Theoretical Development of DnaG Primase as a Novel Narrow-Spectrum Antibiotic Target

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ABSTRACT: The widespread use of antibiotics to treat infections is one of the reasons that global mortality rates have fallen over the past 80 years. However, antibiotic use is also responsible for the concomitant rise in antibiotic resistance because it results in dysbiosis in which commensal and pathogenic bacteria are both greatly reduced. Therefore, narrow-range antibiotics are a promising direction for reducing antibiotic resistance because they are more discriminate. As a step toward addressing this problem, the goal of this study was to identify sites on DnaG primase that are conserved within Gram-positive bacteria and different from the equivalent sites in Gram-negative bacteria. Based on sequence and structural analysis, the primase C-terminal helicase-binding domain (CTD) was identified as most promising. Although the primase CTD sequences are very poorly conserved, they have highly conserved protein folds, and Gram-positive bacterial primases fold into a compact state that creates a small molecule binding site adjacent to a groove. The small molecule would stabilize the protein in its compact state, which would interfere with the helicase binding. This is important because primase CTD must be in its open conformation to bind to its cognate helicase at the replication fork.

INTRODUCTION

The widespread use of antibiotics to treat infections is one of the reasons that global mortality rates have fallen over the past 80 years. However, the concomitant rise in antibiotic resistance is projected to counter those successes with 10 million deaths by infections per year worldwide by 2050. Many of the deadliest pathogens are Gram-negative and Gram-positive bacteria (Table 1). When broad-range antibiotics are taken orally to treat gastrointestinal infections (Benthesicymus cereus, C. burnetti, Cosmarium difficile, E. coli, E. faecium, F. tularensis, V. cholerae), it results in long-term adverse changes to hundreds of gut microbes that play positive roles in human health. The result is a decrease in the immune response, reduced vitamin production, and an increase in antibiotic resistance. Narrow-range antibiotics overcome these disadvantages and have already been developed to combat tuberculosis, hospital-acquired diarrhea, and even antibiotic resistance.

A good example relating to the effects of broad- and narrow-range antibiotics on the gut microbiome is Clostridoides difficile infection (C. difficile). The resulting diarrhea is often acquired at nursing homes or hospitals, where it persists because the organism forms spores that are difficult to disinfect. Broad-range antibiotics lead to a rise of antibiotic resistance when microbes reinhabit the gut as the antibiotic concentration is decreasing but still modestly high. There are two narrow-range antibiotics marketed for C. difficile. Their use prevents dysbiosis, or wide-range loss and imbalance, of gut microbiota, which is a major factor in the recurrence of infection. Fidaxomicin targets RNA polymerase from Gram-positive anaerobes, including staphylococci and enterococci. Its use has been shown to preserve most gut microbiota. Ridinilazole targets the process of cell division from an even narrower range of Gram-positive anaerobes than fidaxomicin. Neither of these antibiotics passes through the intestinal wall, which leads to high concentrations in the gut and favorably high percent of antibiotic inhibition of their targets.

Very few of the over 200 essential bacterial protein-encoding genes have been exploited as drug targets. More than 10 of those genes code for replication enzymes, some of which are under development as targets. The two enzymes in this study are helicase (DnaB) and primase (DnaG). DnaB helicase is the homohexameric enzyme that couples ATP hydrolysis with unwinding upstream DNA at the replication fork (note that the name DnaB helicase is used here for the protein that is named DnaC helicase in Staphylococcus aureus and Bacillus subtilis because, in all other bacteria, DnaB is the name of the replicative helicase, and DnaC is the name of the helicase loading enzyme). DnaG primase is the specialized DNA-dependent RNA polymerase that synthesizes short oligoribonucleotide polymers called primers. It is required because

Received: October 22, 2021
Accepted: February 1, 2022
Published: March 1, 2022
DNA polymerases lack the ability to initiate chain synthesis but are very efficient at elongating from primers. Both are good antibiotic targets because they are encoded by single genes that produce a few dozen copies of an enzyme that plays critical roles during the initiation and elongation phases of DNA replication.32−36

The goal of this study was to identify the DnaG primase residues that are predicted to have the strongest differences between Gram-positive and Gram-negative bacteria. Since bacterial primase has a distinct fold compared to eukaryotic primase, there should be fewer antibiotic side effects. First, the highly conserved primase N-terminal zinc-binding domain (ZBD) was examined as a case study. Then, the primase C-terminal helicase-binding domain (CTD) residues were studied. Although the primase CTD sequences are very poorly conserved, they have highly conserved protein folds. The primase ZBD and CTD were found to have functional differences between Gram-negative and Gram-positive bacteria.

## RESULTS

The goal of this project was to identify residues in DnaG primase that play an important role and that are conserved within a Gram phylum but different between them. The plan was to work out the methods for discovering these residues by reanalyzing how this was accomplished for the highly conserved primase ZBD and then apply those methods to the more challenging primase CTD. The CTD lacks sequence conservation even within the phyla. However, the available solved structures suggest a higher level of structural conservation that showed distinct differences between Gram-positive and Gram-negative organisms.

### Multiple Sequence Alignments

Given the coevolution of the DnaB−DnaG interaction in G. stearothermophilus, S. aureus, and E. coli, the sequences of primase and helicase from select pathogenic bacteria were aligned (Table 1) as the first step to learn which features were conserved and which might be useful for drug target sites. The sequence analysis shows a distinctive separation of primase and helicase in Gram-positive and Gram-negative organisms (Figure 1). Furthermore, the organismal clustering is also mostly conserved: the two Burkholderia species have the same sequence; E. coli, V. cholerae, and Y. pestis are closely related; S. aureus and S. epidermis are closely related; E. faecium and E. faecalis are closely related; and S. pneumoniae and S. agalactiae are closely related. Also conserved is that F. tularensis is the most distantly

| organism       | genera relevance                      |
|----------------|---------------------------------------|
| *Burkholderia mallei* | Burkhorderia glanders<br>7 |
| *Burkholderia pseudomallei* | Burkhorderia melioidosis<br>6 |
| *Coxiella burnetii* | Coxiella Q_fever<br>9 |
| *Escherichia coli* | Enterobacteriaceae diarrhea<br>10 |
| *Francisella tularensis* | Cocacobacillus tularemia<br>11 |
| *Neisseria gonorrhoeae* | Neisseria gonorrhea<br>12 |
| *Pseudomonas aeruginosa* | Enterobacteriaceae pneumonia, sepsis<br>13 |
| *Ralstonia solanacearum* | Ralstonia pathogenic to plants<br>14 |
| *Vibrio cholerae* | Vibrio cholera<br>15 |
| *Yersinia pestis* | Yersiniaciae bubonic plague<br>16 |
| *Bacillus anthracis* | Bacilli anthrax<br>17 |
| *Bacillus cereus* | Bacilli emetic and diarrheal syndrome<br>18 |
| *Clostridium difficile* | Clostridia gastrointestinal infection (C. diff)<br>19 |
| *Enterococcus faecalis* | Bacilli endocarditis<br>20 |
| *Enterococcus faecium* | Bacilli gastrointestinal infection<br>21 |
| *Staphylococcus aureus* | Bacilli MRSA, staph infection<br>22 |
| *Staphylococcus epidermidis* | Bacilli hospital-acquired skin infection<br>23 |
| *Staphylococcus haemolyticus* | Bacilli blood infection<br>24 |
| *Streptococcus agalactiae* | Bacilli neonatal infection<br>25 |
| *Streptococcus pneumoniae* | Bacilli pneumonia, ear & sinus infection<br>26 |

These bacteria have medical and military importance, and several are on the HHS and USDA Select Agents and Toxins list.

![Figure 1. Phylogenetic full-length protein sequence trees for (A) DnaG primase and (B) DnaB helicase.](https://doi.org/10.1021/acsomega.1c05928)
related for both proteins among the Gram-negative organisms and C. difficile among the Gram-positive organisms. As the farthest outliers, it may be difficult to develop reliable homology models for the enzyme from these organisms. One difference between the trees is that B. cereus and B. anthracis have identical primase sequences, whereas their cognate DnaB helicases are not identical but closely related.

There is no structure for the entire primase−helicase complex. The structures of several individual domains have been determined (Figure 2A). Primase consists of three domains: Zinc-Binding Domain (ZBD), RNA Polymerase Domain (RPD), and C-Terminal Domain (CTD). Helicase consists of two domains: N-Terminal Domain (NTD) and the Helicase Motor/Body. The CTD structure has been solved from three different organisms—two Gram-positive and one Gram-negative (Figure 2A). The structures of three multidomain proteins, one of which is a complex, have been solved (Figure 2B). The ZBD−RPD structure from A. aeolicus, a hyperthermophilic organism, suggests that the linker between the two domains is not flexible. The DnaB helicase homohexamer structure from G. stearothermophilus shows as a trimer of dimers. The DnaB−DnaG CTD complex from G. stearothermophilus established the ratio of one CTD for every two DnaB-NTDs.

Zinc-Binding Domain. The primase ZBD domain residues are highly conserved (Figure 3 for 20 organisms; Supporting Information for 40 organisms covering a wider range of classes, Figures S1−S7). The Gram-positive primases are 37% identical over this region, and the Gram-negatives are 47% identical. This 52-residue region encompasses the four zinc-binding residues C, H, C, and C (Figure 3A gray highlighted residues). It is a potential site for the binding of a narrow-range antibiotic because it is the domain responsible for the initiation, which shows distinct phyletic differences. Enzymatically, S. aureus and G. stearothermophilus primase initiate at the triplet sequence 5′-d(CTA),39,44 whereas the E. coli primase initiates from 5′-
rather than 5′adjacent RNA polymerase domain.42,47 This could be achieved with the single-stranded DNA template that is bound to the strategy might be to reduce the ability of the ZBD to interact sequence-specifically.42,47

must have enough surfaces do not form binding pockets.34 A more promising approach would be to reduce the ability of the ZBD to interact sequence-specifically.42,47

The primase ZBD structure has been determined from G. stearothermophilus47 and Aquifex aeolicus.42 They are members of a unique protein family called “zinc ribbons,” in which the zinc is coordinated by three cysteines and one histidine that form a zinc ribbon structure.42,47

The two residues responsible for recognizing the third nucleotide of the initiation sequence are located at the end of one beta strand located in the center of the five-strand antiparallel sheet (Figure 3B). The exposed Ile58 may stack on the key nucleotide, and the buried Phe59 may ensure the beta-sheet maintains its conformation.

Since the single-stranded DNA template is bound much more strongly to the RNA polymerase domain,42,47 the ZBD must have enough flexibility to fold onto the DNA in a sequence-specific manner. With regard to drug binding, beta sheets are challenging drug target sites because their flat surfaces do not form binding pockets.34 A more promising strategy might be to reduce the ability of the ZBD to interact with the single-stranded DNA template that is bound to the adjacent RNA polymerase domain.42,47 This could be achieved by developing molecules that bind to the α-helical bundle or the linker between the ZBD and the RNA polymerase domain. However, the structure of the linker and its flexibility has not been established yet.

DnaG-DnaB Interface. Residues at the DnaG–DnaB interface should be narrow-range antibiotic targets because there are distinct enzymatic differences between genera and even species of bacteria. First, E. coli primase activity is strongly stimulated by its cognate DnaB helicase,48 S. aureus primase is stimulated only about 2-fold by its DnaB helicase,49 and C. difficile primase is only active in the presence of its cognate DnaB helicase.50 The structural basis for these functional differences has not been determined but are likely related to the different affinities of the catalytic subdomain for the ssDNA template and/or the different affinities of primase C-terminal domain for the cognate helicase N-terminal domain. Information about the primase–helicase interface interactions is based on studies of the X-ray structure of thermophilic G. stearothermophilus.47 At room temperature, the binding affinities for thermophilic proteins are much stronger than those from mesophilic bacteria because thermophilic proteins are less prone to unfolding.51

Sequence alignments supported the idea that there are structural differences between Gram-positive and Gram-negative CTDs (Figure 4) despite sharing no identical residues and almost no conserved residues. The residues at interface A (purple highlights in Figure 4) are in the small molecule binding pocket of the S. aureus closed conformation. The orange highlighted residues are at interface B. The purple highlighted residues are buried at interface A. The gray highlighted residues are conserved helix-stabilizing residues, two of which are not conserved in Gram-positive sequences, where the conformational change occurs.

Figure 4. Primase CTD sequence alignment. The primase CTD from the organisms in Table 1 were aligned with numbers corresponding to the S. aureus sequence and with two arrows showing the location of two critical G. stearothermophilus residues. Along the top are the secondary structures of two closed structures and one open. The red box and delta sign show the major structural differences between the open and closed conformations. In the center of the sequences are the few conserved residues (: is highly conserved; . is conserved). Along the bottom are the four open conformations from Gram-negative organisms. The green highlighted residues form the small molecule binding pocket of the S. aureus closed conformation. The orange highlighted residues are at interface B. The purple highlighted residues are buried at interface A. The gray highlighted residues are conserved helix-stabilizing residues, two of which are not conserved in Gram-positive sequences, where the conformational change occurs.

\[d(CTG)\].45 A mutagenesis study of the residues that differ between Gram-positives and Gram-negatives but that are conserved in one or the other of those phyla showed that the S. aureus residues Ile56 and Cys57 (Figure 3A purple highlighted residues) were responsible for initiating from 5′-d(CTA) rather than 5′-d(CTG).46

The primase ZBD structure has been determined from G. stearothermophilus37 and Aquifex aeolicus.42 They are members of a unique protein family called “zinc ribbons,” in which the zinc is coordinated by three cysteines and one histidine that hold together five antiparallel beta strands (Figure 3B). The two or three amino acids between the zinc-chelating residues are “knuckles” with highly conserved sequences. Before and after the zinc ribbon structure are α-helices that form a bundle.42
corollary residues in the Gram-positive sequences but not in the Gram-negative sequences. The residues highlighted in green form the small molecule binding site in the *S. aureus* DnaG closed conformation. The secondary structures of Gram-negative CTDs have many more helix-favoring residues at the site where Gram-positives have a turn. This suggests that Gram-negative CTDs lack the flexibility to change conformation, which indicates there are significant structural differences with the Gram-positive primase CTDs.

*G. stearothermophilus* primase CTD adopts two conformations. Its open conformation binds to two helicase NTDs (Figure 5A). The formation of this complex increases the affinity between DnaB helicase and the DNA polymerase III holoenzyme by 500-fold, which may allow rapid transfer of the short RNA primer to the DNA polymerase. The homology model of *S. aureus* primase CTD sequence in its open conformation (Figure 5B) was created using the *G. stearothermophilus* structure as a template (Figure 5B). The two DnaG-DnaB interfaces are distinct. Interface A involves the extreme C-terminal helical hairpin of DnaG CTD and the five-helix bundle of DnaB NTD 1. In contrast, interface B is between the five-helix bundle of DnaG CTD and the five-helix bundle of DnaB NTD 2.

The other CTD conformation is compact (Figure 5C) and was observed in the solution structures of two Gram-positive primases. The difference from the open conformation is that there is a bend in the longer helix of the terminal hairpin. In the *S. aureus* primase CTD compact conformation, the resulting shorter terminal hairpin forms several weak interactions with its five-helix bundle to form a closed conformation. This forms a groove into which small molecules are able to bind with low millimolar concentrations affinity (Figure 5) to prevent interface B from forming. The currently identified small molecules can serve as seed molecules in the search for others with higher affinity.

**Characterizing the Critical Residues at Interface A through Virtual Mutations.** The PRODIGY program was used on the *G. stearothermophilus* DnaB–DnaG co-crystal to identify the interatomic contacts at interface A within a defined cutoff distance of 5.5 Å (Table 2). Six of the residues were the same as those previously identified as making multiple contacts.

| virtual mutation | ΔΔG (kcal/mol) |
|------------------|----------------|
| Wild-type        | 0.0 (ΔG = −6.2 kcal/mol) |
| T569G            | −0.3 |
| A580G            | 0.0 |
| A581G            | 0.0 |
| L578G            | 0.0 |
| A584G            | 0.0 |
| E565G            | 0.1 |
| I588G            | 0.1 |
| K575G            | 0.2 |
| K591G            | 0.2 |
| K568G            | 0.2 |
| F577G            | 0.2 |
| R582G            | 0.2 |
| K585G            | 0.4 |
| K592G            | 0.4 |
| E572G            | 0.5 |

Table 2. Predicted ΔΔG for Single Virtual Mutations of *G. stearothermophilus* DnaG CTD Interface A

PRODIGY identified the residues at the interface within a defined cutoff distance of 5.5 Å (SI Figure 8).
at the interface: E572T, F577V, L578E, A581K, A584L, and I588V. Nine other residues were more solvent-exposed.

To establish whether there were any residues that played key roles in the interface interaction, each of the 15 G. stearothermophilus residues was virtually mutated singly to glycine using the DeepView-Swiss-PdbViewer, and the strength of the binding interaction between the resulting mutant and helicase NTD 1 was quantified with PRODIGY. This measurement estimates the relative importance of the residues to the overall interaction and would be different from in vitro binding strengths because both interfaces A and B would have to be disrupted. The free energy was ~6.2 kcal/mol for the wild-type primase interface. When each of the 15 residues was singly mutated, three of them weakened ΔG by more than 0.2 kcal/mol: E572, K585, K592 (Table 2). The strongest perturbation was caused by mutating E572. This is the only residue that made contact with multiple adjacent residues and was exposed to solvent.

To determine whether there was an interface A sequence code, all six buried interface residues were simultaneously mutated to glycine (Table 3). The resulting ΔΔG of 0.6 kcal/mol was significant but indicated that each residue makes a modest individual contribution (and an average of 0.1 kcal/mol) to the overall affinity. To determine whether the contributions of those six residues were conserved among Gram-positive organisms, they were virtually mutated to the equivalent sequence in S. aureus. The small value for ΔG indicated that the S. aureus residues were capable of substitution, which was consistent with phyletic conservation.

Since testing the most buried residues did not identify any critical interface residues, the three solvent-exposed interface residues were examined (Table 3). When E572, K585, and K592 were virtually mutated to glycine, the free energy was perturbed by 1.1 kcal/mol, or about 0.4 kcal/mol/residue. The location of these amino acids can be seen in Figure 6. This large contribution per residue was confirmed by a perturbation of 0.7 kcal/mol when only two of those residues were mutated (K585 and K592). To determine the degree of phyletic conservation, K585 was mutated to glutamine, the equivalent residue in the S. aureus. This mutant had nearly the same free energy as the glycine mutant, consistent with a difference in function between Gram-negative and Gram-positive organisms.

**Table 3. Predicted ΔΔG for Multiple Virtual Mutations of G. stearothermophilus DnaG CTD Interface A**

| virtual mutation | ΔΔG | notes |
|------------------|-----|-------|
| E572G, F577V, L578G, A581G, A584G, I588G | 0.6 | all closest contacts to glycine |
| E572T, F577V, L578E, A581K, A584L, I588V | −0.2 | all closest contacts to S. aureus |
| F577G, L578G, A581G, A584G, I588G | 0.2 | closest contacts to glycine, except E572 |
| E572G, K585G, K592G | 1.1 | three most perturbing to glycine |
| K585G, K592G | 0.7 | second & third most perturbing to glycine |
| E572G, K585Q, K592G | 0.3 | two of three most perturbing to glycine, K585 to S. aureus |
| E572G, K585W, K592G | 0.9 | two of three most perturbing to glycine, K585 to E. coli |

“PRODIGY identified the residues at the interface within a defined cutoff distance of 5.5 Å (Supporting Information Figure S8).

**Figure 6.** G. stearothermophilus DnaG CTD key interface A residues—E572, K585, and K592—on the terminal helical hairpin in blue as determined by virtual mutation.

### DISCUSSION

DnaB helicase and DnaG primase are antibiotic targets because they interact to carry out critical reactions during DNA replication. These enzymes are narrow-range targets because Gram-positive and Gram-negative primases and helicase have distinct specificities, which are the result of three domains—the primase zinc-binding domain, the primase CTD, and the helicase NTD. Although the primase zinc-binding domain is responsible for distinct differences between the phyletic substrate specificity, its protein fold lacks a pocket for small molecule binding. On the other hand, Gram-positive primase CTD creates a groove when the C-terminal hairpin folds into its closed conformation. This groove creates a small molecule binding pocket in S. aureus primase that can serve as a model for the C. difficile primase (Figure 8). One of the three critical interface A residues, E572, is near that pocket. Therefore, a promising direction would be to extend the size of the small molecule so that it extends into the adjacent groove to also bind E572. Such binding will favor the closed conformation of primase CTD, which cannot bind to the helicase because the interface A residues are sterically obscured.

### EXPERIMENTAL SECTION

The organism sequences were obtained from UniProt. Clustal Omega was used to create the multiple sequence alignments in a phylib format. SplitsTree used the phylib format from Clustal Omega to create the phylogenetic trees. Chimera was used to examine the 3D model structures from
their PDB IDs. The 3D homology models were created using the modeling program from SWISS-MODEL\(^58\) using template structures from the PDB. PRODIGY\(^59\) was used to predict the amino acids in closest proximity (5.5Å or less) as well as the \(\Delta G\) for the protein−protein binding interaction. PRODIGY has the option to select the temperature, and the \(\Delta G\) values were calculated at 37 \(\degree\)C due to its biological relevance.

DeepView-Swiss-PdbViewer\(^60\) was used to introduce virtual mutations to the wild-type protein, and PRODIGY was used to predict the \(\Delta G\) at 37 \(\degree\)C for each mutation. \(\Delta\Delta G\) was calculated by subtracting the \(\Delta G\) wild-type from the \(\Delta G\) mutation. PRODIGY predicts the binding affinity using the formula reported by Vangone and Bonvin.\(^61\) It counts the number of Interatomic Contacts (ICs) made at the interface of a protein−protein complex within a 5.5 Å distance threshold and classifies them according to the polar/apolar/charged character of the interacting amino acids. This information is then combined with properties on the Non-Interacting Surface (NIS), which was previously shown to influence the binding affinity.\(^62\)

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**ASSOCIATED CONTENT**

* Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c05928.

Multisequence alignment of 40 full helicase sequences (Figure S1); multisequence alignment of 20 full helicase sequences−Gram-positive organisms only (Figure S2); multisequence alignment of 20 full helicase sequences−Gram-negative organisms only (Figure S3); multisequence alignment of 40 full primases sequences (Figure S4); multisequence alignment of 20 full primases sequences−Gram-positive organisms only (Figure S5); multisequence alignment of 20 full primases sequences−Gram-negative organisms only (Figure S6); phylogenetic full-length protein sequence trees for DnaG primase (A) and DnaB helicase (B) (Figure S7); list of DnaG residues at the interface predicted by PRODIGY on the G. stearothermophilus DnaG CTD (Figure S8) (PDF)
Complete contact information is available at:
https://pubs.acs.org/10.1021/acsomega.1c05928

Author Contributions
The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Notes
The authors declare no competing financial interest.

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