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Evolution of the Antibiotic Resistance Protein, FosA, is Linked to a Catalytically Promiscuous Progenitor†

Daniel W. Brown, **Matthew R. Schaab**, **William R. Birmingham**, and **Richard N. Armstrong***
Departments of Biochemistry and Chemistry, Vanderbilt University, Nashville, TN 37232-0146

Abstract

The fosfomycin (1) resistance proteins FosA and FosX in pathogenic microorganisms are related to a catalytically promiscuous progenitor encoded in a *phn* operon in *Mesorhizobium loti*. The *mlr3345* gene product ($FosX_{Ml}$) from *M. loti* has a very low epoxide hydrolase activity and even lower glutathione transferase activity toward **1** and does not confer resistance to the antibiotic. *In vitro* homologous recombination of the *mlr3345* and *pa1129* genes (a *fosA* gene from *Pseudomonas aeruginosa* that does confer robust resistance to **1**) produces recombinant proteins that confer resistance to **1** and indicate that the FosA resistance proteins are functionally and genetically related to *mlr3345*.

Fosfomycin, **1**, is an effective broad-spectrum antibiotic produced by certain strains of Streptomyces (1). Microbial resistance to fosfomycin involves one of three plasmid or genomically encoded resistance proteins, FosA, FosB or FosX that catalyze the reactions shown in Scheme 1 (2,3). All three enzymes are members of the same metalloenzyme superfamily (4). FosA and FosB catalyze the addition of glutathione (GSH) and L-Cys to the antibiotic while FosX catalyses the addition of water (5). The relationship among these proteins and the evolution of resistance mechanisms is a topic of considerable interest in efforts to understand and combat antimicrobial resistance.

Recently we described a protein ($FosX_{Ml}$) encoded in a *phn* operon from *Mesorhizobium loti* that has both tepid FosX and FosA activities (5). The protein, which is probably involved in some aspect of phosphonate catabolism in *M. loti*, does not confer significant resistance to **1** when expressed in *Escherichia coli*. $FosX_{Ml}$ has been proposed, based on its catalytic promiscuity, as a progenitor of genuine fosfomycin resistance proteins (5).

Structure-based sequence alignments of the various FosA and FosX proteins reveal a very limited set of residues that appear to differentiate a FosA-active protein from a FosX-active protein. Several mutations in $FosX_{Ml}$ would appear to be required to confer efficient GSH transferase activity to the enzyme. These include a triple mutation in the GSH binding site (E44G/F46Y/M57S) very near the metal center and two basic residues at the base of the K^+-binding loop as indicated in Figure 1 (6). The remarkable conservation of sequence at both the DNA and protein level in the K^+-binding loop suggests that K^+-dependent FosA enzymes and the monovalent cation-independent, FosX enzymes share a closely related heritage.

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*To whom correspondence should be addressed. Phone: 615-343-2920. FAX: 615-343-2921. Email: r.armstrong@vanderbilt.edu.

SUPPORTING INFORMATION AVAILABLE

Details of the construction and screening of recombinants, purification of proteins and *in vivo* and *in vitro* assays (9 pages). This material is available free of charge via the Internet at http://pubs.acs.org.

In this report we provide evidence that the promiscuous but ineffective $FosX_{Ml}$ can serve as a genetic template for the evolution of biologically effective FosA-like proteins. A rationally designed triple mutant of $FosX_{Ml}$ (E44G/F46Y/M56S) is shown to be sufficient to abolish the fosfomycin hydrolase activity of $FosX_{Ml}$ and to substantially improve its GSH transferase activity. More importantly, *in vitro* homologous recombination of the genes encoding $FosX_{Ml}$ and $FosA_{Pa}$ produces recombinants that are 90% identical in sequence to $FosX_{Ml}$ but confer significant resistance to **1** in *E. coli*. The antibiotic resistance is due to acquisition of GSH transferase activity by the recombinant proteins toward **1** that arises from the selective incorporation of several residues and a loop that are implicated in the catalytic mechanism of $FosA_{Pa}$ (6).

The $FosX_{Ml}$(E44G/F46Y/M57S) mutant was prepared by standard site-specific mutagenesis protocols as previously described (3,6). The resultant purified protein had no detectable fosfomycin hydrolase activity but did exhibit a much-improved GSH transferase activity toward **1** (Table 1). Unlike $FosX_{Ml}$, the triple mutant confers detectable resistance to fosfomycin in *E. coli*. Several attempts to improve the catalytic activity of the mutant $FosX_{Ml}$ by structure-based rational mutagenesis were not successful.

In contrast, homologous recombination between the promiscuous gene and the highly evolved gene yielded new and more effective enzymes. *In vitro* homologous recombination of the genes encoding $FosX_{Ml}$ and $FosA_{Pa}$ was accomplished following the DNA shuffling methodology described by Stemmer and coworkers (7,8) with modifications described here and elsewhere (9). Briefly, the DNA from expression vectors for *mlr3345* (5) and *pa1129* (10) were amplified by PCR using specific primers. The amplified genes were purified and then digested with DNAase1. Gene fragments, 30–50 bp in length, were selected from a 2% agarose gel purification of the digested DNA. The collected fragments were assembled in a primer-less extension reaction by PCR with *Taq* DNA polymerase. The assembled PCR mixture was then amplified using primers specific for the *mlr3345* gene containing NdeI and BamH1 restriction sites to avoid background that could be associated with the *pa1129* gene. The amplified recombinants were digested with NdeI/BamH1 and ligated into the pET20b expression vector. The DNA was used to transform *E. coli* Rosetta (DE3) cells. An initial selection of the transformed cells was done on agar plates containing 100 μg/mL ampicillin, 30 μg/mL chloroamphenicol and 2.5 μg/mL **1**. Individual colonies were picked, cultured and then spread onto plates containing increasing concentrations (0.05 to 1 mg/mL) of **1**. Details of the experimental procedures, enzyme purification and the assays of the enzymes are provided in Supporting Information.

Plasmids from sixteen colonies that grew on plates containing ≥ 0.05 mg/mL fosfomycin were isolated and sequenced. Six unique sequences arose from this selection as indicated in Table 1. The recombinant proteins are approximately 90% identical in sequence to $FosX_{Ml}$ protein. They recapitulate the rational triple mutant protein (mutations 1, 2 and 3) located at positions associated with the GSH binding site of $FosA_{Pa}$ (6). They also transplant the loop region known to be important for K^+ binding and reveal other sites that may be important in FosA activity.

Fourteen of the sixteen recombinants recovered from the screening harbor all five residues that are near to or implicated in the binding of GSH. Mutations 4 and 5 (Table 2), do not appear to be directly involved in the binding of GSH but nevertheless are strictly conserved in the recovered recombinants. Analysis of the DNA sequences of the parent and recombinant genes suggest that all mutations arise from homologous recombination events and not errors in the PCR used to assemble and amplify the genes.

Two of the recombinant proteins were chosen for a more detailed analysis and comparison with the two parent enzymes and the rationally designed triple mutant $FosX_{Ml}$(E44G/F46Y/

M57S). One of the recombinants, S1.1, harbors three of the five mutations thought to be involved in GSH binding while the other, S1.2, contains all five mutations that predominate in the selection (Table 2). The MIC values toward **1** measured in *E. coli* expressing the proteins and the steady-state kinetic parameters of each purified enzyme were measured. The results of these experiments are shown in Figure 2 and Table 1, respectively. The recombinants have no hydrolase activity.

The rationally designed triple mutant exhibits very little growth on agar containing 50 μg/mL **1** while the S1.1 and S1.2 recombinants survive at higher concentrations of the antibiotic. The kinetic data show a selection for proteins that have both an enhanced k_{cat}/K_M^{fos} and k_{cat}/K_M^{GSH}. The principal structural difference between the triple mutant and the two recombinants is the incorporation of the K^+-binding loop region and the two residues (K95 and R98) associated with GSH binding. Although the native $FosA_{Pa}$ enzyme is activated up to 100-fold with 0.1 M KCl, the S1.2 enzyme is activated ≤ 6-fold with 1.0 M KCl suggesting that the transplanted K^+-binding sequence is not fully functional in the recombinants.

The distribution of mutations around the active site of the recombinant protein S1.2 mapped onto the X-ray crystal structure of $FosX_{Ml}$ is illustrated in Figure 3. Of the four residues (Y46, S57, K95, R98) thought to directly interact with GSH, three (Y46, K95, R98) are conserved in all the recombinants. All sixteen of the recombinant proteins also contain the E60K and A63V mutations that are on the periphery of the active site. Whether these mutations contribute to the catalytic properties of the recombinant proteins is not clear at this juncture. Nevertheless, it is notable that all of the mutations are either in the active site or are on loops that flank it.

The results reported here demonstrate that a minimal transfer of sequence information (~10%) from a catalytically robust enzyme to a promiscuous but ineffective protein is sufficient to give rise to new and biologically effective resistance proteins. The structural diversity of the recombinant proteins obtained here was restricted by the use of $FosX_{Ml}$-specific primers in the amplification step. A more diverse and catalytically effective library of enzymes can be anticipated with the inclusion of FosA-specific primers in the amplification step.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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FIGURE 1.

Sequence alignment between the FosA and FosX proteins in the K^+-loop region of FosA. The residues colored in red and green are involved in GSH and K^+ binding, respectively, in FosA. The residues in blue represent a three-residue insertion unique to the FosX proteins. This particular alignment emphasizes the conservation of residues in the K^+-binding loop.

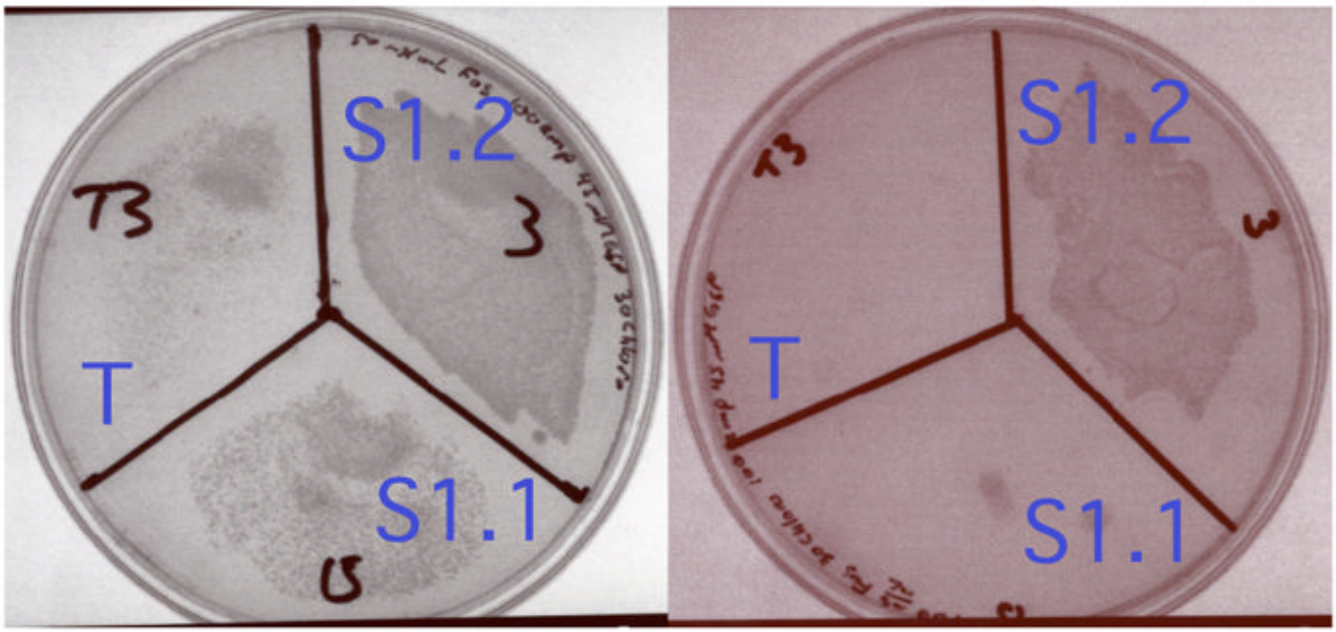


FIGURE 2.
Comparison of the growth of *Escherichia coli* transformed with the expression vectors encoding the triple mutant (T) $FosX_{Ml}$(E44G/F46Y/M57S), and the two recombinants S1.1 and S1.2 on agar plates containing fosfomycin at 50 μg/mL (left) and 100 μg/mL (right).

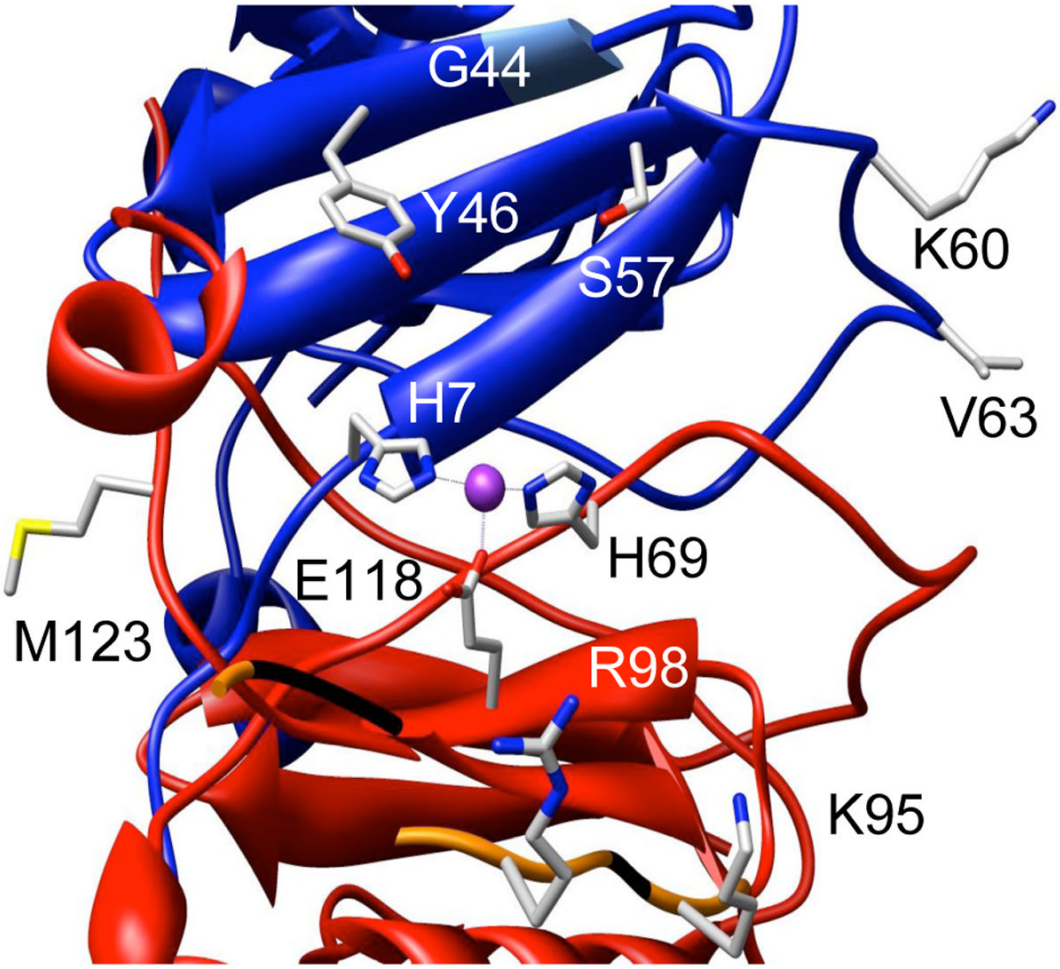
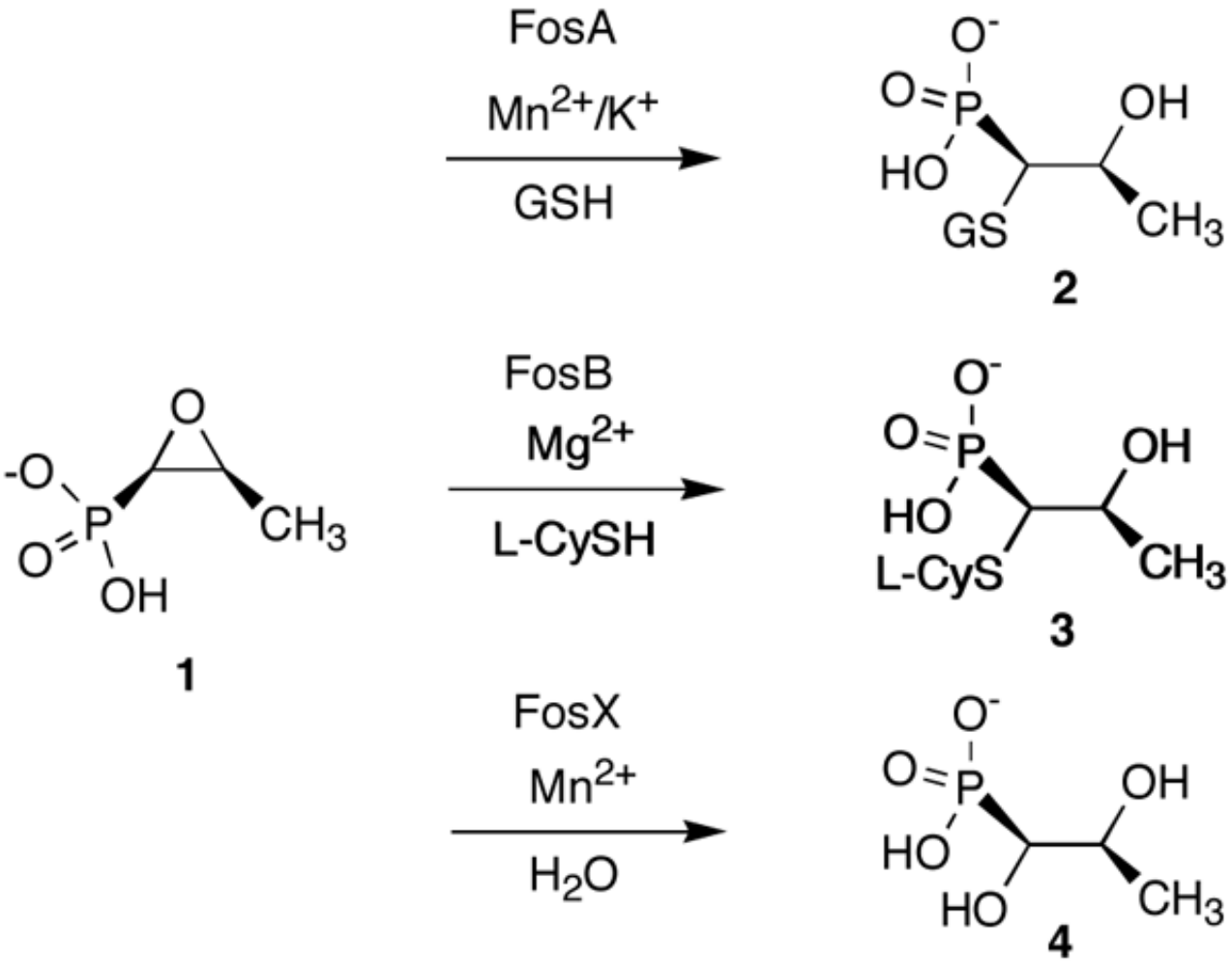


FIGURE 3.

Distribution of key residues near the active site of the recombinant protein S1.2 using the crystal structure of $FosX_{Ml}$ as the template. The Mn(II) ion is shown in purple. Illustrated are the three conserved metal ligands (H7, H69 and E118), the mutations thought to be important in GSH binding (Y46, S57, K95 and R98) and mutant residues of unassigned function (G44, K60, V63, and M123).



Scheme 1.

Table 1

Catalytic and Biological Characteristics of the Parent FosA and FosX Enzymes and Mutants[a]

Enzyme name	k_{cat} (s^{-1})	k_M^{fos} (μM)	k_{cat}/K_M^{fos} ($M^{-1}s^{-1}$)	k_{cat}/K_M^{GSH} ($M^{-1}s^{-1}$)	MIC (mg/mL)
$FosA_{pa}$	180 ± 6	200	$(9.0 \pm 1.4) \times 10^5$	$(4.1 \pm 0.8) \times 10^4$	>20
$FosX_{Ml}$	0.15 ± 0.02[b] > 0.06[c]	300[b]	$(5.0 \pm 0.6) \times 10^2$[b]	<10[c]	<0.01
$FosX_{Ml}$(E44G/F46Y/M57S)	5.0 ± 0.2	600	$(8 \pm 3) \times 10^3$	$(4.9 \pm 0.2) \times 10^2$	0.05
S1.1	19 ± 1	400	$(5 \pm 1) \times 10^4$	$(6.4 \pm 0.5) \times 10^2$	0.075
S1.2	136 ± 4	1100	$(1.2 \pm 0.2) \times 10^5$	$(4.3 \pm 0.2) \times 10^2$	0.15

[a] Kinetic constants k_{cat} and k_{cat}/K_M^{fos} were obtained at [GSH] = 20 mM. k_{cat}/K_M^{GSH} were obtained at [**1**] = 10 mM.

[b] Kinetic constants are for the epoxide hydrolase activity of $FosX_{Ml}$.

[c] Estimate based on ^{31}P-NMR assay (2,5) of the formation of the GSH adduct, **2**.

Table 2

Common Mutations Found in the *in vitro* Recombinant Proteins.[a]

Enzyme name	Mut. 1	Mut. 2	Mut. 3	Mut. 4	Mut. 5	Loop 94–106 (ΔP99/V100/E101)	Mut. 6
S1.1	E44S	F46Y	M57L	E60K	A63V	WKQNRSEGDS	
S1.2	E44G	F46Y	M57S	E60K	A63V	WKQNRSEGDS	T123M
S2.2	E44G	F46Y	M57S	E60K	A63V	WKQNRSEGDS	
S2.8	E44G	F46Y	M57S	E60K	A63V	WKQNRPEGDS	
S3.3	E44G	F46Y	M57S	E60K	A63V	WKQNRSEGDS	E126K
S3.4	E44S	F46Y	M57S	E60K	A63V	WKQNRSEGDS	

[a] Mutations shown in red have been identified as being near to or having direct interactions with GSH in FosA$_{Pa}$ (6). Infrequent mutations are shown in blue. The loop insertion (residues 94-103) is the K^+-binding loop and associated GSH binding residues illustrated in Figure 1 with deletion of P99, V100, and E101. The residue numbering is from the FosX$_{Ml}$ protein, irrespective of any deletions.