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Product of *fosC*, a Gene from *Pseudomonas syringae*, Mediates Fosfomycin Resistance by Using ATP as Cosubstrate

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Pseudomonas syringae **PB-5123, a producer of fosfomycin, is resistant to high concentrations of the antibiotic. Two possible mechanisms of resistance have been detected: (i) impermeability to exogenous fosfomycin, even in the presence of sugar phosphate uptake inducers, and (ii) antibiotic phosphorylation. The gene responsible for this last activity, *fosC*, encodes a ca. 19,000-Da protein and is immediately followed by a second open reading frame, which shows sequence similarities to glutathione *S*-transferases. FosC uses ATP as a cosubstrate in an inactivation reaction that can be reversed with alkaline phosphatase. Other nucleotide triphosphates cannot be substituted for ATP in this reaction. No relationship between *fosC* and the previously described genes of fosfomycin resistance was found.**

Fosfomycin [L-(*cis*)-1,2-epoxy propyl phosphonic acid] is a broad-spectrum antibiotic produced by some strains of *Streptomyces* (15) and by *Pseudomonas syringae* (25). It enters cells by active transport through the L-α-glycerophosphate and the hexose-6-phosphate uptake systems and blocks peptidoglycan biosynthesis through inhibition of the formation of *N*-acetylmuramic acid (18).

Chromosomally encoded fosfomycin-resistant (Fo^r) strains have an impairment in fosfomycin uptake (17) or a low-affinity pyruvyl transferase (31). Plasmid-borne Fo^r in gram-negative bacteria is mediated by a 141-amino-acid polypeptide (FosA) which catalyzes the fusion of fosfomycin and glutathione (for a comprehensive review, see reference 26). Another plasmid-encoded Fo^r determinant (*fosB*) was found in *Staphylococcus epidermidis* (10). The proteins FosA and FosB exhibit 38.3% sequence identity (35).

It has been suggested that the genes encoding antibiotic resistance isolated from nosocomial strains may have originated from antibiotic-producing organisms, because analogous enzymatic activities (2, 33) and protein sequence similarities (27) have been found in both producers and clinical isolates. On the other hand, antibiotic resistance and biosynthetic genes are usually clustered, and consequently, the molecular cloning of resistance genes may also facilitate a molecular characterization of the biosynthetic pathways. In this report we describe the characterization of a DNA fragment from *P. syringae* PB-5123; when this fragment is introduced into *Escherichia coli* it leads to Fo^r. The Fo^r determinant, *fosC*, encodes a 19-kDa polypeptide that probably inactivates fosfomycin through the introduction of a phosphate group in the molecule, with ATP as a donor.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. *P. syringae* PB-5123 was used throughout the study (25). *E. coli* HB101 (23) and *E. coli* JM83 (34) were used as recipients for recombinant plasmids. *E. coli* XL1-Blue (24) was transfected with M13mp18 or M13mp19 phage DNA (34) containing the DNA fragments to be sequenced. Minicell experiments were conducted with *E. coli* AR1062 (F$^-$ *leu minA minB rpsL thi thr*). *E. coli* ESS, a fosfomycin-hypersusceptible strain, was used as an indicator organism in drug bioassay studies. *E. coli* HB101(pUO001) containing *fosA* was used as a positive control of fosfomycin inactivation (1, 32). Plasmids pBR322, pUC18, and pUC19 (23) were used in the cloning experiments.

Culture conditions and MIC determination. Cultures were grown in nutrient broth (Difco) and L broth. Solid media contained 15 g of agar-agar per liter. *P. syringae* and *E. coli* were incubated with aeration at 30 and 37°C, respectively. The MIC was determined in Mueller-Hinton plates (Difco) containing twofold increasing concentrations of fosfomycin seeded with about 200 CFU of the bacteria to be tested.

Fosfomycin uptake assays. Fosfomycin uptake assays were performed with exponentially growing cultures in L broth containing 2 mM glucose 6-phosphate. The cells were washed with warm L broth without inducer and were suspended in this medium, and incubation was continued. [^{3}H]fosfomycin (10.8 mCi/mmol) was added to a final concentration of 300 μg/ml, and incorporation of radioactivity was determined as described previously (13).

In vitro modification of fosfomycin. Extracts of exponentially growing cultures, prepared as described previously (1), were incubated with fosfomycin (radioactively labelled or not) at a final concentration of 300 μg/ml and with the corresponding cosubstrates at 10 mM. The reaction proceeded for 60 min at 37°C and was stopped with 5% trichloroacetic acid (final concentration). After 30 min at 4°C, the supernatants of the reactions were tested by paper chromatography, bioautography, or both methods. In the first case the conditions described by Llaneza et al. (21) were used. In the second case, neutralized portions (10 μl) were adsorbed to antibiogram disks, which were placed onto Mueller-Hinton agar plates seeded with *E. coli* ESS. The zones of inhibition of bacterial growth were compared with those produced by standard fosfomycin solutions.

DNA techniques. Total DNA of *P. syringae* PB-5123 was isolated and purified as described previously (7), except that incubation of the cells with lysozyme was at 4°C for 5 min. Plasmid DNA was obtained by the alkaline method (4) or through isopycnic CsCl gradient centrifugation in the presence of ethidium bromide. Restriction endonucleases and T4 DNA ligase were supplied by Boehringer Mannheim, Mannheim, Germany, and were used as recommended by the supplier. Competent *E. coli* cells were transformed by the $CaCl_2$ method (23). Antibiotic resistance transformants were selected with 50 μg of ampicillin per ml, 30 μg of fosfomycin per ml, and 15 μg of tetracycline per ml.

For DNA-DNA hybridizations, DNA restriction fragments were transferred from agarose gels to nitrocellulose filters (BA 85; Schleicher & Schuell, Keene, N.H.). The DNA probe was labelled with [^{32}P]dCTP (3,200 Ci/mmol, 10 mCi/ml) by nick translation by using a commercial kit (Boehringer Mannheim). High- and low-stringency hybridizations were carried out overnight in aqueous solutions at 68 and 55°C, respectively, by following the method described previously (23). All other DNA techniques were carried out as indicated elsewhere (23).

DNA sequencing and analysis. DNA fragments, which were cloned into bacteriophage M13 derivatives, were sequenced by the dideoxynucleotide chain termination method (24) with ^{35}S-dATP (1,200 Ci/mmol; Amersham Iberica, Madrid, Spain) and a Sequenase 2.0 DNA sequencing kit (U.S. Biochemical Corp., Amersham). A computer-aided database search and sequence analyses were carried out by using the University of Wisconsin Genetics Computer Group program package (9).

Minicell techniques. Minicells were purified by centrifugation through continuous sucrose gradients (10 to 30%), and the proteins were labelled with [^{35}S]methionine (1,103.2 Ci/mmol; Amersham) and were detected by fluorography as described previously (32).

Nucleotide sequence accession number. The GenBank accession number of the DNA sequence reported here is Z33413.

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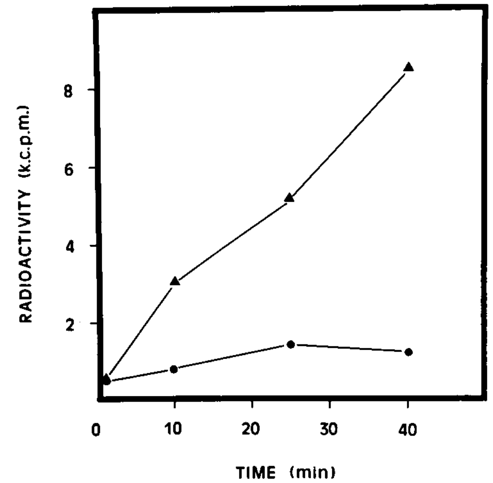

FIG. 1. Incorporation of [^{3}H]fosfomycin into *P. syringae* (●) and *E. coli* HB101(pUO001) (▲) cells.

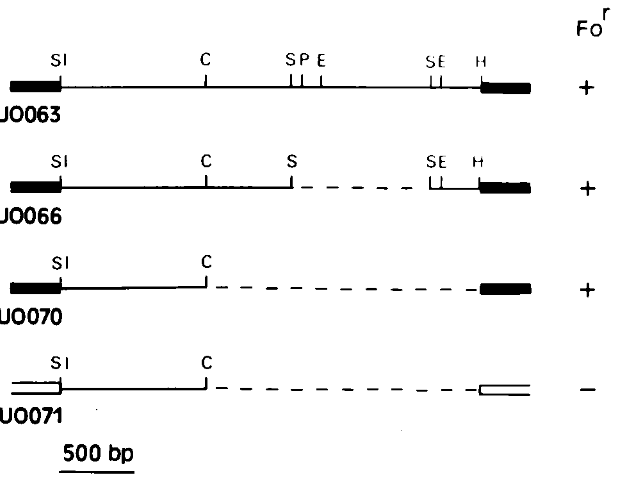

FIG. 2. Structures of some of the plasmids used in the study. Black boxes, pUC18; open boxes, pUC19. Only the relevant restriction sites are shown. Abbreviations are as follows: C, *Cla*I; E, *Eco*RV; H, *Hin*dIII; P, *Pst*I; S, *Sac*II; SI, *Sac*I; Fo^r, fosfomycin resistance.

RESULTS

Fosfomycin resistance in *P. syringae*. The MIC of fosfomycin is 1,024 μg/ml for *P. syringae* PB-5123. This resistance is exerted by at least two mechanisms. One of them is the impermeability of the organism to extracellular antibiotic; no incorporation of [^{3}H]fosfomycin into the cytoplasm was observed, even after previous incubation of the cells with the uptake inducer glucose 6-phosphate (Fig. 1). On the other hand, extracts of *P. syringae* inactivated the antibiotic and altered the mobility of radioactive fosfomycin on paper chromatograms. However, the amount of fosfomycin transformed was not increased by the addition of exogenous glutathione (data not shown), which seemed to indicate that the modifying activity was not analogous to that of FosA. Furthermore, no hybridization of *fosA* to total *P. syringae* DNA was observed, even under low-stringency conditions. These were taken as indications that a new mechanism of fosfomycin inactivation was present in *P. syringae.*

Cloning of a DNA segment of *P. syringae* which confers Fo^r to *E. coli*. Total DNA from *P. syringae* was ligated to plasmid pUC18, and the mixture was used to transform *E. coli* JM83. Selection with ampicillin (50 μg/ml), fosfomycin (30 μg/ml), and glucose 6-phosphate (2 mM) rendered a clone that contained a plasmid, named pUO061, with a 9-kbp *Hin*dIII DNA insert. Deletion mutants of pUO061 were obtained with Fo^r selection generating pUO063 and pUO066 with 2.9- and 1.9-kbp DNA inserts, respectively (Fig. 2). Finally, insertion of a 0.9-kbp *Sac*I-*Cla*I segment into pUC18, but not into pUC19, resulted in Fo^r clones. The putative resistance gene was named *fosC* and is believed to be under the control of the *lacZ* promoter of pUC18 in *E. coli*. When *P. syringae* total DNA was subjected to a Southern blot analysis with *fosC* as a probe, a single band was detected. In contrast, no cross-hybridization was observed between segments containing *fosA* and *fosC* (data not shown).

Organization of the DNA region that contains *fosC*. In the nucleotide sequence of the 1.7-kbp *Sac*I-*Eco*RV fragment of pUO063, two closely located open reading frames (ORFs) were identified along with 40 amino acids of the carboxy-terminal end of a third ORF, located at the beginning of the fragment (Fig. 3). The G+C content of the segment was 58%. ORF1 likely corresponds to *fosC*, as deduced from deletion mutants (Fig. 2) as well as from structural and functional data (see below). The translation start site could be at any of the four ATG triplets that are located between positions 387 and 411 and might stop at either of the two stop codons that are located in tandem at positions 933 and 936. We believe that the third ATG is most likely the triplet start site since a potential ribosome-binding site (GATGA) is located 8 bp upstream of it. In the 387 bp sequenced upstream of ORF1, no consensus *E. coli* promoter was found. Translation of ORF1 yields a protein of between 174 and 182 amino acids with an estimated molecular size of between 19,013 and 19,957 Da, depending on the initial ATG start site.

ORF2 starts four nucleotides downstream of ORF1, at an ATG triplet located at position 941, and terminates at position 1568. It encodes a putative protein of 209 amino acids with a calculated size of 23,758 Da. A potential ribosome-binding site is located 6 bp upstream of the start codon.

Three inverted repeats were found in the sequence. The first one is located upstream of ORF1 and forms a stable 17-bp stem. The second repeat, of 14 bp, is 3′ to ORF1 and expands into the ORF2-coding region. Finally, a third inverted repeat was observed 3′ of ORF2.

To determine if both ORFs were expressed in *E. coli*, minicells containing pUO063 as the only DNA were obtained and the proteins were synthesized and labelled with [^{35}S]methionine. Two polypeptides of 19 and 25 kDa were observed in addition to the proteins encoded by pUC18 (data not shown). These molecular sizes agree with those predicted for ORF1 and ORF2 by sequence analysis.

Characterization of the resistance mechanism encoded by *fosC*. An extensive database search revealed a region of partial homology between the ORF1 translation product and the Mg-ATP binding domains of some eukaryotic kinases and AMP-ATP phosphotransferases (Fig. 4) (29). The ORF2 product presented an overall similarity to the glutathione *S*-transferase of *Schistosoma* species (in 127 amino acids, 23.6% identity and 57.5% conservative substitutions) (16). These findings prompted us to determine if the addition of the corresponding exogenous precursors to extracts of *P. syringae* and *E. coli*(pUO063) had any effect on the antimicrobial activity of fosfomycin. The results indicated that the addition of ATP in the presence of Mg^{2+} resulted in fosfomycin inactivation (Fig. 5, plate 2, disk a) and in a change of migration in paper chromatography (Fig.

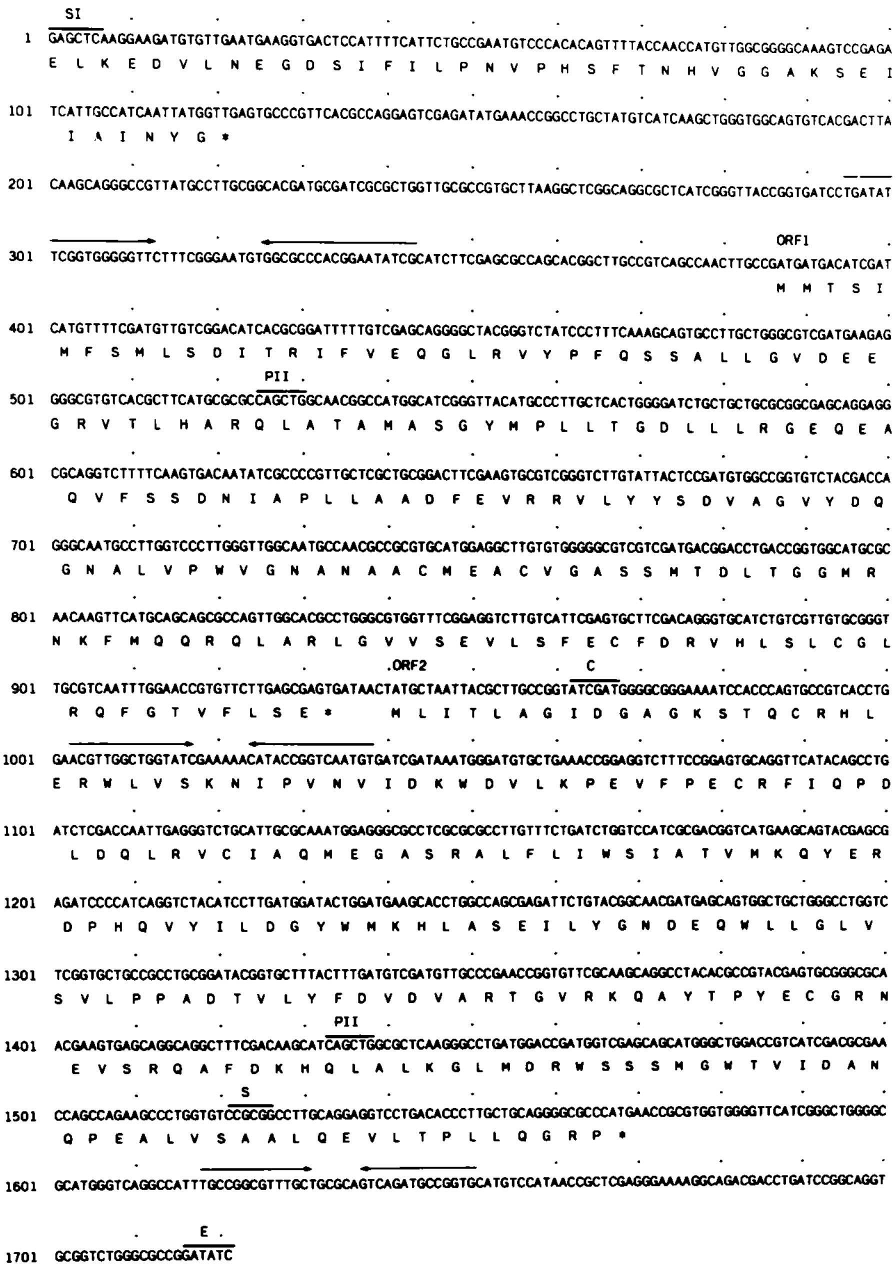

FIG. 3. Nucleotide sequence of the *Sac*I-*Eco*RV DNA fragment that confers Fo^r. The deduced amino acid sequences of FosC (ORF1) and ORF2 are presented under the nucleotide sequence. *, stop codon. Potential inverted repeats are indicated by arrows. Relevant restriction sites are overlined and are abbreviated as follows: C, *Cla*I; E, *Eco*RV; PII, *Pvu*II; S, *Sac*II; SI, *Sac*I.

6). No other nucleotide triphosphates were able to be substituted for ATP (Fig. 5, plate 2, disks b, c, and d). This result suggested that ATP induced the modification of fosfomycin by mediating either its phosphorylation or its adenylylation. To test the first possibility, extracts in which fosfomycin was inactivated as indicated above were treated with alkaline phosphatase, which resulted in restoration of the antimicrobial activity (Fig. 5, plate 2, disk e). Adenylylated fosfomycin (a gift of H. Ono and H. Toguchi, Takeda Chemical Industries, Osaka, Japan) (19) was treated with alkaline phosphatase as well, but no change in its lack of antimicrobial properties was detected. These data suggest that FosC induces fosfomycin phosphorylation with ATP as a substrate, which results in modification of the antibiotic and loss of its antimicrobial activity.

Furthermore, the abilities of the extracts to conjugate glutathione and fosfomycin were tested by using two different substrates: 1-chloro-2,4-dinitrobenzene (a universal substrate for glutathione *S*-transferases) and fosfomycin (the substrate of FosA, the plasmid-encoded resistance protein found in nosocomial bacteria). No activity was found, suggesting a function of the ORF2 gene product other than as a glutathione *S*-transferase.

DISCUSSION

Fosfomycin, acting as a phosphoenolpyruvate analog, binds irreversibly to pyruvyl transferase, an enzyme that catalyzes the formation of *N*-acetylmuramic acid from *N*-acetylglucosamine

FosC	55	S	G	Y	M	P	L	L	T	G	D	L	L	L	R	G	E	Q	71
AK1p	32	Y	G	Y	T	H	L	S	T	G	D	-	L	L	R	A	E	V	47
AKy	28	F	H	A	A	H	L	A	T	G	D	-	M	L	R	S	Q	I	43
AKe	24	Y	G	I	P	Q	I	S	T	G	D	-	M	L	R	A	A	V	39

FIG. 4. Alignment of the Mg-ATP binding motifs of some adenylate kinases with a region of FosC. AK1p, porcine skeletal muscle cytosolic adenylate kinase (14); AKy, cytosolic adenylate kinases from baker's yeast (28); AKe, E. coli adenylate kinase (5).

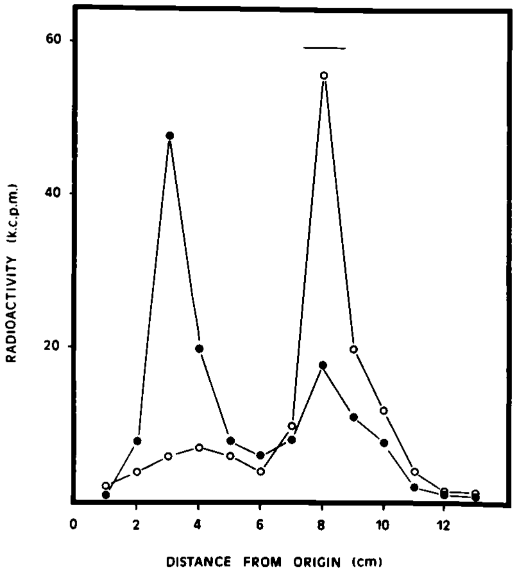

FIG. 6. Detection of plasmid-mediated fosfomycin modification. Cell extracts were incubated with [^{3}H]fosfomycin and ATP and precipitated with trichloroacetic acid, and the supernatants were subjected to paper chromatography as described in the text. (●), *E. coli* JM83(pUO063); ○, *E. coli* JM83. The horizontal line above the graph indicates the zone of migration of pure fosfomycin.

and phosphoenolpyruvic acid (18). It is thus a bacteriolytic antibiotic which blocks peptidoglycan biosynthesis.

Bacteria that produce bactericidal antibiotics must possess protection mechanisms against them (8). Most streptomycetes, including the nonproducers of fosfomycin, are highly resistant to fosfomycin (MIC, 1,024 μg/ml; unpublished data), indicating that probably a general (innate) mechanism is operating in the genus. In contrast, fosfomycin is very active against organisms of the genus *Pseudomonas* and has been used to treat *P. aeruginosa* infections (12). Consequently, the resistance shown by *P. syringae* PB-5123 is peculiar to this strain, as is its ability to produce fosfomycin, and both activities are possibly related.

The fragment of *P. syringae* DNA that conferred fosfomycin resistance to *E. coli* clones showed two complete ORFs and the carboxy-terminal end of another ORF in front of them. Although the possibility that the incomplete ORF is involved in fosfomycin resistance has not been formally excluded, we think that the first complete ORF is *fosC* because of the correlation between structural and functional data. The deduced protein sequence of ORF1 presents zones of similarity to the ATP binding domain of ATP-AMP phosphotransferases, and FosC probably uses ATP as a donor in a reaction in which a phosphoryl group becomes esterified to fosfomycin, which loses its antibiotic properties. The existence of this reaction is confirmed by its reversion with alkaline phosphatase, which restores the antimicrobial activity of fosfomycin.

We can now speculate on the role of *fosC* in its indigenous host. *P. syringae* is not permeable to extracellular fosfomycin. This would be sufficient for resistance in a nonproducer organism such as the chromosomal mutants of *E. coli*. However, this would not protect against the newly produced fosfomycin unless it is produced in an inactive precursor form or unless an enzyme functionally analogous to the target enzyme, but with a lowered affinity for the antibiotic, is synthesized. The first situation is usually a consequence of the addition of a chemical group to one of the precursors, which is eliminated upon passage through the membrane. This occurs with bialaphos (acetylation [22]) and kanamycin (phosphorylation [3]) among others. The genes that catalyze these activities usually confer resistance to the antibiotic when they are cloned in nonproducers and have frequently been used to isolate clusters of antibiotic biosynthesis genes (6, 30). It is possible that the translation product of *fosC*, through the introduction of a phosphoryl group into a fosfomycin precursor, plays a detoxification role during the biosynthesis of fosfomycin by *P. syringae*. In such a case fosfomycin would not be the true substrate of FosC. This would help provide an understanding of the low level of resistance to fosfomycin conferred by *fosC* to *E. coli*, which is not, apparently, due to low-level gene expression in this heterologous host, since FosC is readily observed in minicell studies and since extracts of *E. coli*(pUO063) inactivate fosfomycin at least as efficiently as those of *P. syringae* do (data not shown).

Adjacent to *fosC* and probably transcribed in tandem with it is a second ORF whose deduced protein sequence presents partial overall homology to some glutathione *S*-transferases.

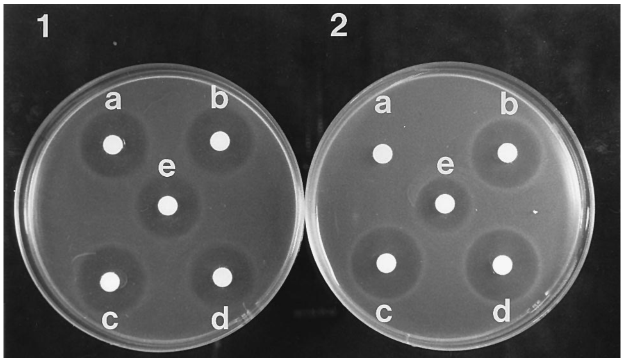

FIG. 5. Bioautography of the reaction products obtained after incubation of cell extracts with fosfomycin and different nucleotide triphosphates. Plate 1, *E. coli* JM83; plate 2, *E. coli* JM83(pUO063). Extracts were incubated with ATP (disk a), CTP (disk b), GTP (disk c), or UTP (disk d) or were incubated with ATP and subsequently with alkaline phosphatase (disk e).

ORF2	Q	V	Y	I	L	-	-	D	G	Y	W	M
Mddh	Q	V	P	I	L	V	-	D	G	E	F	T
α-GSTs	Q	V	P	M	V	E	I	D	G	M	K	L
FosA	S	T	Y	F	L	D	P	D	G	H	K	L
FosB	S	I	Y	F	T	D	I	D	G	H	K	L

FIG. 7. Comparisons between the inferred glutathione-binding site of α-glutathione *S*-transferases (α-GSTs) (11) and similar regions of prokaryotic glutathione *S*-transferases and ORF2. Mddh, *Methylobacterium* sp. dichloromethane dehydrohalogenase (20); FosA, Fo^r protein from *Serratia marcescens* (32); FosB, Fo^r protein from *S. epidermidis* (35).

Furthermore, a D-G dipeptide considered to be the glutathione binding motif (11) was present in it, and it is also present in the Fo^r proteins that use glutathione as a cosubstrate (Fig. 7). In spite of this, the role of this ORF remains unsolved, since extracts of *P. syringae* or *E. coli* clones containing ORF2 did not support typical glutathione *S*-transferase reactions.

In conclusion, a new mechanism of fosfomycin inactivation has been described. This mechanism could possibly help in the localization of the biosynthetic pathway of fosfomycin in *P. syringae.*

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