

Identification of a novel UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) from *Vibrio fischeri* that confers high fosfomycin resistance in *Escherichia coli*

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Abstract

MurA (UDP-*N*-acetylglucosamine enolpyruvyl transferase) is a key enzyme involved in bacterial cell wall peptidoglycan synthesis and a target for the antimicrobial agent fosfomycin, a structural analog of the MurA substrate phosphoenol pyruvate. In this study, we identified, cloned and sequenced a novel *murA* gene from an environmental isolate of *Vibrio fischeri* that is naturally resistant to fosfomycin. The fosfomycin resistance gene was isolated from a genomic DNA library of *V. fischeri*. An antimicrobial agent hypersensitive strain of *Escherichia coli* harbouring *murA* from *V. fischeri* exhibited a high fosfomycin resistance phenotype, with an MIC of 3000 µg/ml. The cloned *murA* gene was 1269 bp long encoding a 422 amino acid polypeptide with an estimated pI of 5.0. The deduced amino acid sequence of the putative protein was identified as UDP-*N*-acetylglucosamine enolpyruvyl transferase by homology comparison. The MurA protein with an estimated molecular weight of 44.7 kDa was expressed in *E. coli* and purified by affinity chromatography. MurA of *V. fischeri* will be a useful target to identify potential inhibitors of fosfomycin resistance in pharmacological studies.

Key words MurA . Fosfomycin . Antibiotic resistance . *Vibrio fischeri* . MIC

Introduction

The cell wall of bacteria is composed of alternating units of N-acetyl glucosamine and N-acetyl muramic acid interconnected by penta-peptide cross links. The peptidoglycan cell wall determines the cell shape and provides the essential rigidity to withstand the high internal osmotic pressure. The first committed step in the synthesis of bacterial cell wall takes place in the cytoplasm, which involves the addition of enolpyruvate from phosphoenolpyruvate (PEP) to the 3'-hydroxyl group of UDP-N-acetylglucosamine (UDP-NAG) with the release of inorganic phosphate (Bugg and Walsh 1992). This reaction is catalyzed by the enzyme UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) (EC 2.5.1.7). MurA is an essential enzyme in *E. coli*, as its inactivation is lethal to the organism due to the loss of cell integrity and susceptibility to osmotic lysis (Brown et al., 1995). This enzyme is a target for the antibiotic fosfomycin which inactivates the enzyme by irreversibly binding to the enzyme forming a covalent adduct with the cysteine 115 residue of *E. coli* MurA (Kahan et al. 1974; Eschenburg et al. 2005). Fosfomycin is a broad spectrum bactericidal antibiotic that is effective against a range of Gram-positive and Gram-negative bacteria. The antibiotic is used to treat uncomplicated urinary tract infections (UTIs) caused by *E. coli* and *Enterococcus faecalis* world wide (Falagas et al. 2008). Some pathogenic bacteria such as *Mycobacterium tuberculosis* and *Chlamydia trachomatis* have been reported be intrinsically resistant to fosfomycin and this ability has been attributed to the cystein-to-aspartate replacement in the active site of MurA enzyme (DeSmet et al. 1999; McCoy et al. 2003).

Bacteria develop resistance to antimicrobial agents through various mechanisms such as the enzymatic modification or degradation of antibiotics, modifications of antibiotic targets or active efflux of antibiotics (Walsh 2000). The resistance to antimicrobial drugs may be intrinsic or develop due to prolonged exposure to sub lethal concentrations of drugs over a period of time. In the majority of bacteria, fosfomycin is transported into cells via the L- α -glycerophosphate (α GP) system (Kahan et al.1974). Fosfomycin resistant mutants can appear spontaneously during the course of antibiotic treatment primarily due to mutations in chromosomal *glpT*, causing failure to transport fosfomycin (Tsuroka and Yamada 1975). The plasmid encoded glutathione S- transferase enzymes FosA and FosB are implicated in fosfomycin resistance in many species of bacteria (Arca et al. 1990; O'Hara 1999). The *fosA* gene is reported to be widely distributed

among members of the Enterobacteriaceae and *Pseudomonas* spp. (Teran et al. 1988). The presence of *fosB* in Gram-positive bacteria such as *Staphylococcus aureus*, *Bacillus subtilis* and the *fosX* gene from *Mesorhizobium loti* and *Listeria monocytogenes* have been studied in detail with respect to their role in fosfomycin resistance (Cao et al. 2001; Etienne et al. 1991; Fillgrove et al. 2003).

The objective of our study was to identify and characterize the gene responsible for natural resistance of a *V. fischeri* strain to fosfomycin. In this report, we describe the identification, cloning and sequencing of a novel *murA* gene from *V. fischeri* that confers elevated resistance to the cell wall inhibiting antibiotic fosfomycin.

Materials and methods

Bacterial strains and plasmids

The bacterial strains and the plasmids used in this study are listed in the Table 1. *V. fischeri* VF107 was grown in a medium consisting of 1% peptone, 0.5% yeast extract and 2% sodium chloride at 30°C. Host *E. coli* strains KAM32 and SG13009 were grown in Luria Bertani (LB) broth at 37°C. LB broth was supplemented with ampicillin (100 µg/ml), kanamycin (25 µg/ml) and 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside) when required. Plasmid extractions were performed using a commercial kit (Qiagen, USA). Electrotransformation was followed for introducing plasmids into *E. coli* host strains.

Cloning and sequencing of *V. fischeri murA*

Genomic DNA of *V. fischeri* VF107 was extracted following the method of Ausubel et al. (1995). About 10 µg of the pure genomic DNA was digested with the restriction endonuclease enzyme *Pst*I, and the fragments ranging in size from 2-10 kb were purified from an agarose gel after electrophoresis. The plasmid vector pSP72 (Promega, USA) was similarly digested, treated with calf intestinal alkaline phosphatase (Fermentas, USA), ligated with the genomic DNA fragments using a LigaFast DNA ligation kit (Promega, USA) and transformed into *E. coli* KAM32 (Chen et al. 2002) The transformants were screened on LB agar plates containing 100 µg/ml each of ampicillin and fosfomycin. A 1.7 kb insert was sequenced by dideoxy chain termination procedure on both strands using vector specific primers.

Determination of minimum inhibitory concentration (MIC)

The MICs of fosfomycin to *V. fischeri* VF107 and *E. coli* KAM32/pSP72/Vf-murA were determined in Mueller Hinton broth following the CLSI microbroth dilution procedure (CLSI 2006). The assay was performed in a 96-well microtitre plate with appropriate uninoculated media blanks. The test strains were grown to an O.D₆₂₅ of 0.4, washed and resuspended in fresh MH broth to yield 10³-10⁴ cells/ml. A serial 10-fold dilution of the stock antibiotic was performed in MH broth in microtitre plate wells and inoculated with the test strains. The plates were incubated at 37°C for 24 h, and the growth was examined visually. The assay was repeated thrice, and the lowest concentration of fosfomycin that inhibited growth was recorded as the MIC for the test strains.

Overexpression and purification of *murA* in *E. coli*

Primers FM-F (5'-ATGGATAGCGCCGAGATTAA-3') and FM-R (5'-TAAGTCGTCGCTATGAACT-3') were used to amplify *murA* for cloning into the expression vector pQE 30 (Qiagen USA). The amplification was done in a 50 µl reaction volume consisting a 1× PCR buffer (10 mM Tris-HCl, 50 mM KCl and 2 mM MgCl₂), 200 µM concentrations of each of the four dNTPs, 25 picomoles of respective primer, 3 U of Taq polymerase (MBI Fermentas) and 500 ng of the pure genomic DNA. The PCR product was purified using a PCR purification kit (Qiagen, USA), cloned into pQE-30 UA cloning vector (Qiagen, USA) and transformed into *E. coli* SG13009[pREP4] to yield SG/pQE30/Vf-murA.

The 6×His-tagged MurA fusion protein was purified by using nickel chromatography columns following the manufacturer's protocol (Qiagen USA). Briefly, the recombinant *E. coli* SG/pQE30/Vf-murA was grown in LB broth containing 100 µg/ml ampicillin and 25 µg/ml kanamycin to an OD₆₀₀ of 0.5, induced with 1 mM IPTG and further incubated for 8 h with shaking. The cells were harvested by centrifugation at 3000 × g for 15 min., and the pellet was lysed using a lysis buffer (6M GuHCl, 0.1M NaH₂PO₄, 0.01M Tris Cl, pH 8.0) and centrifuged at 11,000 g for 15 min. at room temperature to pellet the cellular debris. The supernatant was loaded onto Ni-NTA affinity columns, centrifuged and the column was washed twice with wash buffer (6M GuHCl, 0.1M NaH₂PO₄, 0.01M Tris Cl, pH 6.3). The bound recombinant protein was eluted with elution buffer (6M GuHCl, 0.1 M NaH₂PO₄, 0.01M Tris Cl, pH 4.5), dialyzed against

Tris buffer (10 mM Tris Cl pH 8.0, 0.1% Triton X-100) overnight at 4 °C to remove guanidine hydrochloride. The recombinant protein was electrophoresed on a 12% sodium dodecyl sulphate polyacrylamide gel and visualized by staining with Coomassie brilliant blue.

Sequence analysis

The nucleotide sequence derived was subjected to a GenBank homology search using a standard nucleotide-nucleotide BLAST analysis (Altschul et al. 1997). The deduced amino acid sequence of *V. fischeri* MurA was analyzed for conserved domains at <http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi> (Marchler-Bauer et al. 2007) and was compared with other homologous sequences in GenBank using the CLUSTAL W multiple sequence alignment program (Higgins et al. 1994).

Nucleotide sequence

The nucleotide sequence derived in this study has been deposited in the GenBank with the accession number FJ172353.

Results and discussion

Vibrio fischeri is a marine bacterium that is either free living or lives in symbiotic association with some marine squids and fish (Ruby and McFall-Ngai 1999). Marine environments have diverse species of bacteria and fungi producing varieties of bioactive compounds, including antibiotics (Bernan et al. 1997). Bacteria living in such a competitive environment tend to develop natural resistance to antibiotics as a strategy to overcome the lethal effects of inhibitory compounds produced by competing bacteria in the same niche. Thus, it is likely that antibiotic resistant bacteria are encountered in the marine environment, despite the fact that bacteria are not considered clinically important and the antibiotic resistance phenotype has not evolved due to exposure to chemotherapeutic use of antibiotics. We found that *V. fischeri* VF107 was naturally resistant to the antibiotic fosfomycin that could not be attributed to any plasmid-borne gene. In order to identify the gene responsible for fosfomycin resistance, we created a genomic DNA library of *V. fischeri* in an antimicrobial agent hypersensitive strain *E. coli* KAM32 (Chen et al. 2002) and screened for fosfomycin resistant clones. This yielded *E. coli* KAM32/psp72/Vf-murA harboring a *V. fischeri* genomic fragment of 2400 bp. Sequence

analysis of the gene revealed 2 putative open reading frames (ORFs) consisting of a 258 bp toluene tolerance gene *ttg2F*, followed by a gene encoding UDP-N-acetylglucosamine 1-carboxy vinyl transferase (*murA*). At the 3'end, a partial sequence of the gene encoding 1-acyl-sn-glycerol-3-phosphate acyltransferase was identified. The organization of the *murA* gene in *V. fischeri* was found to be identical with that of *V. salmonicida*, the whole genome sequences of which are currently available. In the genomes of these two species, the *murA* gene is preceded by predicted toluene tolerance genes *ttg2A*, *ttg2B*, *ttg2C*, *ttg2D* and *ttg2F*.

The minimum inhibitory concentrations (MICs) of fosfomycin was determined for the host *E. coli* strain KAM32, *V. fischeri* from which the *murA* gene was cloned and *E. coli* KAM32/pSP72/Vf-*murA*. The results are presented in Table 2. *E. coli* KAM32 was highly sensitive to fosfomycin with an MIC of 32.5 µg/ml. *E. coli* KAM32/pSP72/Vf-*murA* had an MIC of 3000 µg/ml, which was about half of the MIC obtained with *V. fischeri* VF107 (MIC = 6000 µg/ml). The reason for this difference in MIC levels could not be determined in this study.

Sequence analysis of cloned the *murA* gene from *V. fischeri* revealed that the gene is 1269 bp encoding a polypeptide of 422 amino acids with a calculated pI of 5.0. The *murA* gene was amplified, cloned and over expressed in *E. coli* SG13009. The 6×Histidine tagged MurA protein was purified by metal affinity chromatography under denaturation conditions. The pure recombinant MurA exhibited a molecular mass of about 45 kDa (Fig. 1). This value corresponded well with the calculated mass of 44.7 kDa (Fig. 1). At this stage, our attempts to purify native recombinant MurA protein using imidazole in the elution buffer were not successful, possibly due to the affinity tag being hidden in the protein folds.

In *E. coli* fosfomycin inactivates MurA irreversibly by forming a covalent adduct with nucleophilic cysteine 115 present in the catalytic site of the enzyme (Kahan et al. 1974; Marquardt et al. 1994). Later studies have revealed that replacement of Cys-115 with aspartate renders *E. coli* MurA resistant to fosfomycin. Organisms with a Cys→Asp replacement at position 115 in their MurA proteins are naturally resistant to fosfomycin, such as in the case of *Mycobacterium tuberculosis* and *Chlamydia trachomatis* (DeSmet et al. 1999; McCoy et al. 2003). We compared the deduced amino acid sequence of *V. fischeri* MurA with the homologous sequences from *V. salmonicida*, *E. coli* and *M. tuberculosis*. Overall, *V. fischeri* MurA was 95%

identical with the corresponding sequence of *V. salmonicida*, 79% identical with the MurA of *E. coli* K12 and 43% with *M. tuberculosis* MurA (Fig. 2). The Cys-115 residue of *E. coli* MurA corresponds to Cys-116 of *E. coli* K12 and Cys-117 of *M. tuberculosis*. This residue is absolutely conserved in *V. fischeri* MurA. Based on sequence analysis, we could not determine why *V. fischeri* MurA was less susceptible to binding by fosfomycin. A number of highly conserved amino acids could be observed in the enzyme from diverse sources, the functions of which are not known. Speculatively, it is possible that a yet undiscovered sequence variant elsewhere along the peptide is responsible for fosfomycin resistance observed in this study. Further work will be necessary to test this hypothesis.

Our future work will focus on the functional characterization of the MurA protein from *V. fischeri* and determining amino acids responsible for fosfomycin resistance by site directed mutagenesis.

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Table 1 Bacterial strains and plasmids

Strain/Plasmid	Description/genotype	Source/Reference
<i>Vibrio fischeri</i> VF107	Seawater isolate	Laboratory stock
<i>E. coli</i> KAM32	$\Delta acrB$, $\Delta ydhE$, hsd	Chen et al. (2002)
<i>E. coli</i> SG 13009	[pREP4]; NaI ^S , Str ^S , Rif ^S , F ⁻ , RecA ⁺	Gottesman et al. (1981)
KAM32/pSP72/Vf-murA	<i>V. fischeri murA</i> ; Amp ^R	This study
SG/pQE30/Vf-murA	<i>V. fischeri murA</i> ; Amp ^R Kan ^R	This study

Table 2 Minimum inhibitory concentrations (MICs) of fosfomycin to wild type and recombinant bacterial strains determined by micro broth dilution technique

Strain/plasmid	MIC $\mu\text{g/ml}$ of fosfomycin
<i>E. coli</i> KAM32	32.5
<i>V. fischeri</i> VF107	6000
<i>E. coli</i> KAM 32/pSP72 /vf-murA	3000

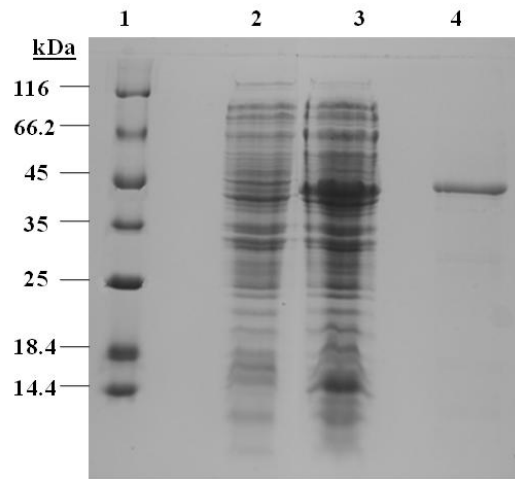


Fig. 1 SDS-PAGE analysis of recombinant MurA protein purified by affinity chromatography. *Lane 1*, Protein molecular weight marker (Fermentas); *lane 2*, *E. coli* SG/pQE30/Vf-murA without IPTG induction; *lane 3*, Recombinant *E. coli* SG/pQE30/Vf-mur induced with 1 mM IPTG; *lane 4*, Pure recombinant MurA

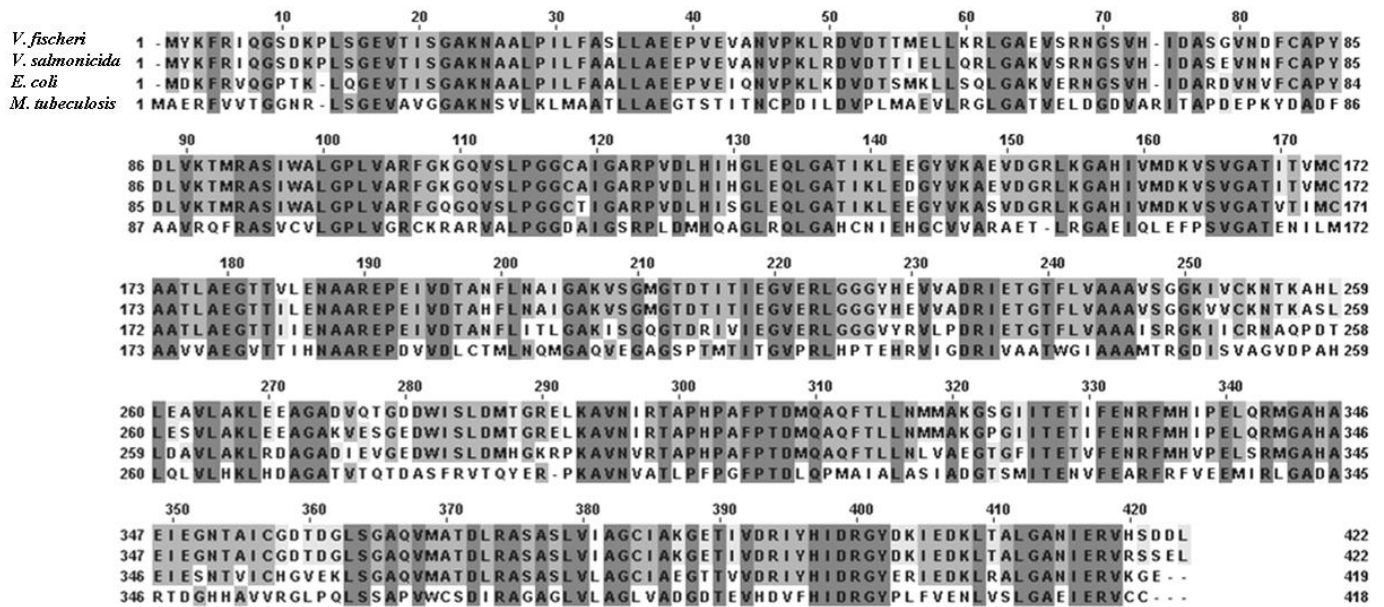


Fig. 2 Multiple sequence alignment of the deduced amino acid sequence of *V. fischeri* Mura with homologous proteins from *V. salmonicida* (GenBank accession number CAQ78196), *E. coli* (GenBank accession number NP_417656), *Mycobacterium tuberculosis* (GenBank accession number CAA65472)