physiology. In Gram-positive bacteria, nitrogen homeostasis is controlled by an operon encoding glutamine synthetase (GS), a dodecameric machine that assimilates ammonium into glutamine, and the GlnR repressor. GlnR detects nitrogen excess indirectly by binding glutamine-feedback-inhibited-GS (FBI-GS), which activates its transcription-repression function. The molecular mechanisms behind this regulatory circuitry, however, are unknown. Here we describe biochemical and structural analyses of GS and FBI-GS-GlnR complexes from pathogenic and non-pathogenic Gram-positive bacteria. The structures show FBI-GS binds the GlnR C-terminal domain within its active-site cavity, juxtaposing two GlnR monomers to form a DNA-binding-competent GlnR dimer. The FBI-GS-GlnR interaction stabilizes the inactive GS conformation. Strikingly, this interaction also favors a remarkable dodecamer to tetradecamer transition in some GS, breaking the paradigm that all bacterial GS are dodecamers. These data thus unveil unique structural mechanisms of transcription and enzymatic regulation.

¹Department of Biochemistry, 307 Research Dr., Box 3711, Duke University Medical Center, Durham, NC 27710, USA. ²Cryo-EM core, Department of Biochemistry and Biophysics, University of North Carolina, Chapel Hill, NC 27514, USA. ³Genome Integrity and Structural Biology Laboratory, National Institute of Environmental Health Sciences, National Institutes of Health, Department of Health and Human Services, Research Triangle Park, NC, USA.

✉email: Maria.Schumacher@Duke.edu
Nitrogen is an indispensable macronutrient for all organisms. Despite its importance, it is not abundantly bioavailable, which often makes it a growth-limiting factor. Because of this, highly regulated systems for nitrogen sensing, acquisition, and utilization have evolved in most organisms. In the cases when nitrogen levels are found to be low, there is an up-regulation of the genes encoding nitrogen acquisition factors. The same holds true when the nitrogen levels increase; the same genes should be down-regulated or repressed to conserve cellular resources. In Gram-positive bacteria, nitrogen homeostasis is not controlled by the NtrBC/o54 systems employed by enteric Gram-negative bacteria. In low G + C Gram-positive bacteria (Firmicutes), nitrogen homeostasis is instead controlled by the key nitrogen metabolic enzyme, glutamine synthetase (GS), and the transcriptional regulator GlnR. Comparative genome analyses indicate that the distribution of GS enzymes in living organisms is flexible, which often makes it a growth-limiting factor. GS is found in all extant life forms and only was the GS in the FBI-GS-TnrA complex tetradecameric, consistent with the Bs FBI-GS-TnrA crystal structure, but the GS in the FBI-GS-GlnR complex also adopted a tetradecameric state, which is consistent with the low-resolution FBI-GS-TnrA C-tail structure provided insight into how TnrA binds GS, but the GS in the structure adopted a tetradecameric state, which has been proposed to be a crystallization artifact. To date, the molecular mechanism by which GlnR binds FBI-GS is unknown. Underscoring the importance of understanding this conserved GS/GlnR circuitry, recent studies have shown that it contributes to virulence in several Gram-positive pathogenic bacteria, including *Listeria monocytogenes* and *Staphylococcus aureus*. Indeed, GlnR was initially identified in *S. aureus* (Sa) as a factor involved in methicillin resistance (femC) and *Listeria monocytogenes* (Nm) depends on the GlnR/GS regulon for the generation of nitrogen compounds, such as glutathione and glutamine, that are important for virulence. In *Paenibacillus polymyxa* (Pp), GlnR also plays a central role in nitrogen fixation. Sa, Lm, and Pp all lack TnrA genes. Hence, GlnR is the key nitrogen regulator in these bacteria. Here we show, using a combination of biochemical, crystallographic, and cryo-EM studies, the molecular mechanism by which the key nitrogen metabolic enzyme, GS, activates the activity of the GlnR regulator of nitrogen homeostasis.

Results

**Bs GS is tetradecameric in Bs GS-TnrA and Bs GS-GlnR complexes.** All bacterial GS structures solved to date have been dodecameres, with the exception of the Bs FBI-GS-TnrA C-tail crystal structure, which contains a tetradecameric GS. However, this oligomeric state was postulated to be a crystallization artifact. Thus, to assess the oligomeric state of Bs GS and the complexes it forms with TnrA and GlnR in solution we analyzed these samples by negative stain electron microscopy (EM). 2D classifications of the top views of the GS oligomers showed that apo Bs GS was dodecameric (Fig. 1A). Strikingly, however, not only was the GS in the FBI-GS-TnrA complex tetradecameric, consistent with the Bs FBI-GS-TnrA crystal structure, but the GS in the FBI-GS-GlnR complex also adopted a tetradecameric state (Fig. 1A). The Bs TnrA and GlnR DNA-binding domains are flexibly attached to their FBI-GS binding C-tails, indicating that these domains may not exist in a single orientation relative to the GS. Indeed, the GlnR and TnrA DNA-binding domains were not discernable in the low-resolution 2D negative stain EM classes. This finding is consistent with a recent cryo-EM study that examined fusion constructs of MBP covalently linked to GS. These data revealed that only a construct with a short linker between the proteins that enabled each MBP to make the same sets of contacts with its attached GS allowed for MBP visualization.

**The FBI-GS-GlnR interaction.** The Bs GS interaction with Bs GlnR is arguably the best characterized. However, data suggest that *Lm*, *Pp*, and *Sa* GS proteins also interact with their GlnR proteins. These data revealed that only a construct with a short linker between the proteins that enabled each MBP to make the same sets of contacts with its attached GS allowed for MBP visualization.
the FBI-GS-GlnR interaction in Bs, Sa, Lm, and Pp proteins by fluorescence polarization (FP) using fluorescently labeled peptides that contain GlnR C-tail residues (Methods). The Sa, Bs, Pp, and Lm FBI-GS proteins bound their corresponding fluorescently labeled GlnR peptides with $K_D$ of 4.2 ± 0.3 μM, 7.2 ± 0.3 μM, 18.7 ± 3.3 μM, and 27.2 ± 3.4 μM, respectively (Fig. 1B).

Structures of FBI-GS-GlnR cryo-EM complexes. Our negative stain EM studies showed that Bs GlnR binding to FBI-GS induces or stabilizes the formation of a GS tetradecamer. To ascertain if this is a conserved mechanism among FBI-GS-GlnR complexes and to elucidate, in detail, the molecular mechanism by which low $\alpha_3$-strands and $\beta$-strands and $\alpha$-helices. Helix $\alpha_3$ divides each subunit into a larger C-domain and a smaller, N-domain (Fig. 3B). Both GS dodecamers and tetradecamers form double-ring structures whereby the two rings are held together by interactions between C-terminal helical "thongs", $\alpha_{14}$ and $\alpha_{15}$, with $\alpha_{14}$ and $\alpha_{15}$ from the neighboring subunit in the other ring (Fig. 3B-C). The GS active sites, which are formed at the dimer interfaces in each ring, are composed of five key regions: the E flap (Bs residues 300–306), the Y loop (residues 365–373), the N loop (residues 231–242), the $\beta$-hairpin loop (residues 148–158) and the D50$'$ loop (residues 52–66), the latter loop being the only active site region contributed from the adjacent GS subunit (Fig. 4A).

GlnR C-tail binds within the FBI-GS active site. Interestingly, the GlnR binding site in FBI-GS shows overlap with the TnrA C-tail site (Supplementary Fig. 5A). This is consistent with previous data showing that mutations in Bs GlnR C-tail residues 301–306 impaired interaction with both TnrA and GlnR. These data were obtained using a fluorescence polarization (FP) assay (Supplementary Fig. 5A, B). In the FBI-GS-GlnR structures, each GS pore binds two closely juxtaposed but non-interacting GlnR C-tails as each pore contains two active sites, one from each ring (Fig. 3C).

Fig. 1 8s GS forms tetradecamers in the presence of TnrA and GlnR. A 2D class averages of negative stain EM images of apo 8s GS, 8s FBI-GS-TnrA, and 8s FBI-GS-GlnR complexes. Right shows a close-up of the top views revealing that apo 8s GS is a dodecamer, while 8s FBI-GS-TnrA and 8s FBI-GS-GlnR are tetradecamers. B Fluorescence polarization (FP) based assays analyzing 8s FBI-GS, Sa FBI-GS, Pp FBI-GS, and Lm FBI-GS binding to their corresponding fluorescently labeled GlnR C-tails with $K_D$ of 4.2 ± 0.3 μM, 7.2 ± 0.3 μM, 18.7 ± 3.3 μM, and 27.2 ± 3.4 μM, respectively. The curves are representative curves from three technical repeats. The error bars represent SD. Data were presented as mean values ± SD. The source data are provided in the Source Data file.
proton from ammonium while the E flap contains the catalytic glutamate (asterisk, Fig. 4A). Thus, it is striking that these GS regions provide the majority of the interactions with GlnR (Fig. 4B–F and Supplementary Fig. 6). GlnR contacts are also provided by GS residues on thong helix α14′ from the adjacent dimer and α15″ from the neighboring subunit of the other ring. Thus, the GlnR binding site within the GS active site is positioned at the nexus of the double-ring oligomerization region (Fig. 4B–F).

A consensus GlnR binding motif for FBI–GS interaction. The FBI-GS-GlnR structures show similar overall GlnR binding modes (Fig. 4B–F). The GlnR C-tails in all but the Lm structure contains a glycine at the N-terminal region of the bound peptide (XG(E/D)XSRF). This glycine binds proximal to the E flap, allowing its amide nitrogen to H bond with the carbonyl oxygen of the GS glycine residue in the E flap. The corresponding residue in Lm GlnR is glutamine and the density corresponding to this N-terminal GlnR peptide region in the Lm structure is poorly resolved (Fig. 4F). Arg62′ (Bs numbering) from the D50′ loop interacts with an acidic residue that is generally conserved in GlnR C-tails (XG(E/D)XSRF). The GlnR acidic residue also interacts with GS residue Arg316. Arg316 is a key catalytic residue and must move to the active site during catalysis. This relocation would be prevented by GlnR binding.

The SRF motif is the most conserved region of the GlnR C-tails (Fig. 4B–F). The GlnR arginine of this motif makes contact with residues on α15′ and stacks with the side chain of the aromatic residue in the GS D50′ loop in the Pp, Sa, and Bs structures. In the Lm structure this arginine contacts an α14′ acidic residue. Residues in α14′ and α15′ also make extensive hydrophobic contact with the GlnR SRF phenylalanine residue. Finally, the
serine from the SRF motif acts as a brace, anchoring the peptide across the dimer, by making an H bond to backbone atoms of E flap residues (Fig. 4B–F).

Comparison of apo and transition state structures of Bs, Pp, Lm, and Sa GS. Previous GS structures from E. coli, M. tuberculosis, Salmonella typhimurium, and Helicobacter pylori were all dodecameric as was our apo Bs GS structure.24,29–32,48. However, our EM data showed that GlnR binding results in the formation of a tetradecamer in several GS. Whether the GS tetradecamer only forms in the presence of TnrA and GlnR is unclear. Thus, we next obtained cryo-EM structures of apo Pp, Lm, and Sa GS to resolutions of 3.16, 2.85, and 2.13 Å, respectively (Supplementary Table 2). These structures revealed that, like the Bs apo GS, the Lm, Sa, and most of the Pp apo GS states are dodecamers. However, a small percentage of tetradecamers were evident in the apo Pp GS sample (Supplementary Fig. 7). But, due to the small number and limited side views of tetradecamers in the data, we were unable to generate a 3D reconstruction of the apo Pp GS tetradecamer. Nonetheless, this finding, along with our FBI-GS-GlnR structures, clearly indicates that the current paradigm that all bacterial GS enzymes are dodecamers needs revision.

Interestingly, overlays of the individual GS subunits show that the apo GS subunits are similar to those in the FBI-GS-GlnR complexes (rmsds of 0.5–0.8 Å for overlay of 400 corresponding Ca atoms between GS proteins). In this conformation, key active site residues are not properly positioned for catalysis. In particular, the Arg62 side chain is rotated into the active site preventing the proper positioning of the catalytic Asp50’ side chain and Arg316, which assists in catalysis, is moved out of the active site. Hence, conformational changes in the apo states would be needed to generate an enzymatically active conformation unless these enzymes employ a catalytic mechanism different from other GS. In fact, the electron density for the active site regions of the apo GS structures are poorly defined indicating flexibility in these regions. This suggests that they could undergo conformational changes during catalysis.

To visualize the active, transition state (TS) conformations of the GS proteins we reacted them with L-methionine-S-sulfoximine (MSO) in the presence of ATP and solved the structures. In GS proteins this reaction leads to the formation of a stable TS analog, L-methionine-S-sulfoximine phosphate (Met-Sox-P), and ADP. In these structures, the Met-Sox-P methyl group occupies the ammonium substrate binding site, thus mimicking the GS transition state and preventing further reaction. Structures of the Pp, Lm, and Sa GS-Met-Sox-P-ADP complexes were obtained to 1.98, 3.50, and 2.95 Å resolution, respectively (Fig. 5A and Supplementary Table 3). These structures were all dodecameric and densities for Met-Sox-P and ADP were clearly observed in each enzyme (Fig. 5A). The subunit and oligomeric structures of each TS structure were also the same and were similar to our Bs TS structure (rmsds of 0.5–0.7 Å for overlay of 400 corresponding Ca atoms in each subunit) (Fig. 5B). The GS TS structures all display the same contacts to the Met-Sox-P and ADP. Hence, the contacts in the Pp structure will be described here due to its higher resolution (Fig. 5A, right panel). In the complexes, the ADP adenine N6 is read by the carbonyl oxygen of
Cryo-EM FBI-GS-GlnR structures reveal GlnR binds in the GS active site. A Cartoon diagram of the Pp GlnR C-tail (red) binding to GS. GlnR binds in the GS active site between two subunits and at the nexus of the oligomer interface between stacked rings. In this figure one GS subunit is colored green and the other salmon. The active site regions are colored blue and labeled. B Density for GlnR peptide and contacts with GS in the Pp FBI-GS(12)-GlnR complex (map contoured at 0.09 σ). Residues making contacts are labeled and different GS subunits are denoted as GSI, GSII, and GSIII, underscoring that three GS subunits participate in GlnR contacts. C Density for GlnR peptide and contacts with GS in the Pp FBI-GS(14)-GlnR complex (map contoured at 0.06 σ). D Density for GlnR peptide and contacts with GS in the Sa FBI-GS-GlnR complex (map contoured at 0.15 σ). E Density for GlnR peptide and contacts with GS in the Bs FBI-GS-GlnR complex (map contoured at 0.65 σ). F Density for GlnR peptide and contacts with GS in the Lm FBI-GS-GlnR complex (map contoured at 0.55 σ).

Leu326. The side chains of Tyr199 and Arg329 make stacking interactions with the adenine base and Ser247 makes a hydrogen bond to the adenine N1 atom. Contacts to the Met-Sox-P phosphate are provided by Mg2+ ions and the side chains of GS residues Arg314, Arg329, Arg333, Arg334, and His243. The Arg296 side chain interacts with the Met-Sox carboxyl moiety and the Met-Sox-P amide nitrogen interacts with the Glu132 side chain as well as the carbonyl of Gly239. Catalytic E flap residue Glu302 contacts Asp52´ and is close to the Met-Sox-P methyl group. The Glu302–Asp52´ interaction would shield the Met-Sox-P from attack by bulk solvent as well as facilitate proton abstraction of the ammonium to form ammonia. The resultant ammonia would then be in an ideal position to attack the γ-glutamyl phosphate intermediate. Thus, the GSI-α-Met-Sox-P-ADP complexes mimic the tetrahedral adduct, formed before the generation of the glutamine and inorganic phosphate products.

Structures indicate a conserved two-state model for low G + C Gram-positive GS. Strikingly, while the TS structures are similar to each other, they show significant differences, both global and local, when compared to the corresponding apo and FBI-GS-GlnR bound states (as apo and FBI-GS-GlnR conformations are the same, the state will be referred to as apo/FBI-GS-GlnR) (Fig. 5B). The largest local structural changes between the GS TS and apo GS/FBI-GS-GlnR-bound states are within the active site loops regions (rmsd of apo GS/FBI-GS-GlnR versus Met-Sox-bound GS subunits is 2.0–2.5 Å for 400 corresponding Ca atoms), but the rmsds are 0.8 Å when the active site loops are not included. Moreover, superimposition of the TS dodecamer onto the apo GS or FBI-GS-GlnR bound oligomers (comparing Ca atoms of the dodecamers) results in rmsds of >3.0 Å, indicating that the catalytically induced structural changes are transmitted between subunits, causing significant alterations in the oligomeric structure (Fig. 5B). Although all GS active site loops are altered upon TS formation, residues in the D50´ loop undergo the most dramatic structural rearrangements (Fig. 6A–C). In the TS state, Asp62´ and Arg316 are repositioned to provide an optimal active site architecture. Thus, our structural analyses indicate that GS enzymes from low G + C Gram-positive bacteria adopt two distinct states, the active, TS conformation (herein termed the “A” state) and the conformation adopted by apo GS/FBI-GS-GlnR, termed the “I” or inhibited state. This is distinct from GSI-β enzymes the structures of which have revealed moderate active site loop movements between TS and apo states while taut and relaxed designations for GSI-β structures have been described, they involve structural changes in loops caused by metal binding.48

High GlnR concentrations can inhibit GS. The structural comparisons show that GlnR binding favors an inhibited conformation, which is also adopted by the apo state. This further suggested that at high concentrations GlnR may bind, albeit weakly, to the apo state and possibly inhibit GS activity. Hence, we performed enzyme assays to test this hypothesis and found that indeed, the addition of high concentrations of GlnR had an inhibitory effect on GS activity (Fig. 5C). Thus, the GS-GlnR interaction may be used as a possible route in the design of antimicrobials.

As noted, although the apo GS structures are not optimally configured for catalysis, their active site regions are not confined and appear flexible. Hence, these regions could readily transit to the active conformation during substrate binding and catalysis. Glutamine binding, however, stabilizes the I state. But the formation of the FBI-GS state depends on the intracellular concentrations of glutamine, which have been measured to range from 0.3 to 3 mM.49 Thus, in the absence of GlnR, the
The multiple measurements (from three to six) with the error bar representing SD. Two-way ANOVA using the software GraphPad Prism 9 was performed.

Length proteins were utilized. GS activity was measured (as described in methods) in the presence and absence of GlnR. The results are the average of all three cases (Sa, Lm, and Pp, Bs, Lm, and Sa GS bind glutamine identically. Thus, we used ITC and measured a K_d of 0.5 mM for glutamine binding to Sa GS (Supplementary Fig. 8). This relatively weak affinity suggests that glutamine might dissociate when its intracellular concentrations are low. The addition of GlnR would lock in the inhibited state. Thus, our combined data indicate that GlnR functions as a negative allosteric regulator of GS, possibly providing another level of regulation during conditions of nitrogen excess.

**Structure and function analyses of DNA binding by GlnR homologs.** Our FBI-GS-GlnR C-tail structures reveal the mode of GlnR binding to GS. However, to gain insight into the possible conservation of the DNA-binding mechanism among GlnR homologs and how FBI-GS binding to GlnR activates its DNA-binding function, we performed biochemical and structural analyses on GlnR–DNA interactions. Our previous Bs GlnR–DNA structure shows that Bs GlnR binds a palindromic DNA site as a dimer. Sequence homology among GlnR proteins exists primarily within the DNA-binding domain and, to a lesser extent, the last ~10 residues comprising the GS-binding domain (Fig. 7A). The linker region connecting these domains varies in both sequence and length but is expected to play a key role in GlnR function as its length and conformation would impose restrictions on the ability of GlnR DNA-binding domains, when bound to FBI-GS, to form a DNA-binding active state. The 122 residue Sa and Lm GlnR proteins have shorter linkers than Pp and Bs GlnR, which have 135 and 137 residues, respectively, and hence may not function the same in enabling DNA binding and autoinhibition as the longer GlnRs.

Thus, to determine if the C-tail of the shorter Sa GlnR is autoinhibitory, as observed for the Bs GlnR, we measured DNA binding by FL Sa GlnR and truncated Sa GlnR, the latter protein lacking C-tail residues (Methods). These experiments, which revealed K_d of 9.9 ± 0.9 nM and 75 ± 8.0 nM for the truncated and FL Sa GlnR protein, respectively, support the presence of the Sa GlnR C-tail inhibits DNA binding (Fig. 8A). We also showed that a fluoresceinated Sa GlnR C-tail peptide binds to truncated Sa GlnR, providing support for direct autoinhibition by the GlnR C-tail (Supplementary Fig. 9).

**Lm and Sa GlnR-DNA structures.** We next obtained structures of Sa GlnR and Lm GlnR bound to a 21 bp glnR consensus operator site to 2.35 and 3.45 Å, respectively, by X-ray crystallography (Methods; Supplementary Table 4). The GlnR structures are similar to each other and the Bs GlnR (rmsd = 0.6–0.8 Å for overlay of 67 corresponding Ca atoms). Each GlnR subunit can be divided into three regions, a variable N-terminal region, a MerR-like winged-HTH (with topology: α1-α2-β1-β2), and a short helical domain (Fig. 7A-B). The latter region forms two α-helices, α3-α4, in the Sa GlnR-DNA, but in the Lm GlnR-DNA structure, this region contains just one helix (Fig. 7C). Residues 2–73 and 2–83 were visible for the Lm and Sa GlnR proteins respectively. The Lm and Sa GlnRs form comparable dimers to the Bs GlnR and dock on the DNA similarly (Fig. 7C). As observed in the Bs structure, both DNA sites bound by Sa and Lm GlnR are bent inward but the DNA from the Lm complex is
slightly less bent (~21°) compared to the Sa and Bs structures (~25°) (Fig. 7C). However, like Bs GlnR, the Sa and Lm GlnR surfaces that contact the DNA are both electropositive, indicating electrostatics as a general contributor to GlnR DNA binding (Fig. 7D).

**GlnR-DNA operator contacts.** The GlnR DNA used for crystallization contains four conserved bps (bolded) in each half-site, 5'-CGTGTGAGATAACTGACACG-3' (where the consensus binding site is N,G,T,N,T,N,A,T,N,T,N,A,T,N,N,A,T,N). These conserved bps are contacted in the major groove by Lm and Sa GlnR residues that are completely conserved among GlnRs; a conserved tyrosine (Sa Tyr32/Lm Tyr31) makes hydrophobic contacts to the DNA thymine (T3) methyl group, an arginine (Sa Arg31/Lm Arg30) specifically reads the G4 base and makes hydrophobic interactions with the methyl moiety of T5 and finally, the side chain methylene carbons of a conserved arginine (Sa Arg31/Lm Arg30) contacts the T7' methyl group (Fig. 8B-C).

Previous binding studies showing that substitution of G4 to any other nucleotide abrogates GlnR binding and substitution of conserved nucleobases 3, 5, and 7 leads to significant reductions in binding are consistent with the structures. Phosphate contacts help anchor the GlnR proteins to the DNA and are mostly conserved. However, GlnR proteins contain different wing residues, which our structures show make distinct contacts to the minor groove; wing residues Bs Arg46 and Sa Lys48 make nonspecific base contacts in the minor groove, but the corresponding Lm GlnR residue, His47, makes hydrophobic contacts to a ribose. The lack of minor groove base contacts by Lm GlnR may explain the slightly reduced DNA bend observed in this structure as the minor groove base contacts by Bs Arg46 and
Sa Lys48 are facilitated by the more bent DNA conformation (Fig. 8B). To test the role of this wing residue in operator DNA-binding affinity, we mutated the Sa GlnR Lys48 residue to arginine and histidine, which are found in Bs and Lm GlnR and performed FP studies. The Kds determined in these experiments were 9.9 ± 0.8 nM, 8.0 ± 0.6 nM, and 11.6 ± 0.6 nM for WT Sa GlnR(1-87), Sa GlnR(1-87)K48R, and Sa GlnR(1-87)K48H, respectively (Supplementary Fig. 10; Methods). Thus, these data indicate that the different wing contacts by GlnR proteins do not significantly impact operator binding affinity.

Mechanism for GS mediated DNA-binding activation of GlnR.
A notable feature of the GlnR-DNA structures is the small GlnR dimer interface. These interfaces are formed by hydrophobic contacts between residues in the N-terminal domains. The Sa GlnR dimer is generated by interacting N-terminal loops, while the Lm and the B. subtilis N-terminal regions fold into distorted helices that interact. Thus, the GlnR dimer interfaces created upon DNA binding show some variability. Nonetheless, all these interfaces bury significantly less surface area (∼300–400 Å² BSA) than the >2000 Å² shielded by physiologically relevant dimers. Indeed, most GlnR proteins have been reported to be monomeric and while a few studies suggest some GlnR proteins may dimerize or exist in an equilibrium between monomer and dimers, these experiments were typically performed at high, nonphysiologically relevant concentrations. Clearly, control of GlnR dimerization and hence specific DNA binding would be an efficient mechanism of gene regulation. Thus, these data combined with our FBI-GS-GlnR structures indicate a mechanism for FBI-GS activation of GlnR binding. Specifically, our data show that the C-terminal residues of GlnR form a distorted helix when bound to FBI-GS while our GlnR-DNA structures revealed residue 84 as the last ordered/visible residue in GlnR-DNA complexes. The flexible connection between these domains would thus be minimally comprised of ∼30 residues (calculated as the number of residues between the first and last residues visible in the Sa GS-GlnR and Sa GlnR structures; the B. subtilis and L. monocytogenes disordered linkers are >40 residues), which if fully extended could span >90 Å. Two C-tails

Fig. 7 GlnR-DNA structures. A Multiple sequence alignment of the B. subtilis, S. aureus, L. monocytogenes, and P. putida GlnR proteins with secondary structural elements (from B. subtilis, S. aureus, and L. monocytogenes) shown above the alignments. Residues in the N-terminal domains are highlighted in yellow, those in the wHTH are in pink and those in the C-terminal region are green. C-tail residues that bind FBI-GS are highlighted in red. Residues not observed in either the GlnR-DNA or FBI-GS-GlnR structures are not highlighted and form the flexible linker connecting domains. Secondary structural elements are indicated over the sequence alignments. B–D Structure of the Sa GlnR-DNA complex, with one subunit colored as in A and the other colored gray. C Superimpositions of the Sa, B. subtilis, and L. monocytogenes GlnR-DNA complexes, which are colored slate, salmon, and cyan, respectively. D Electrostatic surface diagrams of Sa, B. subtilis, and L. monocytogenes GlnR proteins bound to DNA. Blue and red represent electronegative and electropositive surfaces, respectively. The figure was generated as a charged smoothed potential in PyMOL (Sa GlnR-DNA charge potentials levels range from −86 to +86, B. subtilis GlnR-DNA from −73.7 to +73.7, and the L. monocytogenes GlnR-DNA from −81.3 to +81.3).
bound by FBI-GS in each side pore are separated by only 7 Å, which would juxtapose the extended linkages between the domains, increasing the local concentration of the GlnR DNA-binding domains, facilitating dimer formation on operator DNA (Fig. 9).

Discussion
Glutamine synthetases are one of the most ancient enzymes and are essential for all organisms. Thus, these enzymes have been extensively studied. These investigations include multiple efforts to target GS function in the development of herbicides and therapeutics; plant GS are currently targeted by the herbicide glufosinate and GS enzymes have been proposed as promising drug targets in the treatment of Mycobacterial infections and cancer. Despite extensive studies on GS, our work shows that much remains to be learned regarding the structural and functional aspects of these enzymes as well their regulation. Indeed, long-held dogma in the field has been that bacterial GS form dodecamers. Using electron microscopy, we show here that some Gram-positive bacterial GS enzymes can exist in a tetradecameric state and that this state is favored by binding to the master transcription regulator of nitrogen metabolism, GlnR.
The FBI-GS-GlnR circuitry represents a unique regulatory paradigm in which a key metabolic enzyme directly transduces nutrient availability to the transcription regulator (GlnR). Our studies on the GS and GlnR proteins from *B. subtilis*, *S. aureus*, *P. polymyxa*, and *L. monocytogenes* show that the GS enzymes form large oligomers composed of two stacked rings, and exist in two distinct states, a state optimal for catalysis and an inactive state. However, while all bacterial GS form oligomers composed of two stacked rings, there has been no known function for this double-stacked structure and the side cavities between rings. Our high-resolution structures of *Pp*, *Bs*, *Lm*, and *Sa* GS-GlnR complexes revealed a role for this pore, which is to function as a cavity for GlnR C-tail binding. In this way, GS functions as a GlnR chaperone, facilitating the folding of the GlnR C-tail and leading to GlnR DNA-binding activation.

It is currently unclear why GlnR binding to some GS proteins favors the tetradecameric form over the dodecameric and why some GS can form tetradecamers in their apo state. However, the data point to the helical thongs, α14 and α15, as being important in determining the oligomeric state. In particular, our structures show that the deep insertion and conserved folding of the GlnR C-tail into the central GS pore region occurs at the nexus where the two rings join, namely α14 and α15. This insertion could enhance and/or stabilize subunit exchange favoring the tetradecamer. In fact, our finding that Gram-positive GS (GSI-o) enzymes can exist in dodecameric and tetradecameric states was unusual but is consonant with data showing that GS proteins, in general, exist in an equilibrium between monomeric and oligomeric conformations. Indeed, size exclusion chromatography (SEC) analyses on GS from *E. coli*, *Ruminococcus albus 8*, *Neurospora crassa*, and the plant *Phaseolus vulgaris* revealed the presence in solution of both monomers and higher-order oligomers.53–56 We have also observed monomers of GS in equilibrium with higher-order oligomers in SEC experiments on *Bs* GS.39 Subunit exchange between oligomers would impair GS activity as the active sites are formed between two GS subunits and because GS enzymes are cooperative23,53 such disruption could impact the activity of the entire oligomeric complex.

Our data show that the tetradecamer form is favored in the presence of GlnR and TnrA in some GS, indicating that these regulators shift the equilibrium towards the tetradecameric state. Because GS undergoes transitions from monomeric to oligomeric states, monomer dissociation could result in an unoccupied site within each stacked layer that could be fit by the insertion of an additional GS subunit (Fig. 9). The distinct residues in the GS interfaces and/or the binding affinities of the GlnR C-tail for its GS partner may contribute to this phenomenon. In this regard, it is interesting to note that while the *Lm* GlnR peptide binds with lower affinity to its FBI-GS, the most ordered part of its C-tail is its SRF motif, which makes contacts to α14 and α15. In addition, GS sequence alignments show that residues in α14 and the connection between α14 and α15 that contact GlnR and contribute to the oligomer interface show a higher degree of sequence variability than the rest of the protein and thus could determine whether a given GS can form a tetradecamer (Supplementary Fig. 6). However, testing the hypothesis that α14-α15 residues determine GS oligomeric states and can be influenced by GlnR binding is complicated by the fact that mutation of these residues may also impact GlnR binding. Thus, future studies will be required to dissect the possible roles of α14 and α15 in GS oligomer selection.

Our data show that FBI-GS-GlnR complexes not only influence the GS oligomeric state but also favor the inhibited state, providing another level of regulation to the system. Interestingly, other GS-binding polypeptides have recently been identified and characterized. For example, the *Methanosarcina mazei* GS was shown to bind a 23 residue ORF encoded peptide, called sP26,57 the soybean GS binds the C-terminal domain of nodulin 26 (Nod26), a key symbiosome protein that is involved in nitrogen fixation58 and the cyanobacterial GS interacts with small proteins called IFs that inhibit its activity.69–63 The molecular bases for these interactions are currently unknown. But these findings show that protein-protein interactions are utilized to regulate GS enzymes of prokaryotes and eukaryotes. Interestingly, while no structural information is available, data on the GS-sP26 interaction indicate that sP26 activates GS activity by shifting its equilibrium from the monomer to the higher-order oligomer state.57 Like other GS enzymes, the *M. mazei* GS must form an oligomer for its activity as its active sites are formed between two subunits in the oligomer. These studies further underscore the importance of the GS oligomeric state for its activity.

A key function of the FBI-GS-GlnR interaction is an activation of the DNA-binding activity of GlnR. GlnR is monomeric in the absence of GS and operator DNA. Our combined data unveil a molecular mechanism for FBI-GS-mediated activation of GlnR whereby the juxtapositioning (7 Å) of two GlnR monomers bound in adjacent active sites facilitates GlnR dimer contacts on DNA. Specifically, our GlnR-DNA structures reveal that each protein has a short, ordered C-terminal region after the wTH region that is connected, via an extended linker of ~30 or more residues to the FBI-GS-binding domain. By contrast, the DNA-binding activity of TnrA, which is abrogated by its interaction with FBI-GS, has only four flexible residues connecting its FBI-GS and DNA-binding domains. Hence, even if fully extended, two TnrA subunits would be unable to interact on a DNA to form a DNA-binding dimer. Thus, the longer linker between the FBI-GS and DNA-binding regions in GlnR differentiates GlnR from TnrA and leads to the FBI-GS interaction facilitating dimer formation in GlnR rather than preventing or disrupting dimer formation.

In conclusion, our combined data provide detailed, high-resolution molecular snapshots of the nitrogen regulatory machinery from multiple non-pathogenic and pathogenic low G+C Gram-positive bacteria. These data show that these GS proteins are unusual multitasking proteins that function as enzymes and transcription regulators and unveil conserved mechanisms of activation of these enzymes and modes of GlnR binding to DNA. The findings also reveal unexpected features of this unique metabolic regulatory circuitry. First, our data indicate that the FBI-GS-GlnR interaction facilitates dimer formation of the weak GlnR dimer on the DNA by the close binding of two GlnR C-tails within each GS active site. Second, we showed that the FBI-GS-GlnR interaction also impacts GS function by stabilization of the GS inactive form. Third, our work breaks the current dogma that bacterial GS always form dodecamers. Finally, these studies also provide a template for the development of specific therapeutics, modeled from the GlnR-FBI-GS interaction, against Gram-positive pathogens.

**Methods**

**Protein purifications.** The genes encoding *Bs* GS, *Sa* GS, *Lm* GS, *Pp* GS, *Sa* full length (FL) GlnR, *Lm* FL GlnR, *Pp* FL GlnR, *Sa* GlnR(1–87), *Lm* GlnR(1–87), and *Pp* GlnR(1–87) were purchased from GenScript Corporation and subcloned into pET15b such that an N-terminal His-tag was expressed on each protein for purification (Piscataway, NJ, USA; http://www.genescrit.com). *Escherichia coli* C41(DE3) cells were transformed with these expression vectors. Cells with each expression construct were grown at 37 °C in an LB medium with 0.10 mg/mL ampicillin to an OD600 of 0.3–0.4, then induced with 0.30 mM isopropyl β-D-thiogalactopyranoside (IPTG) at 15 °C overnight. Cells were harvested by centrifugation and then resuspended in Buffer A (50 mM Tris-Cl pH 7.5, 300 mM NaCl, 5% (v/v) glycerol, 5 mM MgCl2, 0.5 mM β-mercaptoethanol (β-ME), with 1X protease inhibitor cocktail and DNase I (~10 μL of 100 μg/mL DNase I per reconstitution). The resuspended cells were then disrupted with either a sonicator or microfluidizer and cell debris was removed by centrifugation (18,000 × g, 4°C, 60 min). For each protein, the supernatant was loaded onto a cobalt NTA column.
The column was washed with 300–500 mL of 2 mM imidazole in Buffer A and eluted in steps with 5, 10, 20, 30, 40, 50, 100, 200, 300 mM, and 1 M imidazole in Buffer B. All proteins were analyzed by SDS-PAGE. Protein concentration was determined using the BCA assay. The His-tags were removed in all the proteins used in crystallography experiments by thrombin digestion overnight at room temperature (rt) to generate a colorimetric product read at an absorbance of OD570. For these assays, we utilized the Biovision colorimetric GS activity kit (Cat K2056-100). In this crystal, after reaction initiation, the absorbance was measured immediately at 570 nm using a Molecular Devices SpectraMax M5.

Glutamine synthase (GS) enzyme assay. To corroborate GS enzyme activity, we utilized the Biovision colorimetric activity kit (Cat K2056-100). In this sensitive assay, the ADP generated from GS activity is utilized in a subsequent reaction in the presence of an ADP converter, developer mix, and ADP probe to generate a colorimetric product read at an absorbance of OD570. For these assays, the Sa, Pp, and Lm GS proteins were the first buffer exchanged into the GS Assay Buffer from the kit. The protocol included with the kit was used for the assays and the absorbance was measured immediately at 570 nm using a Molecular Devices SpectraMax M5, after reaction initiation. In these experiments, Pp and Sa GS were present at 300 pM and the Lm GS at 600 pM. GS was added at a concentration of 10 nM. The measurements were done in kinetic mode at rt at 5 min intervals. One unit of GS activity was defined as the amount of enzyme that produces 1 μmol of ADP per min at pH 7.2 at 37 °C. The sample size for Sa, Lm, and Pp were 6, 4, and 3, respectively, and performed as independent experiments (on different days). To analyze the data, we performed Two-way ANOVA using the software GraphPad Prism 9.0. The two independent factors were GS and GlnR for each bacterial species. The P value for all the source of variation (interaction, row factor, and column factor—as specified in the software) are statistically significant (<0.0001) in all three cases (Sa, Lm, and Pp). The error bars represent the standard error of the mean.

Fluorescence polarization (FP) binding experiments. To measure DNA binding to Sa FL GlnR and GlnR(1-87), a fluorescence-activated version of the 21-mer DNA site used for crystallization was obtained. For the experiment, increasing concentrations of the proteins were titrated into the sample cell containing 1 nM of the DNA in a buffer of 25 mM Tris pH 7.5, 100 mM NaCl, and 5 mM MgCl2. To this, the protein:DNA complex was created in Phenix65, and replacement of solvent molecules were added. The structure contains two GlnR dimer-DNA complexes in the asymmetric unit (ASU); the GlnR subunits in the complexes can be superimposed with rmsds of 0.25 Å (for 77 Ca atoms). See Supplementary Table 4 for data collection and refinement statistics. A Sa GlnR monomer bound to a final 0.5-mer was added to the GlnR-DNA structure. Crystallographic symmetry generates the GlnR dimer and duplex DNA in this crystal. After one round of refinement, the Lm residues were substituted. All the nucleotides of the DNA site in the ASU were visible in the structure but only GlnR residues 2–73 could be traced in the density. Molprobity (version 4.5.1) was used to check and validate the GlnR-DNA structures. The Lm GlnR-DNA crystals were highly anisotropic, possibly explaining the slightly elevated R values.

Negative stain EM. For the negative stain EM experiments, purified Bs GS was in a buffer consisting of 25 mM Tris pH 7.5, 200 mM NaCl, 5% (v/v) glycerol, and 1 mM MgCl2. The Bs GS-TnR and Bs GS-GlnR samples were in a buffer consisting of 25 mM Tris pH 7.5, 200 mM NaCl, 5% (v/v) glycerol, 1 mM MgCl2, and 5 mM glutamine. Bs GS was mixed 1:1 stoichiometrically with Fl TnrA and Fl GlnR to generate the GS-TnR and GS-GlnR sample complexes. Samples were diluted to a final concentration of 20–50 μg/mL and prepared for negative stain using buffer instead of water for the washes. Negatively stained specimens were imaged in a Tecnai 12 electron microscope (FEI Company) equipped with a LaB6 electron source and operated at 120 kV. Images were automatically collected under low-dose conditions using EPU (FEI Company) at a nominal magnification of 67,000×. Unprocessed EM images (1–3 mm) were recorded on a US$4000 CCD camera (Gatan) with a pixel size at the specimen level of 1.77 Å. Images were processed with EMAN2 (2.91) to produce 2D classes.

Isotothermal titration calorimetry (ITC) binding of GS to glutamine. ITC experiments were performed using a VP-ITC system (MicroCal Inc., Northampton, MA, USA) to analyze glutamine binding to GS. The Ts GS was used for the studies as it could be dialyzed and concentrated to high concentrations without noticeable precipitation. For ITC, the Ts GS sample was dialyzed into the ITC buffer (25 mM Tris pH 7.5, 150 mM NaCl and 5 mM MgCl2) and the t-glutamine (t-glutamine is used in all studies and is denoted as glutamine) was dissolved in the same dialysis buffer. The ITC experiments were performed with glutamine (in the syringe) at the concentration of 50 mM and Ts GS at a hexamer concentration of 42 µM (in the sample cell). Glutamine was titrated into the sample cell containing Ts GS at 25 °C, and the resulting thermogram was fitted with Origin version 7.0 (MicroCal LLC).
**Cryo-EM sample and grid preparation.** Cryo-EM flow diagrams for processing and final structure analyses are provided in Fig. 2 and Supplementary Figs 1–4.

Bs GS14-Q-GlnR peptide. Purified Bs GS was buffer exchanged into Buffer B (12.5 mM Tris pH 7.5, 20 mM glutamine, 5 mM MgCl₂, 150 mM NaCl, 2.5% (v/v) glycerol, 1 mM BME) using a 10 kDa MWCO spin filter (Millipore). Next, GS (1.0 mg/mL) was mixed with 2.5 mM Bs GlnR C-tail peptide (Genscript; sequence: QQAGRFVKQ-LMGKDLRQP) and incubated at rt for 30 min. For grid preparation, Quantifoil R1.2/1.3 Cu 300 (Quantifoil) holey gold grids were cleaned for 180 s using a PELCO easiGlow glow discharge cleaning system and 3 μL of the sample were applied at 95% humidity and 22 °C. Following a 10 s incubation period, the grids were blotted for 1.5 s and plunged frozen into liquid ethane using a Leica EM GP2 (Leica Microsystems). All grids were stored in liquid nitrogen until imaging.

Lm GS14-Q-GlnRt peptide. Purified Lm GS was exchanged into Buffer B using a 10 kDa MWCO spin filter (Millipore). Next, Lm GS (0.9 mg/mL) was mixed with 2.5 mM Lm GlnR C-tail peptide (Genscript; sequence: QQAGRFVKQ-LMGKDLRQP) and incubated at rt for 30 min. For grid preparation, Quantifoil R1.2/1.3 Cu 300 (Quantifoil) holey carbon grids were cleaned for 100 s using a PELOC easiGlow glow discharge cleaning system and 3 μL of the sample were applied at 95% humidity and 22 °C. Following a 10 s incubation period, the grids were blotted for 1.5 s and plunged frozen into liquid ethane using a Leica EM GP2 (Leica Microsystems).

Pp GS14-W-Q-GlnR peptide and Pp GS14-Q-GlnR peptide. Purified Pp GS was exchanged into Buffer B using a 10 kDa MWCO spin filter (Millipore). Next, Pp GS (0.8 mg/mL) was mixed with 2.5 mM Pp GlnR C-tail peptide (Genscript; sequence: KRPGQVSLIQGELSRFFNNR) and incubated at rt for 30 min. For grid preparation, Quantifoil R1.2/1.3 Cu 300 (Quantifoil) holey carbon grids were cleaned for 100 s using a PELOC easiGlow glow discharge cleaning system and 3 μL of the sample were applied at 95% humidity and 22 °C. Following a 10 s incubation period, the grids were blotted for 1.5 s and plunged frozen into liquid ethane using a Leica EM GP2 (Leica Microsystems).

Sa GS12-Q-GlnRt peptide. Purified Sa GS was buffer exchanged into Buffer B using a 10 kDa MWCO spin filter (Millipore). Next, GS (0.75 mg/mL) was mixed with 5 mM Sa GlnR C-tail peptide (Genscript; sequence: KPIGETLPINGRDSLFRF) and incubated at rt for 30 min. For grid preparation, UltraAulfoil R1.2/1.3 Cu 300 (Quantifoil) holey gold grids were cleaned for 180 s using a PELOC easiGlow glow discharge cleaning system and 3 μL of the sample were applied at 95% humidity and 22 °C. Following a 10 s incubation period, the grids were blotted for 1.5 s and plunged frozen into liquid ethane using a Leica EM GP2 (Leica Microsystems).

Pp GS12 apo. Purified Pp GS was exchanged into Buffer B using a 10 kDa MWCO spin filter (Millipore) and concentrated to 0.9 mg/mL. For grid preparation, Quantifoil R1.2/1.3 Cu 300 (Quantifoil) holey carbon grids were cleaned for 100 s using a PELOC easiGlow glow discharge cleaning system and 3 μL of the sample were applied at 95% humidity and 22 °C. Following a 10 s incubation period, the grids were blotted for 1.5 s and plunged frozen into liquid ethane using a Leica EM GP2 (Leica Microsystems).

**Data availability.** The structural data referenced in this study can be found in the Protein Data Bank under the accession codes 4LNI, 4B4E, and 4G0B. The structural data generated in this study have been deposited in the Protein Data Bank under the accession codes 7TEA, 7TEC, 7TDP, 7TEN, and 7TDV for the crystal structures. The structural data generated in this study by cryo-EM have been deposited in the Protein Data Bank under the codes 7TPE, 7TFE, and 7TFD for the apo GS structures and 7TFC, 7TBF, 7TFA, 7TF6, and 7TF9 for the FBI-GS-GlnR structures.

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Author contributions

B.A.T. made samples for cryo-EM data analyses and solved cryo-EM structures. J.V.P. collected and processed cryo-EM data, R.S. purified proteins, and performed ITC and enzyme assays. N.L. and V.D.N. purified proteins and N.L. contributed to cryo-EM data analyses. M.J.B. performed negative stain analyses, B.D. solved the Su GS TS structure. R.G.B. contributed to experimental design and manuscript writing. M.A.S. performed FP, solved Pp TS GS, Lm GS TS, Lm GlnR-DNA, and Su GlnR-DNA crystal structures, designed experiments, and wrote the paper.

Competing interests

The authors declare no competing interests.

Additional information

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Correspondence and requests for materials should be addressed to Maria A. Schumacher.

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