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Effects of Nucleobase Amino Acids on the Binding of Rob to Its Promoter DNA: Differential Alteration of DNA Affinity and Phenotype

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Abstract

Nucleic acid binding proteins have been studied extensively, but the nature of the interactions that control their affinity, selectivity, and DNA and RNA functions is still not well understood. To understand the nature and functional consequences of such interactions, we introduced nucleobase amino acids at specific positions of the transcriptional regulator Rob protein *in vivo* and succeeded in demonstrating that an alteration of the protein–DNA affinity can affect specific phenotypes associated with Rob protein–DNA interactions. Previously, we inserted different nucleobase amino acids in lieu of Arg40; this residue is known (via X-ray crystallography) to interact with the *micF* DNA promoter A-box residue Gua6. The interactions predominantly involved Watson–Crick-like H bonding. The present study focused primarily on the *micF* DNA promoter B-box; the crystallographically determined interaction involves H bonding between the agmatine moiety of Arg90 within an HTH motif of Rob and a phosphate oxygen anion to the 5′-side of Thy14. We had two main goals, the first of which was to demonstrate enhanced Rob-binding to the *micF*

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Supporting Information

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Tables of the experimental yields, figures regarding the reactions, and experimental methods for preparing and studying proteins with nucleobase amino acids (PDF)

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promoter DNA and the functional consequences resulting from the interaction of *micF* DNA with Rob analogues containing Arg90 nucleobase mimics. The second was to explore the possible functional consequences of enhancing the protein–DNA affinity with nucleobase replacements, which mechanistically mediate interactions differently than those reported to be operative for specific protein–DNA interactions. Nucleobase replacement at position 90 with Arg isosteres enhanced the Rob protein–*micF* DNA affinity in parallel with increasing antibiotic and Hg²⁺ resistance, while aromatic amino acid replacements increased the affinity but not the antibiotic or Hg²⁺ resistance. The demonstration of an increased affinity through strong base stacking interactions was notable.

Graphical Abstract

A non-coding RNA stress response gene found in *Escherichia coli* and related bacteria, *micF*, post-transcriptionally controls the expression of the outer membrane porin gene *ompF*.^{1–3} The *micF* gene encodes a non-translated 93 nucleotide anti-sense RNA that binds its target *ompF* mRNA and regulates OmpF expression by inhibiting translation and inducing mRNA degradation. The *ompF* and *ompC* genes are regulated by the OmpR–EnvZ two-component signal transduction system.^{4–6} These genes are responsible for synthesizing their corresponding porins, which associate as homotrimers in the outer cell membrane and form pores that enable small hydrophilic molecules to enter cells by simple cross-membrane diffusion.⁷ Reducing the level of OmpF porin has been shown to result in bacterial multidrug resistance.^{4,7,8} The expression of MicF is controlled both by environmental and internal stress factors, and increasing MicF concentration decreases the cellular OmpF level.⁹ The *micF* gene has a complex promoter region that contains binding sites for several transcriptional regulators belonging to the AraC/XylS family.^{10,11} The best known are

MarA, SoxS, and Rob.^{12–14} Rob has been proposed as a general transcriptional cofactor;¹⁵ in a *rob* deletion strain, but not in SoxS or MarA, the expression of *MicF* is not activated by cationic peptide antibiotics.¹⁶ There is some evidence that MarA can repress the *rob* gene through steric hindrance.¹⁷

The X-ray crystal structure of Rob bound to the *micF* promoter has previously been determined.¹⁸ The Rob protein consists of two domains. The N-terminus is homologous with transcriptional regulator MarA.¹⁹ This domain contains two helix–turn–helix (HTH) motifs that are connected by a rigid central helix that fixes their relative orientation and is responsible for DNA binding. The C-terminal domain (CTD) is unique to Rob and is not yet well understood. Recently, it has been suggested that the CTD regulates the activity of Rob and protects it from degradation by Lon protease.²⁰

Rob, SoxS, and MarA have similar mechanisms of DNA binding. They all bind to an asymmetric DNA sequence ~20 base pairs in length, which is part of the gene promoter region.^{11,21,22} These binding sites include two conserved sequences, namely, the A-box and B-box (Figure S1), that are separated by seven base pairs with variable sequences. Residues in the HTH motif of Rob determine the shape and electrostatic interactions with the A-box sequence. Trp36 can make a van der Waals contact with Cyt7, whereas Arg40 can form a H bond with the O⁶ atom of Gua6. The Rob A-box sequence interactions compare well with data found for MarA in complex with its promoter.¹⁸

The second HTH motif in all members of the AraC/XylS family of transcriptional factors contains a cluster of highly conserved polar residues, which can bind within the B-box in cognate DNA promoters.¹⁸ Two conserved residues (Arg90 and Lys94) were found as ligands during the crystallographic study of the Rob–DNA complex.¹⁸ Despite the high homology between Rob and MarA, some differences were found in regard to Rob's contacts with the B-box. In the case of MarA, Arg96 located in helix α F forms direct H bonds with two purines, Gua15 and Ade7' (numbered as in the *micF* structure).²³

However, these two purines are not conserved in the binding sites for Rob, Mar, and Sox. The contacts between Mar and the B-box of *mar* DNA more likely include water-mediated H bonds.²³ In the case of Rob Arg90, which is also situated within helix α F and located in close proximity to *micF* DNA, binding with the DNA backbone is mediated via a H bond with the 5'-phosphate of Thy14.¹⁸

In this study, we have extended our analysis of the interaction of Rob protein with its *micF* promoter DNA by utilizing several purine nucleobase amino acids as analogues of a key Rob amino acid. The protein–DNA crystal structure suggests that residues Arg40 and Arg90 in adjacent HTH structures within Rob interact directly with the A-box and B-box of the *micF* promoter DNA, respectively. The relevant interactions are illustrated in Figure 1. By molecular modeling and the use of side-chain-altered analogues of arginine (norarginine and homoarginine, Figure 2), we determined that there can be some flexibility in the length or conformation of the side chain at this position. Accordingly, it seemed possible that the nucleobase amino acid that is structurally related to guanine (1) might well serve as a rigid isostere of Arg90 (Figure 2) and increase the affinity of Rob for its DNA promoter. We

demonstrated that this replacement did enhance the *micF*-Rob affinity, and that three nucleobase analogues of 1 (Figure 2, 2–4) had the expected effects on the protein–DNA affinity. By studying amino acid analogues that are predominantly capable of either base pairing or base stacking interactions, we found that these gave distinctly different results when introduced into position 40 or 90. These observations supported the conclusion that different DNA sequences exhibited preferences for different types of protein interactions and demonstrated the ability of aromatic amino acids derived from nucleobases to utilize base stacking interactions to stabilize cognate protein–DNA interactions. Finally, by the use of assays that quantify the reported ability of Rob to induce resistance to macrolide antibiotics and heavy metal ions such as Hg^{2+} , we demonstrated that the nucleobases in Rob that reinforce *micF*DNA binding using the same strategies as wild type resulted in enhanced antibiotic and heavy metal resistance, while those that increased binding by other molecular mechanisms did not enhance the resistance.

MATERIALS AND METHODS

Synthesis of Amino Acids.

The syntheses of amino acids not commercially available are discussed in the Supporting Information.

Biochemical Experiments.

Site-Directed Mutagenesis of the Rob Gene.—Rob gene mutagenesis corresponding to position Arg90 was done using a modified Quik-ChangeTN site-directed mutagenesis protocol. The pETRobwt plasmid, which has the wild-type *rob* gene, and the primers used were 5′-CTCTC-AACAG-ACATT-TACCC-TCGCA-TTCAAGAAG-CAGTTTG-3′ for Rob90-Leu and 5′-CTCTCA-ACAGA-CATTT-ACCTA-GGCAT-TCAAGAAG-CAGTTTG-3′ for Rob proteins containing nucleobase amino acids. Primer oligonucleotide phosphorylation was carried out in a reaction mixture (20 μL total volume) that had 100 pmol of primer, 1 mM ATP, 70 mM Tris buffer (pH 7.6), 10 mM MgCl_2 , 5 mM DTT, and 1 unit of T4 polynucleotide kinase. These were incubated at 37 °C for 1 h and then chilled on ice. Following the incubation, 40 μL of deionized water, 240 μL of 5 M NH_4OAc , and 750 μL of cold ethanol were added. The combined solution was mixed, incubated (−20 °C for 20 min), and centrifuged ($12\,000 \times g$ for 20 min). The pellet was washed with 70% ethanol, air-dried, and then dissolved in 50 μL of RNase-free water.

The polymerase chain reaction (PCR) was carried out using 50 μL (total volume) of 35 mM Tris-HCl (pH 8.0) which contained 300 ng of template, 14 pmol of primer, 10 nmol of dNTPs, 12 mM KOAc, 5 mM DTT, 0.05% Triton X-100, 0.05 mM EDTA, 2.5 U of Pfu polymerase, and 20 U of Taq DNA ligase. The programming for the terminal cyclers was as follows: pre-incubation at 95 °C for 2 min; 18 cycles for 1 min at 95 °C, 1 min at 45 °C, and 12 min at 65 °C; and then a final extension at 72 °C for 7 min, after which the reaction mixture was allowed to cool to room temperature. Next, 1 μL of restriction endonuclease DpnI was added, and the reaction mixture was incubated at 37 °C for 2 h. The sample was then denatured at 95 °C for 1 min, followed by two cycles at 95 °C for 1 min, 50 °C for 1 min, and 70 °C for 12 min. DH5 α high efficiency competent cells ($>10^7$ cfu/ μg) were

transformed using 5 μ L of the PCR product for each 50 μ L of cell suspension. The transformants were placed on the LB agar, prepared with 60 μ g/mL kanamycin, and incubated at 37 °C for 18–24 h.

Preparation of Cells and Overexpression of Rob Proteins.—The individual wild-type and modified *rob* genes were introduced into plasmids and co-transformed with pTECHPly-OP in BL-21(DE-3) competent cells. Single colonies of these cells were cultured in a 2YT medium, which had been supplemented with 30 μ g/mL chloramphenicol and 60 μ g/mL kanamycin; the isolated cells were maintained at –80 °C (15% glycerol stocks) until they were used. The initial growth of the cultures for *in vivo* expression employed 5 mL of the 2YT medium, which was supplemented with two antibiotics, until an OD₆₀₀ value of ~0.5 was reached. Next, 3 mL aliquots of each culture were mixed with 3 mL of 30% glycerol. These aliquots were maintained at –80 °C until further use in *in vivo* assays. Following the transfer of the samples into flasks with 30 mL of the 2YT medium supplemented with the two antibiotics, protein production was activated by the addition of IPTG to a 1 mM concentration. At the same time, the amino acids to be incorporated into Rob were added to the culture containing the plasmid harboring the modified *rob* gene; the final amino acid concentration was 2–3 mM. Following incubation at 37 °C overnight, the cultures were centrifuged (3500 $\times$ *g* at 4 °C for 10 min). Cell pellets were resuspended in a lysis buffer consisting of 50 mM Tris-HCl (pH 8.0) supplemented with 10 μ g/mL lysozyme, and three freeze–thaw cycles were carried out. The samples from the last frozen step were centrifuged at 15 000 $\times$ *g* at 4 °C for 1 h. Samples of the supernatants containing the culture lysates were transferred to fresh tubes and maintained on ice until they were used for protein purification.

Protein Purification.—For Rob protein purification, the culture lysates were diluted ~3-fold with 100 mM Tris-HCl (pH 8.3) and applied to a 100 μ L Strep-Tactin Sepharose column. Then, they were equilibrated and washed with 2 mL of the same buffer. The bound proteins were eluted with 0.5 mL of the same buffer, which was supplemented with 2.5 mM desthiobiotin. Finally, the samples were concentrated and desalted using a 10 kDa filtration device (Amicon Ultra, Millipore Corp). The samples were stored in 50 mM Tris-HCl (pH 7.4) that contained 30% glycerol. Samples were analyzed by 15% polyacrylamide-SDS gel electrophoresis following Coomassie R-250 staining (Figure S2). Defined amounts of a commercial sample of lysozyme were used for calibration to estimate the amount of protein in the individual Rob samples.

EMSA.—Electrophoretic mobility shift assays were carried out using *micF* and *micFmutB* promoter DNA sequences. 5′–³²P-end-labeled DNA substrates were prepared by ³²P-ATP labeling in 50 μ L reaction mixtures with 1 pmol of oligonucleotides 5′-TGACAGCACTGAATGTCAAAG-3′ and 5′-TGACAATACTGAATGTCAAAG-3′ for the *micF* and *micFmutB* promoter DNAs, respectively, as well as 3.4 pmol of 5′- γ -³²P-ATP (6000 Ci/mmol, 10 μ Ci/ μ L), 70 mM Tris buffer (pH 7.6) containing 10 mM MgCl₂, 5 mM DTT, and 1 unit of T4 PNK (Promega). Following incubation for 2 h at 37 °C, the radiolabeled oligonucleotides were purified on MicroSpin G-25 columns (GE Healthcare) and mixed with 5 pmol of the corresponding complementary oligonucleotides. The reaction

mixtures were incubated at 90 °C for 5 min and allowed to cool slowly to 37 °C (~30 min). The final ^{32}P -DNA substrates were diluted 2-fold using a $2 \times$ DNA binding buffer (40 mM Tris (pH 8.0), 100 mM NaCl, and 10% glycerol). The final concentration of the ^{32}P -labeled DNA substrates was 6 nM. For EMSA, Rob samples were first diluted using a DNA binding buffer to a concentration of 500 nM. Next, four successive 2-fold dilutions were then carried out. Then, 3 μL of each dilution was mixed with 3 μL of the ^{32}P -labeled DNA substrate, and the reaction mixtures were incubated for 15 min at ambient temperature. Next, 3 μL of the native electrophoresis loading buffer (0.05% xylene cyanol FF and 0.05% bromophenol blue in 50% glycerol) was added to each sample. The samples were then loaded on 10% native polyacrylamide gels, and electrophoresis was run for 1.5 h at 80 V. The amount of bound and free DNA was analyzed using a phosphorimager. The BC_{50} value was determined on the basis of an Excel program using the initial linear part of the curve to represent the percent of bound DNA relative to total DNA in samples vs the concentration of protein in the samples.

Antibiotic Sensitivity Assay.—Cultures of BL-21(DE-3)-Pyl with different amounts of plasmids, pET28b (negative control), pETRobwt (positive control), and pETRob90 were prepared as described above after a cotransformation of the BL-21(DE-3) strain. Before each assay, fresh cultures were prepared by adding ~10 μL aliquots of the glycerol stocks to 3 mL of an LB medium (pH 6.9) supplemented with kanamycin (60 $\mu\text{g}/\text{mL}$) and chloramphenicol (30 $\mu\text{g}/\text{mL}$). The cultures were incubated for about 3–4 h at 37 °C until the OD_{600} value was about 0.5. For the assays, the cultures were diluted to an OD_{600} value of 0.01 for the pET28b and pETRobwt cultures using an LB medium (pH 8.8), and to an OD_{600} value of 0.3 for the pETRob40 cultures. The LB medium was also supplemented with kanamycin (60 $\mu\text{g}/\text{mL}$), chloramphenicol (30 $\mu\text{g}/\text{mL}$), and IPTG (1 mM). For the assays of Rob samples with different amino acids in position 90, the corresponding amino acids were added to the medium to a final concentration of 2 mM prior to each assay. Then, 96-well plates containing the antibiotics roxithromycin and clarithromycin at 50–3.12 $\mu\text{g}/\text{mL}$ dilutions were prepared in an LB medium with chloramphenicol, kanamycin, and IPTG. Each dilution of the macrolides was mixed with an assay solution of BL-21(DE-3)-Pyl cultures and incubated overnight at 37 °C. For each culture, the OD_{600} values were measured and the IC_{50} values were calculated. In the case of the ampicillin sensitivity study, an LB medium of pH 6.9 was used for the assay, and all the cultures were tested at multiple concentrations of the antibiotic dilutions between 0.2 and 25 $\mu\text{g}/\text{mL}$.

Assay for Resistance to Hg^{2+} .—Assay cultures were prepared as described above using an LB medium (pH 8.0) supplemented with kanamycin, chloramphenicol, and IPTG. Six dilutions of HgCl_2 (100–3.12 $\mu\text{g}/\text{mL}$) were prepared using the same medium in 96-well plates. Tested samples of the BL-21(DE-3)-Pyl cultures were mixed with each dilution of HgCl_2 ; the final concentrations of HgCl_2 in the assay were 50–1.56 $\mu\text{g}/\text{mL}$. The final 96-well plates were incubated overnight at 37 °C. For each culture, the OD_{600} values were measured and the IC_{50} values were calculated.

RESULTS AND DISCUSSION

Figure 1 is an analysis of the structural elements in the Rob protein and its *micF* DNA promoter that have been identified by X-ray crystallographic analysis as important to the

binding of these two species. These include the following two arginine residues, each within a separate helix–turn–helix motif, which have been shown by X-ray crystallography to associate with different elements in the *micF* promoter DNA¹⁸ (Figure S1): Arg40, in which the side chain is not fully extended (5.94 Å between C^α of Arg and the side chain NH₂ group that coordinates O⁶ of Gua-6), and Arg90, which is slightly more fully extended and makes contact with the phosphate ester oxygen adjacent to Thy14 (6.09 Å between C^α of Arg and the side chain NH₂ group). We initially introduced norarginine and homoarginine into position 90 and found that the binding of these modified Rob proteins to *micF* DNA was essentially unaltered, with values of 63 ± 11 nM for Rob-wt, and 56 ± 16 and 47 ± 8 nM for Rob analogues containing norarginine and homoarginine, respectively (Table 1 and Figure 3). This suggested that small changes in the positions of key functional amino acids might be tolerated functionally, perhaps by adjustments in the side chain conformation. Accordingly, specific (nucleobase) amino acids (Figure 2) were incorporated singly into both of these Rob positions to determine whether the molecular behaviors suggested by the crystal structure could also be used to enhance the implied interactions selectively by the use of such conformationally constrained nucleobase amino acids.

As shown in Table 1, the introduction of guanine nucleobase amino acid 1 at position 90 in lieu of arginine produced a protein–DNA interaction at least as strong as that induced by wild-type Rob (BC₅₀ 39 ± 2 vs 63 ± 11 nM), which is consistent with the possibility of having the exocyclic NH₂ group of 1 interact directly with the phosphate oxygen to the 5′-side of Thy14 (Figure 1, lower panel).¹⁸ The atoms in arginine and the guanine nucleobase amino acid 1, which are colored in red in Figure 2, highlight this possibility. Modified Rob proteins containing nucleobases 2 and 3 at position 90, which are capable of the same interaction, also exhibited strong *micF* DNA binding (Table 1 and Figure 4). Thus, Rob90–2 exhibited a BC₅₀ value of 38 ± 5 nM, while that for Rob90–3 was 41 ± 4 nM. In comparison, the binding of Rob90–4 to *micF* DNA was weaker (BC₅₀ 80 ± 10 nM), presumably reflecting the effects of the absence of an exocyclic 2-NH₂ group (Table 1). The obvious effect of the presence or absence of the 2-NH₂ group in 1–4 in modified Rob proteins on the interaction with *micF* DNA strongly supports the thesis that compounds 1, 2, and 3 likely behave as conformationally constrained arginine analogues.

The preceding data support the interpretation that the side chains of Arg90 and its close structural congeners, such as norarginine and homoarginine, have a range of lengths appropriate for supporting the Rob interaction with the Thy14 5′-phosphate oxygen atom via H bonding (Figure 1, bottom panel), and verify that conformational constraints, such as those imposed by the bicyclic ring structures in nucleobase amino acids 1–3, can produce enhanced DNA substrate binding. Perhaps as importantly, the data obtained for the Rob proteins containing norarginine and homoarginine help to define the range of differences in the distances for individual amino acids that can be accommodated in the Rob–*micF* DNA complexes by adjustments of the side chain conformations. This may ultimately prove to be exceptionally helpful in the design of new nucleobase analogues as the constituents of proteins.

It is interesting to note that the preferred interactions between two arginine residues (Arg40 and Arg90) and *micF* DNA, both of which involve residues that are part of helix–turn–helix

motifs, involve different types of DNA binding partners, namely, the O⁶ functionality of nucleotide Gua6 (for Arg40) and a phosphodiester O adjacent to Thy14 (for Arg90). This was true even though the distances between the C^α atom and the coordinating side chain NH₂ group were quite similar for each (5.94 and 6.09 Å for Arg40 and Arg90, respectively) and the replacement of Arg90 with Arg analogues that had shorter and longer side chains (norArg and homoArg, respectively) did not seem to alter the nature of DNA binding (Table 1). We showed previously²⁴ that a cytidine nucleobase amino acid placed at Rob position 40 enhanced binding to Gua6, putatively by forming a Watson–Crick-like H bond with Gua6. It is possible that the introduction of adenine amino acid 5 into Rob position 90 permits a Watson–Crick-like interaction (BC₅₀ 56 ± 12 nM) with Thy14 (Table 1).

Another approach to understanding the nature of the molecular interaction of Arg90 with the B-box of *micF*DNA involved the alteration of the DNA itself. As shown in Figure 5, Cyt17 was altered to a deoxyguanosine (*micFmutB*), affording a DNA strand locally very rich in purine nucleotides. While not known to be involved in the Rob–*micF*DNA interaction, as shown in Figure 5 and Table 2, this alteration of the DNA structure caused a modest decrease in the affinity of wild-type Rob for its DNA target (63 nM BC₅₀ for *micF*DNA vs 83 nM BC₅₀ for *micFmutB* DNA). This limited change was entirely consistent with the earlier finding^{24,25} that structural alterations of A-box nucleotides had a much greater effect on Rob affinity for the DNA promoter than changes in the B-box nucleotides. A more interesting finding involved the modified Rob containing nucleobase 1 at position 90 in lieu of arginine (Rob90–1). As shown in Figure 5 and Table 2, the replacement of Arg90 by nucleobase amino acid 1 enhanced the interaction with *micF*DNA (BC₅₀ 39 ± 2 nM for Rob90–1 vs 63 ± 11 nM for Rob-wt) but greatly lowered Rob affinity for *micFmutB* DNA (BC₅₀ 220 ± 20 nM for Rob90–1 vs 83 ± 3 nM for Robwt). Significantly, when Arg90 was replaced with naphthylalanine, giving Rob90-Naph, the affinities for *micF* and *micFmutB* DNAs both increased significantly and to about the same extent (BC₅₀ 34 ± 6 nM for *micF* vs 45 ± 4 nM for *micFmutB*). These findings strongly support the hypothesis that guanine nucleobase amino acid 1 can substitute for arginine in position 90, actually increasing the binding to *micF*DNA; this was also suggested by the data in Figure 4. However, the data also suggest that Rob containing naphthylalanine may be capable of binding to the DNA by an alternative mechanism, whereas Rob90–1, which was designed to be more rigid in proximity to position 90 than Rob-wt, cannot easily accommodate binding to more purine-rich *micFmutB* while simultaneously maintaining the contact it employs in binding to the B-box of Rob-wt.

The preceding data involving Rob90 substituted with naphthylalanine suggest strongly that Rob containing this amino acid can bind to DNA by a different mechanism than Rob containing 1. While we noted the possibility of weak base stacking in an earlier publication dealing with position 40 of the Rob protein,²⁴ the affinities measured in this study were significantly greater and suggested the need for a systematic comparison. Accordingly, we prepared several modified Rob proteins substituted at either position 40 or position 90 with the same amino acids. This included amino acids 1 and 5 (Figure S3) as well as naphthylalanine and phenylalanine at position 90 (Figure S4), the latter of which had previously been introduced into position 40.²⁴ A summary of the BC₅₀ values calculated from the EMSA assays is shown in Table 3. As shown, all substitutions with the same amino

acids gave stronger affinities at position 90 than at position 40 in spite of the reported²⁵ lesser importance of the B-box interaction with Rob. The lower value for amino acid 1 at position 90 was no surprise, given its resemblance to arginine (*vide supra*) and greater conformational rigidity than the canonical amino acid Arg. The results obtained for Rob90–5 and Rob90-Phe as well as Rob90-Naph are consistent with a binding mechanism involving the base stacking of these Rob proteins to the (purine-rich) B-box of the *micF* DNA. However, it should be noted that the molecular basis of the observed binding of Rob90–5 to *micF* DNA may also reflect the Watson–Crick-like binding of 5 to Thy14 (*vide supra*). The loss of affinity of Rob90–1 when the DNA promoter was made more purine-rich on the target strand (*micF* → *micFmutB*) also supports the interpretation that the binding mechanism was impacted by the change in the DNA target structure.

In a recent publication,²⁴ we demonstrated that the introduction of nucleobase amino acids capable of DNA-target H bonding into Rob position 40 enhanced the affinity of this protein for *micF* DNA, while those lacking such H bonding properties exhibited a decreased DNA affinity. Furthermore, the modified Rob proteins displaying enhanced affinity also exhibited a greater resistance to the macrolide antibiotics roxithromycin and clarithromycin than that of wild-type Rob. Increased antibiotic resistance to certain antibiotics is a known phenotype of altered Rob expression, apparently mediated at the level of expression of the export protein OmpF.^{4,7–9} Accordingly, it seemed of interest to determine the effects of the alteration of Rob position 90 on antibiotic resistance.

As shown in Figure 6, the overexpression of Rob90–1, Rob90–2, and Rob90–3 in *E. coli* increased the bacterial resistance to roxithromycin 2- to 3-fold as compared with that of wild-type Rob, while the overexpression of Rob90–4 in *E. coli* resulted in a resistance to roxithromycin approximately as great as that of wild-type Rob. In comparison, Rob90-Naph, which bound to *micF* promoter DNA roughly twice as strongly as Rob-wt and at least as strongly as Rob90–1 (Table 2), induced not dramatically more roxithromycin resistance than that of wild-type Rob and only about one-half that of Rob90–1.

In addition to its phenotypic effect on antibiotic resistance, the overexpression of Rob has also been shown to confer a diminished sensitivity to certain heavy metals.²⁶ To test the phenotypic effects of the modified Rob proteins further, we established an assay in which HgCl₂ inhibited the growth of *E. coli* and became more tolerant to this salt when plasmid-borne Rob-wt was overexpressed (Figure S5). This assay was then used to test the ability of several modified Rob proteins to affect the sensitivity of *E. coli* to Hg²⁺ (Figure 7). As shown in Figure 7, the IC₅₀ value of Hg²⁺ in *E. coli* expressing Rob90–1 was 2–3-fold greater than that of Rob-wt (21 ± 2 vs 7.6 ± 3 μg/mL, respectively). The overexpression of Rob 90–2 also resulted in an increase in *E. coli* tolerance to Hg²⁺, but Rob90–3, Rob90–4, and Rob90–5 produced about as much tolerance to Hg²⁺ as Rob-wt (10.7 ± 1.5 , 8.8 ± 2.5 , and 8.1 ± 1 μg/mL, respectively). For two of them, Rob 90–4 and Rob 90–5, this was consistent with their similar affinities for the *micF* promoter DNA (Table 1). Notably, Rob90-Naph had greater effect on Hg²⁺ tolerance than that of overexpressed wild-type Rob, and the other modified proteins tested were not significantly greater than that of the wild type.

There are two important conclusions from the preceding experiments. The first is that Rob proteins containing nucleobase amino acids can exhibit enhanced binding to their DNA promoter relative to that of the wild type, and that this binding can produce phenotypic responses *in vivo* greater than that accessible by the use of the native system. The second is that the use of nucleobase amino acids as mimics of natural amino acids can be a promising molecular strategy for studying DNA binding in native systems. While nucleobase amino acids permit the use of non-natural interactions to enhance protein–DNA binding, including one which seems to involve a stacking mechanism, the present altered mechanism has not been found to induce significant enhancements in the phenotypic responses mediated by the native system. It seems likely that the phenotypic response relies both on Rob–DNA affinity and also on the specific mode of the molecular interactions between the protein and its DNA target. Finally, there is also an important implication of these studies. While the present work is focused on mimicking and enhancing the properties of canonical amino acids with nucleobase amino acids that have predictable H bonding properties, it seems logical to anticipate that the enhanced protein–DNA binding with nucleobase amino acids could enable altered sites of DNA binding by stabilizing interactions not normally accessible to proteins composed entirely of canonical amino acids. While these studies were not designed to provide insight into the way in which Rob chooses its preferred binding site on the DNA, once the preferred mode of binding is known this does potentially suggest strategies for altering the affinity and selectivity of binding.

An additional feature of these studies that is worth noting is that, while the introduction of nucleobase amino acids in a fashion envisioned to produce stabilizing interactions that are associated with increases in the binding affinity are supported by statistical analysis, the increases are not large. This may be intrinsic to dynamic systems that support modified functions analogous to the wild type, although further study is required to provide further insight and systems that employ simpler responses may be required for this purpose.

Electrophoretic gel shift assays using modified DNA substrates were performed with both Rob and MarA to study the importance of the promoter B-box for protein–DNA interactions (Figure S1).¹⁸ Two conserved thymidines, Thy4' and Thy5' (complementary to Ade18 and Ade19 on the sense strand), were shown to contribute, albeit modestly, to the sequence-specific interaction of MarA with both *micF* and *mar* promoter DNAs through van der Waals contacts with the C⁵ methyl groups. This was established through the replacement of the thymidine residues with uridine and (5-propynyl)-uridine.¹⁸ However, no analogous change was observed for the interaction of Rob with modified *micF* promoter DNAs. In an earlier study, single-base mutational analysis of positions 6–9 (A-box) of the *micF* promoter DNA led to 7- to 100-fold decreases in binding affinity by wild-type Rob, but single base mutational changes at promoter DNA positions 15 or 16 (immediately adjacent to the B-box) resulted in only ~3-fold diminution of Rob binding.²⁵ These findings led to the logical conclusion that the Rob–*micF* promoter DNA interaction depended more importantly on Rob interactions with the A-box of *micF* DNA rather than the B-box.

The observations noted above are not inconsistent with these previous reports,^{18,25} which were based on rather limited examples of protein binding to structurally altered DNA promoters. The only DNA structural change in the present report is outlined in Figure 5 and

Table 2. A single base change (Cyt17 to Gua17) of the promoter sense strand in the B-box along with a change of the complementary nucleotide at position 6' of the antisense strand had only a minimal effect on the Rob-*micF*DNA affinity (BC₅₀ values of 63 vs 83 nM, respectively, Table 2). In a recent report from our laboratory, it was also shown that altering nucleotides 6 and 7 of the *micF* promoter DNA from GC to AT (A-box) resulted in a loss of Rob affinity from 63 to 2500 nM.²⁴ Thus, these experiments also suggest that the A-box protein-DNA interactions may be more important than those involving the B-box.

However, these more recent studies were intended to explore the effects of changing single amino acids in Rob (arginine residues 40 or 90) to nucleobase amino acids.²⁴ The introduction of H bonding nucleobase amino acid mimics for the canonical arginine residues at either position afforded examples leading to an enhanced Rob-*micF*DNA affinity with the concomitant enhancement of phenotypic changes such as antibiotic resistance. However, while little if any base stacking effects were noted for the nucleobase amino acids introduced into the A-box at Rob position 40,²⁴ the effects of the species capable of base stacking were clearly more pronounced at position 90 of Rob, which interacts with the B-box of the *micF* DNA (Table 3); some of these produced fairly substantial changes in the Rob-*micF*DNA affinity. Thus far, none of these enhanced affinities resulting from the changes in Rob 90 seem to have led to the enhanced properties of Rob in conferring a resistance to antibiotics or Hg²⁺.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

wt	wild type
no	no Rob protein sequence in the vector

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Figure 1. Partial structures of the Rob protein–*micF* promoter DNA structure,¹⁸ illustrating the H bonding interactions between the Arg40 of Rob and the O⁶ of Gua6 in the *micF* DNA A-box (upper panel) and between the Arg90 of Rob and a phosphate oxygen anion at the 5′-end of Thy14 in the *micF* promoter DNA B-box (lower panel).

Figure 2. Structures of the nucleobase and other α -amino acids used for the preparation of modified Rob proteins.

Figure 3. EMSA of Rob90wt, Rob90-norarginine, and Rob90-homoarginine, which illustrates the similar affinities of all three for the *micF* promoter DNA.

Figure 4.

The EMSA of Rob90-1, Rob90-2, and Rob90-3 that illustrate the enhanced binding of these proteins to *micFDNA* relative to wild type Rob, while Rob90-4 and Rob90-5 exhibit diminished binding.

Figure 5.

Electrophoretic mobility shift assay (EMSA) analysis of the binding of wild-type Rob to *micF* DNA and *micF-mutB* DNA compared to the binding of modified Rob90-1 and Rob90-Naph proteins to *micF-mutB* DNA.

Figure 6. Resistance of *E. coli* cultures overexpressing Rob-wt, Rob analogues containing nucleobase (1–5) amino acids, or naphthylalanine and homoarginine at position 90 to inhibition by the macrolide antibiotic roxithromycin (pH 8.9). For Rob90–1 vs Rob-wt, $p < 0.05$.

Figure 7.

Resistance of *E. coli* cultures overexpressing Rob-wt and Rob analogues containing nucleobase (1–5) amino acids or naphthylalanine, homoarginine, and leucine at position 90 to inhibition by HgCl₂. For Rob90-1 and Rob90-2 vs Rob-wt, $p < 0.05$; for Rob90-3 vs Rob-wt, $p > 0.05$.

Table 1.Comparison of the Bindings of Different Rob Samples with the Mutations in Position 90 with *micF*^a

DNA binding, BC ₅₀ (nM)	
Rob sample	<i>micF</i>
Rob-wt	63 ± 11
Rob90-1	39 ± 2
Rob90-2	38 ± 5
Rob90-3	41 ± 4
Rob90-4	80 ± 10
Rob90-5	56 ± 12
Rob90-norArg	56 ± 6
Rob90-homoArg	47 ± 8

^aFor Rob-wt vs Rob90-1, Rob-90-2, Rob-90-3, and Rob90-4, $p < 0.05$. For Rob-wt vs Rob90-5, $p > 0.05$.

Table 2.

Comparison of the Binding of Different Rob Samples with Mutations in Position 90 with *micF* and *micFmutB* DNA Substrates^a

Rob sample	DNA binding, BC ₅₀ (nM)	
	<i>micF</i>	<i>micFmutB</i>
Rob-wt	63 ± 11	83 ± 3
Rob90-1	39 ± 2	220 ± 20
Rob90-Naph	34 ± 6	45 ± 4

^aFor Rob-wt (*micF* vs *micFmutB*), $p < 0.05$. For Rob90-1 (*micF* vs *micFmutB*), $p < 0.05$. For Rob90-Naph (*micF* vs *micFmutB*), $p > 0.05$.

Table 3.Comparison of the Binding of Different Rob Samples with Mutations in Positions 40 and 90 with *micF*^a

Rob sample	DNA binding, BC ₅₀ (nM)	
	position 40	position 90
Rob-wt	63 ± 11	63 ± 11
Rob-1	140 ± 7	39 ± 2
Rob-5	104 ± 13	56 ± 12
Rob-Naph	75 ± 5	34 ± 6
Rob-Phe	140 ± 15 ^b	70 ± 6

^aFor Rob-1, Rob-5, Rob-Naph, and Rob-Phe (position 40 vs 90), $p < 0.01$.^bPreviously reported in ref 24.