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ORIGINAL RESEARCH PAPER

Identification of a novel fosfomycin-resistant UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) from a soil metagenome

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Abstract A soil metagenomic library was constructed and screened for clones that conferred fosfomycin resistance. A novel protein with 46 % identity to UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) from *Desulfuromonas acetoxidans* DSM 684 (GenBank accession number: ZP_01311756) was identified. Multiple sequence alignment revealed that the novel protein was a natural MurA, in which an aspartic acid instead of a cysteine was located in the active site. An Asp120Cys mutant of *Escherichia coli* was constructed from the subclone through site-specific mutagenesis, and minimum inhibitory concentration of fosfomycin for the resistant subclone and its mutant were determined. These results showed that fosfomycin resistance was a result of the aspartic acid in the active site. Analysis of all existing MurA sequences revealed that MurAs with an active site aspartic acid that can confer fosfomycin resistance occur in ~14 % of bacteria.

Keywords Fosfomycin resistance · Metagenomics · Soil · UDP-*N*-acetylglucosamine enolpyruvyl transferase

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Introduction

Fosfomycin is a broad-spectrum antibiotic that exhibits its bactericidal activity against many Gram-positive and Gram-negative bacteria through inhibition of UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA). MurA catalyzes the first step in bacterial peptidoglycan synthesis (Kahan et al. 1974). Fosfomycin has principally been used to treat uncomplicated urinary tract infections. In recent years, fosfomycin has been identified as a potentially effective agent for treating multidrug-resistant pathogens such as *Pseudomonas aeruginosa* (Falagas et al. 2009).

Multiple mechanisms have been described for fosfomycin resistance. One such mechanism involves reduced uptake of fosfomycin owing to a defect in one of its transporters (GlpT or UhpT) caused by mutations in the structural genes (Kahan et al. 1974). Amino acid substitution or over-expression of the target enzyme MurA, as well as plasmid-encoded fosfomycin-modifying enzymes such as FosA and FosB, have also been shown to confer resistance (Arca et al. 1990; O'Hara 1993; Horii et al. 1999; Takahata et al. 2010). Moreover, some bacteria expressing MurA with an active site aspartic acid (instead of cysteine) were intrinsically resistant to fosfomycin, including *Mycobacterium tuberculosis*, *Chlamydia trachomatis*, and *Borrelia burgdorferi* (De Smet et al. 1999; McCoy et al. 2003; Jiang et al. 2011).

The soil microbial community contains a high diversity of bacteria, >99 % of which are considered unculturable using standard methods (Daniel 2005). This community may serve as a reservoir for antibiotic resistance genes that could be transferred to pathogenic bacteria (Allen et al. 2010). Functional metagenomics, which involves directly cloning microbial DNA into a host organism followed by function-based screening, allows us to identify novel genes based on the expressed activity (Uchiyama and Miyazaki 2009). In recent years, various new antibiotic resistance genes have been identified through functional screening of metagenomes from different soil samples (Allen et al. 2009; Donato et al. 2010; Lang et al. 2010; Torres-Cortes et al. 2011).

In this study, we identified a novel MurA with aspartic acid in the active site through functional screening of a soil metagenomic library, and the distribution of MurA proteins containing active site aspartic acids in bacteria was also evaluated.

Materials and methods

Sample collection and DNA extraction

Soil samples (5–10 cm) were collected from several locations below trees, transported to the laboratory at 4 °C and then stored at −20 °C until use. Total DNA was extracted from one soil sample as described previously, with some modifications (Zhou et al. 1996). Soil (5 g) was mixed with 13.5 ml DNA extraction buffer (100 mM Tris/HCl [pH 8.0], 100 mM sodium EDTA [pH 8.0], 100 mM sodium phosphate [pH 8.0], 1.5 M NaCl, and 1 % cetyltrimethylammonium bromide) and 100 µl 10 mg proteinase K/ml in a 50 ml sterile plastic centrifuge tube, followed by 30 min incubation at 37 °C with shaking (225 rpm). Next, 1.5 ml 20 % SDS was added to each sample and the mixture was incubated at 65 °C for 2 h with gentle end-over-end inversions every 20 min. Following centrifugation at 6,000×*g* for 10 min at room temperature, the supernatants were collected and transferred into a new 50 ml centrifuge tube and mixed with an equal volume of chloroform/isoamyl alcohol (24:1 v/v). The aqueous phase was recovered by centrifugation at 16,000×*g* for 10 min and precipitated with 0.6 vol 2-isopropanol at room temperature for 1 h. The pellet was washed with cold 70 % ethanol and dissolved in 200 µl sterile deionized water. Extracted DNA was examined by 1 % (w/v) agarose gel electrophoresis.

Metagenomic library construction

Soil metagenomic DNA was fractionated in a preparative pulsed-field gel for 16 h. DNA in the size range of 36–48 kb was excised and then electroeluted and dialyzed against 0.5× TE buffer for 24 h. The purified DNA was end-repaired and ligated into the pCC2FOS fosmid vector, packaged into phage, and introduced into *Escherichia coli* EPI300 using the Copy Control Fosmid Library Production Kit (Epicentre Biotechnologies, Madison, WI, USA).

Screening for fosfomycin-resistant clones and subcloning

All clones from the soil library were plated on Luria–Bertani (LB) agar plates containing 12.5 µg chloramphenicol/ml and 128 µg fosfomycin/ml. Plates were incubated at 37 °C for 24 h, and a positive clone was isolated. For subcloning, the fosmid DNA from the resistant clone was extracted (EZNA Plasmid Mini Kit I, Omega Bio-Tek, Doraville, GA, USA) and partially digested with *Sau*3AI (Takara Bio, Dalian, China). DNA fragments of 1–5 kb were recovered from an agarose gel (EZNA Gel Extraction Kit, Omega Bio-Tek, Doraville, GA, USA) and ligated into *Bam*HI-digested and alkaline phosphatase treated pHSG298 (Takara Bio, Dalian, China). The ligation products were transformed into *E. coli* DH5α (Invitrogen) and

spread on LB-agar plates containing 50 μg kanamycin/ml and 128 μg fosfomycin/ml. One fosfomycin-resistant subclone was selected following 24 h of incubation at 37 °C.

Sequence analysis

The positive subclone was sequenced from both directions using the M13 primers. Sequences were assembled using SeqMan software (DNAStar). Putative open reading frames (ORFs) were identified with ORF Finder and BLAST (http://www.ncbi.nlm.nih.gov). Multiple sequence alignments were carried out using Clustal X, version 2.0 (Larkin et al. 2007), in combination with Genedoc (http://www.psc.edu/biomed/genedoc).

Site-directed mutagenesis

The MutanBEST kit (Takara Bio, Dalian, China) was used for in vitro site-directed mutagenesis according to the manufacturer's instructions. The plasmid containing the fosfomycin-resistant subclone, designated pFMS1, was used as template. Point mutations were introduced into the plasmid using the following primers: 5′-GTTTCCTGGCGGTTGTGCCATTGGAGCCC-3′ and 5′-CCTATTGATACCTTGCCAAACCGA-3′ (where the underlined sequences correspond to the Asp to Cys substitution). The amplified products were then end-repaired, 5′-phosphorylated, and self-ligated prior to being transformed into *E. coli* DH5α. The recombinant plasmid was designated pFMS1 M. The introduced mutations were verified by sequencing, as above.

Determination of minimum inhibitory concentration (MIC)

The MICs of fosfomycin for the resistant subclone and its mutant strain were determined using the agar dilution method on Mueller–Hinton agar containing 25 μg glucose 6-phosphate/ml, according to Clinical and Laboratory Standards Institute (Clinical and Laboratory Standards Institute (CLSI) 2010) guidelines. *E. coli* DH5α carrying vector pHSG298 was used as a negative control and *E. coli* ATCC 25922 was used as the quality control strain.

Nucleotide accession number

Sequence data obtained from this work were deposited in GenBank with the accession number JN629036.

Results and discussion

Cloning a fosfomycin resistance gene from a soil metagenome

To clone a fosfomycin resistance gene, a soil metagenomic fosmid library was constructed. This library, named IMCAS-F012, contains ~200,000 clones with an average insertion size of ~36 kb. Following screening on fosfomycin-containing LB agar plates, one resistant fosmid clone was obtained. To determine the gene responsible for the resistance, the fosmid DNA was partially digested using *Sau*3AI and ligated into pHSG298, resulting in the subclone library. One fosfomycin-resistant subclone was selected and confirmed by sequencing.

Sequence analysis of the gene conferring fosfomycin resistance

The fosfomycin-resistant subclone harbored a DNA fragment of 1,544 bp, in which one ORF of 1,245 bp (from ATG to TAA) encoding 414 amino acids was identified. Compared with other characterized MurA protein sequences, the putative protein sequence was ~30 aa shorter at the *N*-terminus. An alternative start codon, GTG, was then located upstream of the original ATG codon. We therefore concluded that the complete ORF was 1,335 bp in length, encoding a total of 444 aa. BLAST analysis indicated that the deduced protein had the highest homology to the MurA protein from *Desulfuromonas acetoxidans* DSM 684 (GenBank accession number: ZP_01311756) (46 % identity). Therefore, the cloned fragment likely contained a putative MurA protein, which was designated UoMurA (MurA from uncultured organism).

Mechanism of resistance conferred by the UoMurA protein

The newly identified UoMurA protein was compared with homologous sequences from *M. tuberculosis*,

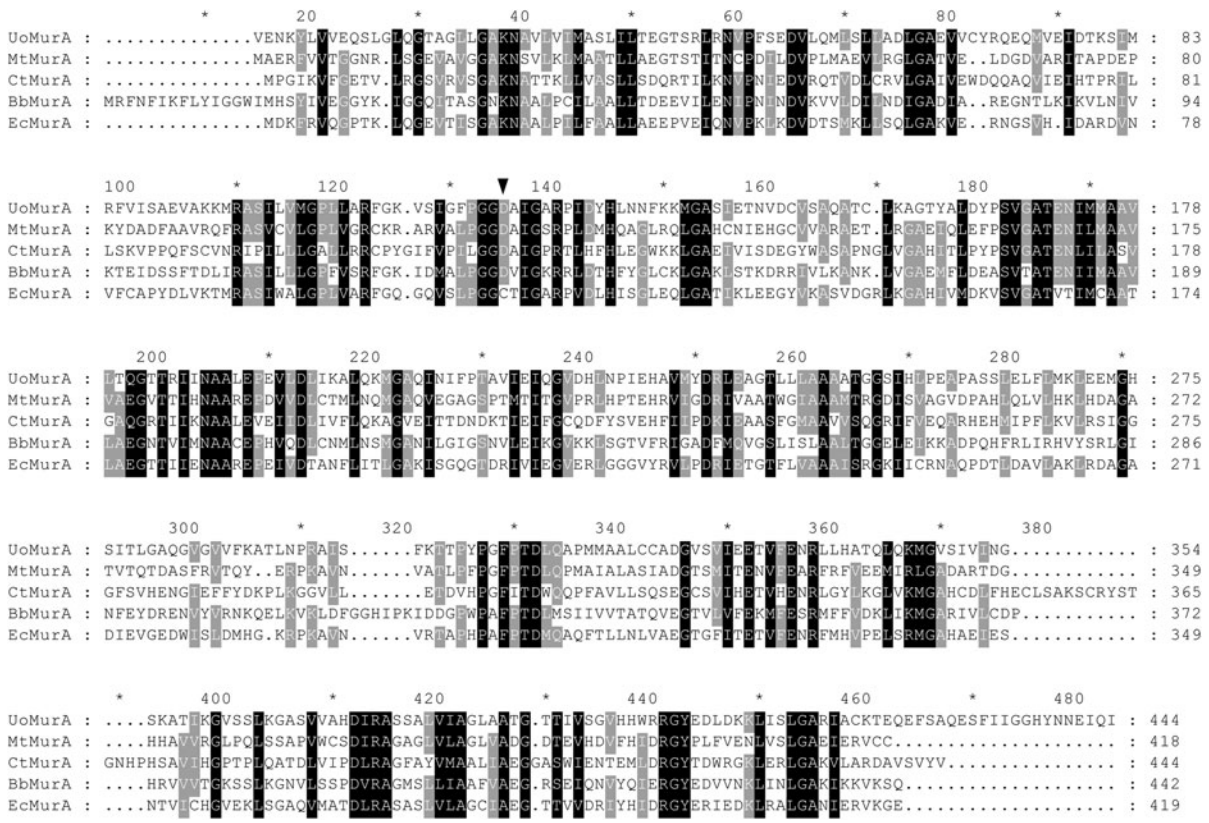

Fig. 1 Multiple sequence alignment of the deduced amino acid sequence of MurA identified from an uncultured organism in this study (UoMurA, GenBank accession number: AEY79929) with homologous proteins from *M. tuberculosis* (MtMurA, GenBank accession number: CAA65472), *C. trachomatis* (Ct MurA, GenBank accession number: AAN28945), *B. burgdorferi* (BbMurA, GenBank accession number: AAC66824), and *E. coli* (EcMurA, GenBank accession number: AAC76221). Residues identical to the consensus are *shaded*, and the active site Asp/Cys residue is indicated by an *arrowhead*

C. trachomatis, *B. burgdorferi*, and *E. coli*. This showed that an aspartic acid residue was present in the active site of UoMurA, in place of a cysteine residue (Fig. 1). In *E. coli*, fosfomycin inactivates MurA by covalently binding to Cys115 in the catalytic site of the enzyme (Skarzynski et al. 1996). Later works have shown that a Cys115Asp substitution in MurA rendered *E. coli* resistant to fosfomycin (Kim et al. 1996). Some organisms, such as *M. tuberculosis*, *C. trachomatis*, and *B. burgdorferi*, are intrinsically resistant to fosfomycin because of the active site aspartic acid in MurA (De Smet et al. 1999; Jiang et al. 2011; McCoy et al. 2003). The UoMurA protein identified in the present study contained the aspartic acid substitution in MurA, thus explaining its resistance to fosfomycin.

To confirm that the fosfomycin resistance conferred by the UoMurA was caused by the Cys to Asp substitution in the active site, a site-directed Asp120Cys mutant of UoMurA was constructed. The MICs of the subclone and the mutant strains were individually determined. These results showed that the MIC of the mutant strain decreased dramatically (>128-fold) compared with the primary subclone strain (Table 1). However, the MIC of the mutant strain increased fourfold compared with the vector control, which was likely caused by the elevated expression of the fosfomycin target protein.

Table 1 Minimum inhibitory concentration (MIC) of fosfomycin to wild type and mutant strains

Strain/plasmid	MIC (μg/ml)
E. coli DH5α/pFMS1	>512
E. coli DH5α/pFMS1M	4
E. coli DH5α/pHSG298	1

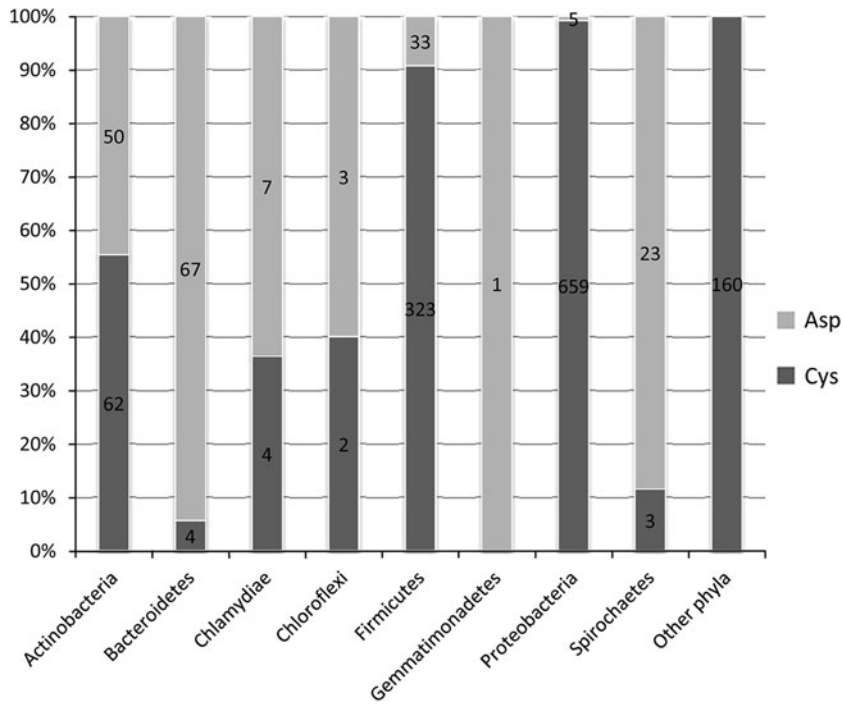

Fig. 2 Distribution of MurAs with an active site aspartic acid in different bacterial phyla. The numbers of bacterial species with cysteine and aspartic acid in the active site are shown

Distribution of aspartic acid-containing MurAs in bacteria

To investigate the distribution of MurA proteins with active site aspartic acids in bacteria, the sequences of all bacterial MurAs from UniProtKB (http://www.uniprot.org/help/uniprotkb) (October, 2011) were retrieved and compared. Of the 1,406 bacterial species analyzed, 189 species belonging to eight different bacterial phyla contained MurA with an active site aspartic acid (Fig. 2).

In the phylum Actinobacteria, the MurA protein of nearly half of the examined bacterial species had aspartic acid in the active site, with most of the species belonging to the genera *Corynebacterium*, *Mycobacterium*, and *Rhodococcus*. Additionally, ~94 % of bacterial species from the phylum Bacteroidetes had MurA proteins with aspartic acid at the active site, which was consistent with previous findings that showed all *Bacteroides* species displayed fosfomycin resistance (Garcia et al. 1977). In the phylum Chlamydiae, all species from the genera *Chlamydia* and *Chlamydophila* harbored MurAs with an active site aspartic acid. Except for the genus *Brachyspira*, all other bacterial species from the phylum Spirochaetes had aspartic acid in the active site. About 9 % of bacterial species from the phylum Firmicutes had the aspartic acid substitution, with most of these species distributed in the genus *Lactobacillus*. Interestingly, only five species in the Proteobacteria phylum had aspartic acid at the active site, with 654 (>99 %) species of this phylum harboring cysteine.

Conclusion

To our knowledge, this is the first report of a fosfomycin resistance determinant being obtained from soil using a functional metagenomic method. The UoMurA identified here represents a new member of UDP-*N*-acetylglucosamine enolpyruvyl transferases, as it contains aspartic acid in its active site and displays only 46 % identity to known MurA proteins. Sequence analysis showed that MurA proteins with an active site aspartic acid, which is likely to confer fosfomycin resistance, are distributed widely in various bacterial phyla. These results are important when considering the clinical usage of fosfomycin.

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