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NOTE TO THE EDITOR

Identification of a novel fosfomycin resistance gene (*fosA2*) in *Enterobacter cloacae* from the Salmon River, Canada

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Abstract

Aims: To investigate the occurrence of fosfomycin-resistant (fos^R) bacteria in aquatic environments.

Methods and Results: A fos^R strain of *Enterobacter cloacae* was isolated from a water sample collected at a site (50°41′33·44″N, 119°19′49·50″W) near the mouth of the Salmon River at Salmon Arm, in south-central British Columbia, Canada. The strain was identified by PCR screening for plasmid-borne, *fosA*-family amplicons, followed by selective plating. Sequencing of the resistance gene cloned using PCR primers to conserved flanking DNA revealed a new allele (95% amino acid identity to *fosA*), and I-*Ceu* I PFGE showed that it was chromosomally located. In *Escherichia coli*, the cloned DNA conferred a greater resistance to fosfomycin than its *fosA* counterpart.

Conclusions: Gene *fosA2* conferred fosfomycin resistance in an environmental isolate of *Ent. cloacae*.

Significance and Impact of the Study: The repurposing of older antibiotics should be considered in the light of existing reservoirs of resistance genes in the environment.

Fosfomycin [(2*R*, 3*S*)-3-methyloxiran-2-yl] phosphonic acid is a broad spectrum antibiotic used extensively in Europe and Africa for treatment of urinary tract infections (Falagas and Kopterides 2007). Fosfomycin resistance (fos^R) was first reported in 1980 in a clinical strain of *Serratia marcescens* wherein a plasmid-borne transposon, Tn2921, carried the *fosA* gene (Navas *et al.* 1990). FosA is a glutathione-*S*-transferase, with two other variants known in *Pseudomonas aeruginosa* (Rife *et al.* 2002; Yatsuyanagi *et al.* 2005). Given a renewed interest in the use of fosfomycin for treating infections caused by multidrug-resistant pathogens, e.g. lower urinary tract infections caused by ESBL-producing *Escherichia coli* (Falagas *et al.* 2010), and the emerging recognition of environmental microbiota as potential reservoirs that facilitate the traffic and persistence of resistance genes, we investigated the occurrence of fos^R bacteria in aquatic environments. We report here the identification and characterization of *fosA2*, a novel fos^R gene, in *Enterobacter cloacae*.

Water samples (500 ml) from the Salmon River in south-central British Columbia, Canada, were filtered through sterile, 0·22-μm membranes (GVHP 04700; Millipore Corp., Billerica, MA) to retain bacteria. Cells were resuspended in 5 ml physiological saline, and 1·5 ml was mixed with 1·5 ml 2× Luria–Bertani (LB) broth and incubated at 37°C overnight. Two millilitres of the mixed culture was then archived (25% glycerol final) at −80°C, while total DNA was extracted from the remainder (GIAamp DNA mini kit; Qiagen Inc., Mississauga, ON) for PCR with primer pair fosAF1/fosAR1 (designed in this study from *Ser. marcescens fosA*, Fig. 1). This generated a 346-bp product (Fig. 1) similar to *fosA* when sequenced (MacroGen, Inc., Seoul, Korea). To recover the source organism, an aliquot of the stored culture was plated on MacConkey agar containing 200 μg ml^{-1} fosfomycin (Sigma). A fos^R strain, Sam066F1, was isolated and identified by 16S rRNA gene sequence analysis, as described by Xu *et al.* (2007), as *Ent. cloacae*, a clinically significant pathogen associ-

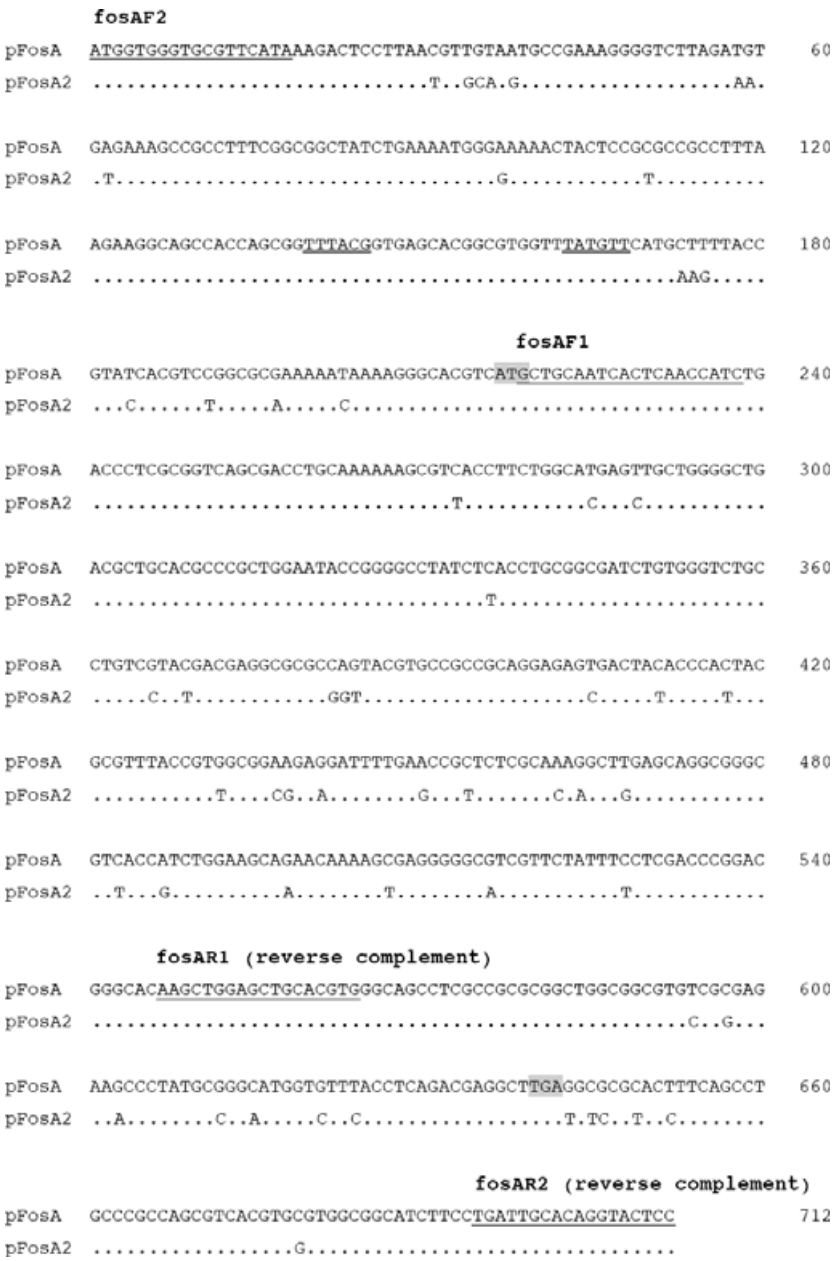

Figure 1 Nucleotide sequences of 712-bp inserts of pFosA (top) and pFosA2 (bottom). Dots indicate identical positions. *Escherichia coli* promoter-like sequences are double underlined, and the putative start and stop codons are highlighted. Sequences of PCR primers are underlined and named as in the text.

ated with urinary tract and respiratory tract infections. Susceptibility to other antibiotics was determined by the disc diffusion method on Mueller–Hinton agar using antimicrobial discs (BD Diagnostic Systems, Sparks, MD, USA): amikacin (30 μg); ampicillin (10 μg); ciprofloxacin (5 μg); chloramphenicol (30 μg); erythromycin (15 μg); gentamicin (30 μg); imipenem (10 μg); kanamycin (30 μg); rifampin (5 μg); streptomycin (10 μg); sulfisoxazole (300 μg); tetracycline (30 μg); and trimethoprim (5 μg). The results showed that Sam066F1 was also resistant to ampicillin, erythromycin and rifampicin.

Primer pair fosAF2/fosAR2 (designed in this study from Tn*2921* sequences flanking *fosA*, Fig. 1) was used to generate a 712-bp product from Sam066F1 by PCR. This amplicon was ligated into pGEM-T and introduced into *E. coli* JM109 (Promega, Madison, WI, USA) with selection on LB agar containing ampicillin (100 μg ml^{-1}) and fosfomycin (200 μg ml^{-1}). The sequence of the insert in the resulting recombinant plasmid was 91% identical to Tn*2921* (Fig. 1). It included an open reading frame of 426 bp, encoding a 141-residue polypeptide with 95% identity to FosA from *Ser. marcescens* owing to six substitutions G56Q, A74E, F80L, H82Q, K83R and V91I. The

deduced protein is more similar to FosA than other fos^R proteins (Fig. S1). We therefore designated the recombinant plasmid, pFosA2, the new gene, *fosA2*, and its product, FosA2.

To assess the fos^R activity conferred by the cloned fragment, the corresponding 712-bp *fosA* region from Tn*2921* (ColE1::Tn*2921*/*E. coli* AB1157, gift of D. Mazel, Institute Pasteur) was similarly amplified with primer pair fosAF2/fosAR2, cloned into pGEM-T in the same orientation and expressed in *E. coli* JM109. Etest (AB Biodisk, Solna, Sweden) assays indicated that cells with pFosA2 had a greater resistance to fosfomycin (MICs of >1024 μg ml^{-1}) than those with pFosA (192 μg ml^{-1}) or with an empty pGEM-T vector only (0·064 μg ml^{-1}). As *fosA2* appears to be associated with a high level of resistance, allele-specific primers that can distinguish it from *fosA* should be employed in future PCR-based strain characterizations.

FosA-associated fos^R in *Ent. cloacae* has been reported to be plasmid-encoded (Alvarez *et al.* 1987). Gene *fosA2* was not transferable by transformation of plasmid preparations from Sam066F1 or by conjugation, suggesting a chromosomal location. This was supported by southern hybridizations of total DNA restricted by I-*Ceu* I endonuclease or S1 nuclease (Sanchez-Cespedes *et al.* 2009) and resolved by pulsed-field gel electrophoresis: a *fosA2* probe colocalized to one of the eight expected I-*Ceu* I fragments that hybridized with a 23S rRNA gene probe (i.e. fragments derived from chromosomal DNA) while both probes colocalized to a compression band corresponding to chromosomal DNA after S1 treatment (Fig. S2). While it is unclear whether *fosA* (plasmid) or *fosA2* (chromosomal) is ancestral, as *fosA2* is still flanked by Tn*2961* sequences, the results of the present study show that this resistance gene is mobile in *Enterobacter* and that allelic variation in fos^R level occurs.

The identification of a novel allele conferring enhanced fos^R in a multidrug-resistant pathogen species in a rural river in British Columbia shows clearly that resistance determinants are pervasive and that any interest in reviving use of old antibiotics would be well served by a thorough surveillance of potential resistance genes and their carriers in the environment, and a greater appreciation of the role of environmental reservoirs in the epidemiology of antibiotic resistance.

The sequence of the 712-bp region including *fosA2* is available as GenBank accession EU487198.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 Phylogenetic tree of fosfomycin resistance proteins.

Figure S2 Location of *fosA2* gene identified by pulsed field gel electrophoresis.

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