

STRUCTURE, FUNCTION AND EVOLUTION OF FOSFOMYCIN RESISTANCE PROTEINS IN THE VICINAL OXYGEN CHELATE SUPERFAMILY

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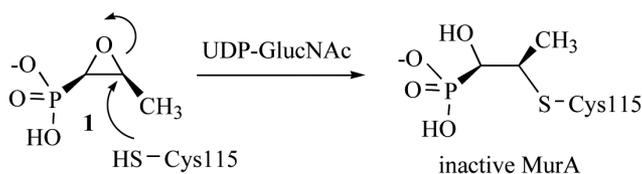
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ABSTRACT

The Vicinal Oxygen Chelate (VOC) superfamily embodies a functionally diverse set of enzymes that catalyze both acid-base and electron transfer chemistries [1]. A subset of these enzymes is known to confer microbial resistance to the antibiotic fosfomycin by three different mechanisms. The resistance proteins FosA (a glutathione transferase), FosB (a thiol transferase) and FosX (an epoxide hydrolase) are found in both Gram-negative and Gram-positive pathogenic microorganisms. These proteins have been proposed to be evolutionarily related to a catalytically promiscuous progenitor (FosX_{MI}) encoded in a *phn* operon in *Mesorhizobium loti* [2]. We recently reported that more robust FosA activity could be evolved by homologous recombination experiments with a FosA gene and the gene encoding the promiscuous FosX_{MI} [3]. This report is incorrect. The “evolved” proteins that were characterized appear not to be the result of homologous recombination but rather due to random mutations in a mutant gene that contaminated the original recombination experiments. This paper first summarizes what is known about the evolutionary relationships among these proteins and then points to new lines of investigation, particularly with respect to Gram-positive microorganisms.

INTRODUCTION

Fosfomycin, (*1R,2S*)-epoxypropylphosphonic acid, **1**, is a natural product that has potent, broad-spectrum antimicrobial activity against both Gram-positive and Gram-negative microorganisms [4, 5]. Fosfomycin acts by the covalent inactivation of the enzyme UDP-N-acetylglucosamine-3-enolpyruvyltransferase or MurA. The MurA enzyme catalyzes the first committed step in peptidoglycan biosynthesis, the addition of pyruvate to the 3'-hydroxyl group of UDP-N-acetylglucosamine (UDP-GlucNAc), providing the three-carbon unit that ultimately links the glycan copolymer and the peptide units of the cell wall. The inactivation of MurA occurs only in the presence of UDP-GlucNAc and involves the alkylation of an active site cysteine residue (C 115 in the *E. coli* enzyme) Scheme 1 [6, 7]. The structure of the covalently inhibited enzyme has been reported in [8].



Scheme 1. Inactivation of MurA by fosfomycin.

A decade after the introduction of fosfomycin into the clinic, a plasmid-mediated resistance to the antibiotic was observed in clinical isolates obtained from patients treated with the drug [9–11]. Additional investigations indicated that the resistance gene encoded a 16 kDa polypeptide that catalyzed the addition of glutathione (GSH) to the antibiotic, rendering it inactive [12, 13].

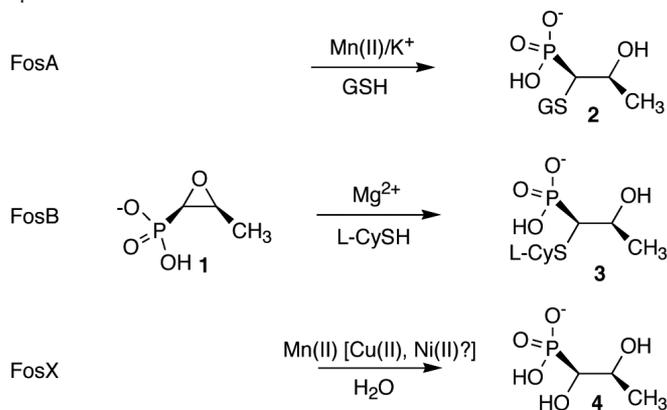


Figure 1. Reactions catalyzed by the fosfomycin resistance proteins FosA, FosB and FosX. The FosA catalyzed reaction with GSH requires the monovalent cation (K^+) for optimal activity. The FosB reaction is optimal with L-cysteine as the nucleophile to form adduct **3** but may use alternative nucleophiles not yet characterized.

 Structure, Function and Evolution of Fosfomycin Resistance Proteins

The fosfomycin hydrolase enzymes (FosX) appear to use Mn(II) in most instances, but with some enzymes, Cu(II) and Ni(II) also work as well as, if not better than, Mn(II). The diol, **4**, is formed by addition of water at C1.

In the period between 1996 and 1999, we established that this resistance protein (now termed FosA) was an Mn(II) and K⁺-dependent GSH transferase that catalyzed the formation of the GSH adduct (**2**) at C1 of the antibiotic, as illustrated in Figure 1 [14, 15]. Moreover, we discovered that there are additional mechanisms of resistance catalyzed by enzymes in the same superfamily [2, 16–18].

An analysis of primary sequence information and available three-dimensional structural data indicates that currently known fosfomycin resistance proteins are evolutionarily related and fall into three basic categories as illustrated in Figures 1 and 2. Although the resistance protein identified was encoded on a multi-drug resistance plasmid, numerous others have been identified encoded in the genomes of human pathogens.

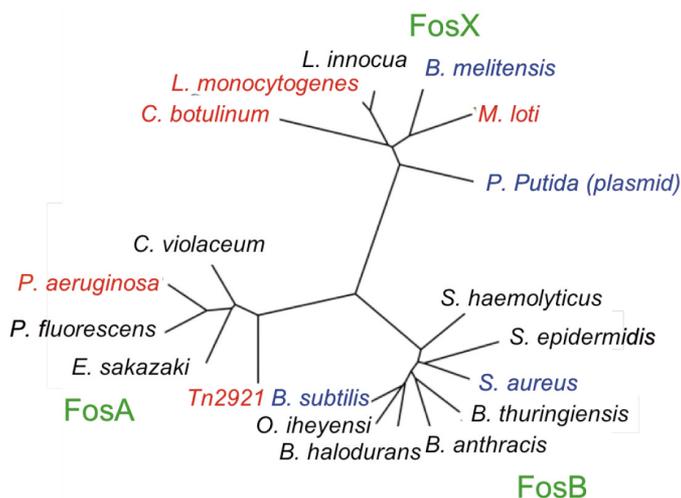


Figure 2. Sequenced-based segregation of the subfamilies of the fosfomycin resistance proteins FosA, FosB and FosX. The three subfamilies are shown in green. The gene products shown in red have been expressed and characterized biochemically, and the crystal structures of the proteins have been determined. The gene products shown in blue have been expressed and characterized biochemically.

MATERIALS AND METHODS

The materials and methods for this work have been reported previously [3, 17] and will not be reiterated here.

FUNCTIONAL AND EVOLUTIONARY RELATIONSHIP BETWEEN FOSA AND FOSX

The FosA and FosX proteins share about 30–35% sequence identity. The FosA proteins that have been characterized do not catalyze the hydration of fosfomycin, and the highly evolved FosX enzymes such as those from *Listeria monocytogenes* and *Pseudomonas putida* do not catalyze the addition of GSH to fosfomycin. However, the first protein to be discovered that catalyzed the hydration of fosfomycin was also found to catalyze the addition of GSH to the antibiotic. This protein (FosX_{Ml}) is encoded by a gene in an operon in *Mesorhizobium loti* that probably functions in phosphonate catabolism. The FosX_{Ml} enzyme is not a good resistance protein but could be an evolutionary progenitor of genuine resistance proteins that have FosA, FosB or FosX activities. To examine this possibility we set out to evolve a robust FosA enzyme from the gene encoding FosX_{Ml} by either rational, structure-based mutagenesis or homologous recombination.

The rational design strategy was guided by structural comparisons of the FosA and FosX proteins and computational prediction of the GSH binding site in FosA. The principal differences in or near the active sites of the two proteins include three residues near the metal center and a loop located adjacent to the active site that serves to help bind GSH and the K⁺-ion in FosA (Figure 3). In a recent report we described the construction of a triple mutant (E44G/F46Y/M56S) in the FosX_{Ml} protein [3]. The FosX_{Ml}(triple mutant) lost all fosfomycin hydrolase activity and gained considerable GSH transferase activity. Replacement of the loop region of the triple mutant of FosX_{Ml} with that of the FosA from *Pseudomonas aeruginosa* (FosA_{Pa}) resulted in a FosX_{Ml}(triple+loop mutant) and a very large loss of GSH transferase catalytic activity (unpublished results).

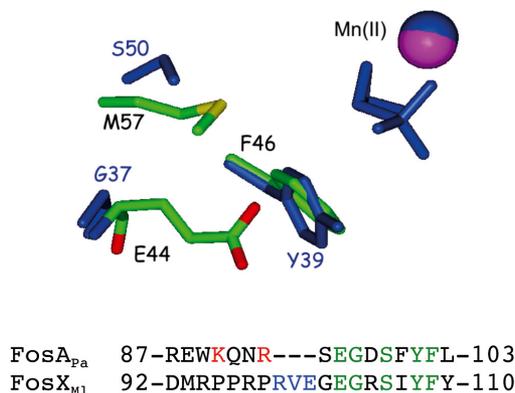


Figure 3. (Top) Overlay of the FosA_{Pa} (blue) and FosX_{Ml} structures illustrating the residues near the substrate also shown in blue. The Mn(II) ions are shown as blue and purple spheres. (Bottom) Sequence alignment of the loop region of FosA_{Pa} and FosX_{Ml}. Residues highlighted in red are involved in GSH binding, those in green are K⁺-binding residues and those in blue are the insert unique to FosX.

In the same paper [3] we also reported what appeared to be successful homologous recombination (gene shuffling) experiments between the FosX_{MI} and FosA_{Pa} genes resulting in six different recombinant proteins [3]. Two of the proteins were characterized in detail both with respect to their steady-state kinetics and their ability to confer resistance to fosfomycin in *E. coli*. In a subsequent effort to extend these studies, we attempted to reproduce the initial experiments using freshly prepared and sequenced DNA samples for FosX_{MI} and FosA_{Pa}. After numerous additional experiments we found that we were unable to reproduce the original results. This failure prompted a detailed analysis of the nucleotide sequences of the six recombinants. The sequence analysis shows that the recombinants were not the result of homologous recombination but strongly suggest that they were due to contamination of one of the original DNA samples (most likely the FosX_{MI} plasmid) with the plasmid encoding the FosX_{MI}(triple+loop mutant). A correction to this effect is being prepared for publication in *Biochemistry*.

The six variants isolated in the antibiotic screen most likely arose either from “self recombination” of the FosX_{MI}(triple+loop mutant) or recombination between fragments of the FosX_{MI} gene and those of the FosX_{MI}(triple+loop mutant). The gene shuffling process requires the use of the polymerase chain reaction (PCR), which is prone to errors in base incorporation. The six variants that were isolated are probably due to errors in the PCR steps of the homologous recombination process. That said, it is important to indicate that we have no direct evidence that this is the case.

Although it is clear that the FosA/B/X proteins are evolutionarily related based on sequence and structure, it is less obvious that the FosX_{MI} protein is a promiscuous progenitor of true fosfomycin resistance proteins. It is clearly a promiscuous enzyme, being able to use both water and GSH as the nucleophile. However, any direct evolutionary connection remains experimentally obscure, perhaps due to evolutionary drift among the genes.

WHAT IS THE THIOL SUBSTRATE FOR FOSB?

In contrast to the FosA and FosX proteins, the FosB resistance enzymes have not been fully characterized. For example, there is no three-dimensional structure of a FosB from any source. One of the most important unanswered questions with respect to the FosB proteins is the identity of the thiol substrate. Gram-positive microorganisms such as *Bacillus subtilis* and *Staphylococcus aureus* do not make glutathione suggesting that it is not the native thiol substrate for FosB. We originally reported that L-cysteine was the most likely candidate substrate for the FosB in *B. subtilis* based on its relatively high concentration (0.1 mM) in the organism. The enzyme does catalyze the reaction but the efficiency of the reaction is not very impressive with a $k_{\text{cat}}/K_M^{\text{fos}}$ of about $10^3 \text{ M}^{-1}\text{s}^{-1}$ and a $k_{\text{cat}} = 5 \text{ s}^{-1}$.

A very recent paper [19] reported a newly discovered thiol that is a derivative of L-cysteine. The compound is found in significant concentrations in many Gram-positive microorganisms, particularly, in strains of *Bacillus*. The concentrations are comparable to or greater than that of L-cysteine. Although the function of this newly discovered molecule is not known, it may well be the natural substrate for FosB. This remains a very fertile area of investigation.

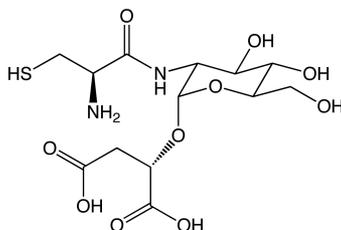


Figure 4. Chemical structure of bacillithiol.

ACKNOWLEDGEMENT

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