

High-Level Fosfomycin Resistance in Vancomycin-Resistant *Enterococcus faecium*

Technical Appendix

Supplementary Methods

Cloning and Purification of MurA

Wild-type and C119D UDP-N-acetylglucosamine enolpyruvyl transferase (*murA*) genes were synthesized (Genscript, Piscataway, NJ, USA), cloned into the pE-SUMOstar prokaryotic expression vector (LifeSensors, Malvern, PA, USA), and transformed into *Escherichia coli* BL21 (DE3) pLysS competent cells (Promega, Madison, WI, USA). Transformed *E. coli* BL21 (DE3) were grown overnight at 37°C in Power Prime Broth (AthenaES, Baltimore, MD, USA) containing 100 mg/L of ampicillin. Overnight cultures were diluted 1:50 in fresh Power Prime Broth, grown to midlog phase (optical density = 0.3 at 600 nm), before protein expression was induced for 4 h at 37°C by addition of 1 mmol/L isopropyl β-D-1-thiogalactopyranoside. Cells were harvested by centrifugation, suspended in 50 mmol/L sodium phosphate buffer (pH 7.8) containing protease inhibitor (lysis buffer), and lysed by using a French press.

Cell supernatants were mixed with prepared TALON Metal Affinity Resin (Clontech Laboratories, Inc., Mountain View, CA, USA), loaded onto a gravity-flow column, and washed with 50 mmol/L sodium phosphate (pH 7.8) containing 0.3 mol/L NaCl and 1 mmol/L β-mercaptoethanol. Bound protein was eluted with 100 mmol/L sodium phosphate (pH 6.0) containing 0.6 mol/L NaCl, 240 mmol/L imidazole, and 1 mmol/L β-mercaptoethanol. The protein was then exchanged into 25 mmol/L Tris-HCl (pH 7.5) by using an NAP-25 column (GE Healthcare, Chicago, IL, USA). Protein concentration was determined by using a Bradford assay with bovine serum albumin as the standard. Purity was assessed by using sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Purified proteins were stored in 25% glycerol at –80°C.

MurA Steady-State Kinetic Assays

MurA (100 nmol/L) was incubated with various concentrations of UDP-N-acetylglucosamine (UNAG) (0–3 mmol/L) or phosphoenolpyruvate (PEP) (0–1 mmol/L) in 50 mmol/L Tris-HCl buffer (pH 7.5) at 37°C for 10 min. Reactions were initiated with 300 µmol/L PEP (for various concentrations of UNAG) or 3 mmol/L UNAG (for various concentrations of PEP) at 37°C for 20 min. Reactions were then quenched, and inorganic phosphate was quantified by using the Malachite Green Phosphate Assay Kit, (BioAssay Systems, Hayward, CA, USA) per the manufacturer’s recommendations. Data were fitted to Michaelis-Menten equations by using GraphPad Prism version 6 (GraphPad Software, San Diego, CA, USA).

Fosfomycin Inhibition Assay

For inhibition assays, 100 nmol/L of wild-type or mutant MurA was incubated with various concentrations of fosfomycin and 3 mmol/L UNAG at 37°C for 10 min. Reactions were initiated with 300 µmol/L PEP, and inorganic phosphate was quantified as described. The concentration of fosfomycin that resulted in 50% inhibition was determined by using GraphPad Prism version 6.

Technical Appendix Table. Characteristics of strains and plasmids used in the study of high-level fosfomycin resistance in vancomycin-resistant *Enterococcus faecium**

Strain or plasmid	Fosfomycin MIC, mg/L	Description/use
Clinical strain		
<i>Enterococcus faecium</i> 2014–7	>1,024	<i>murA</i> ^{C119D}
<i>E. faecium</i> 2014–195	>1,024	<i>murA</i> ^{C119D}
<i>E. faecium</i> 2015–149	>1,024	<i>murA</i> ^{C119D}
<i>E. faecium</i> 2016–78	>1,024	<i>murA</i> ^{C119D}
<i>E. faecium</i> 2016–194	64	<i>murA</i> ^{WT}
Transformant		
<i>E. faecium</i> D344S (pTCV-lac ^{C119D})	>1,024	<i>murA</i> ^{C119D}
<i>E. faecium</i> D344S (pTCV-lac ^{WT})	512	<i>murA</i> ^{WT}
<i>E. faecium</i> D344S (pTCV-lac)	128	Control
Host strain		
<i>E. faecium</i> D344S	NA	NA
<i>Escherichia coli</i> SM10	NA	Plasmid transfer
<i>E. coli</i> TOP10	NA	Plasmid transfer
<i>E. coli</i> BL21 (DE3)	NA	Protein expression
Plasmid		
pTCV-lac	NA	NA
pE-SUMOstar	NA	Cloning of <i>murA</i> ; erythromycin and kanamycin resistant Expression of <i>murA</i> ; ampicillin resistant

*murA, UDP-N-acetylglucosamine enolpyruvyl transferase; NA, not applicable; WT, wild type.

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MurA_2014-7      MEEIIVRGGNQLNGTVRIEGAKNAVLPILAASLLAEEGITTLDNVPILSDVFTMNQVIRH 60
MurA_2014-195   MEEIIVRGGNQLNGTVRIEGAKNAVLPILAASLLAEEGITTLDNVPILSDVFTMNQVIRH
MurA_2015-149   MEEIIVRGGNQLNGTVRIEGAKNAVLPILAASLLAEEGITTLDNVPILSDVFTMNQVIRH
MurA_2016-78    MEEIIVRGGNQLNGTVRIEGAKNAVLPILAASLLAEEGITTLDNVPILSDVFTMNQVIRH
MurA_2016-194   MEEIIVRGGNQLNGTVRIEGAKNAVLPILAASLLAEEGITTLDNVPILSDVFTMNQVIRH
MurA_consensus  MEEIIVRGGNQLNGTVRIEGAKNAVLPILAASLLAEEGITTLDNVPILSDVFTMNQVIRH
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MurA_2014-7      LNVVDVDFDEQKNQVTIDASRQLEIEAPYEYVSQMRASIVVMGPLLARNGHAKVAMPGGDA 120
MurA_2014-195   LNVVDVDFDEQKNQVTIDASRQLEIEAPYEYVSQMRASIVVMGPLLARNGHAKVAMPGGDA
MurA_2015-149   LNVVDVDFDEQKNQVTIDASRQLEIEAPYEYVSQMRASIVVMGPLLARNGHAKVAMPGGDA
MurA_2016-78    LNVVDVDFDEQKNQVTIDASRQLEIEAPYEYVSQMRASIVVMGPLLARNGHAKVAMPGGDA
MurA_2016-194   LNVVDVDFDEQKNQVTIDASRQLEIEAPYEYVSQMRASIVVMGPLLARNGHAKVAMPGGDA
MurA_consensus  LNVVDVDFDEQKNQVTIDASRQLEIEAPYEYVSQMRASIVVMGPLLARNGHAKVAMPGGDA
*****

MurA_2014-7      IGKRPIDLHLKGFQALGAKIIQKNGYIEAIADELIGNTIYLDFFPSVGATQNIMMAAVKAK 180
MurA_2014-195   IGKRPIDLHLKGFQALGAKIIQKNGYIEAIADELIGNTIYLDFFPSVGATQNIMMAAVKAK
MurA_2015-149   IGKRPIDLHLKGFQALGAKIIQKNGYIEAIADELIGNTIYLDFFPSVGATQNIMMAAVKAK
MurA_2016-78    IGKRPIDLHLKGFQALGAKIIQKNGYIEAIADELIGNTIYLDFFPSVGATQNIMMAAVKAK
MurA_2016-194   IGKRPIDLHLKGFQALGAKIIQKNGYIEAIADELIGNTIYLDFFPSVGATQNIMMAAVKAK
MurA_consensus  IGKRPIDLHLKGFQALGAKIIQKNGYIEAIADELIGNTIYLDFFPSVGATQNIMMAAVKAK
*****

MurA_2014-7      GTTIIENVAREPEIVDLANILNKMGAVQYVAGTETMRIEGVDHLHAVNHSIVQDRIEAGT 240
MurA_2014-195   GTTIIENVAREPEIVDLANILNKMGAVQYVAGTETMRIEGVDHLHAVNHSIVQDRIEAGT
MurA_2015-149   GTTIIENVAREPEIVDLANILNKMGAVQYVAGTETMRIEGVDHLHAVNHSIVQDRIEAGT
MurA_2016-78    GTTIIENVAREPEIVDLANILNKMGAVQYVAGTETMRIEGVDHLHAVNHSIVQDRIEAGT
MurA_2016-194   GTTIIENVAREPEIVDLANILNKMGAVQYVAGTETMRIEGVDHLHAVNHSIVQDRIEAGT
MurA_consensus  GTTIIENVAREPEIVDLANILNKMGAVQYVAGTETMRIEGVDHLHAVNHSIVQDRIEAGT
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MurA_2014-7      FMVAAAMTQGNVLIADAISEHNRPLISKLIEMGAEIEEEGGVVRVIGPKHILPTDVKTMP 300
MurA_2014-195   FMVAAAMTQGNVLIADAISEHNRPLISKLIEMGAEIEEEGGVVRVIGPKHILPTDVKTMP
MurA_2015-149   FMVAAAMTQGNVLIADAISEHNRPLISKLIEMGAEIEEEGGVVRVIGPKHILPTDVKTMP
MurA_2016-78    FMVAAAMTQGNVLIADAISEHNRPLISKLIEMGAEIEEEGGVVRVIGPKHILPTDVKTMP
MurA_2016-194   FMVAAAMTQGNVLIADAISEHNRPLISKLIEMGAEIEEEGGVVRVIGPKHILPTDVKTMP
MurA_consensus  FMVAAAMTQGNVLIADAISEHNRPLISKLIEMGAEIEEEGGVVRVIGPKHILPTDVKTMP
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MurA_2014-7      HPGFPTDMQAQMTAIIQLVAEGTSVVTETVFENRFQHLEEMRRMNAHVKIDGNVAIMDGNH 360
MurA_2014-195   HPGFPTDMQAQMTAIIQLVAEGTSVVTETVFENRFQHLEEMRRMNAHVKIDGNVAIMDGNH
MurA_2015-149   HPGFPTDMQAQMTAIIQLVAEGTSVVTETVFENRFQHLEEMRRMNAHVKIDGNVAIMDGNH
MurA_2016-78    HPGFPTDMQAQMTAIIQLVAEGTSVVTETVFENRFQHLEEMRRMNAHVKIDGNVAIMDGNH
MurA_2016-194   HPGFPTDMQAQMTAIIQLVAEGTSVVTETVFENRFQHLEEMRRMNAHVKIDGNVAIMDGNH
MurA_consensus  HPGFPTDMQAQMTAIIQLVAEGTSVVTETVFENRFQHLEEMRRMNAHVKIDGNVAIMDGNH
*****

MurA_2014-7      ELQGAEVYATDLRAAAALVLAGLKANGITRVRNLNLYLDRGYNFHIKLQQLGADVERVDM 420
MurA_2014-195   ELQGAEVYATDLRAAAALVLAGLKANGITRVRNLNLYLDRGYNFHIKLQQLGADVERVDM
MurA_2015-149   ELQGAEVYATDLRAAAALVLAGLKANGITRVRNLNLYLDRGYNFHIKLQQLGADVERVDM
MurA_2016-78    ELQGAEVYATDLRAAAALVLAGLKANGITRVRNLNLYLDRGYNFHIKLQQLGADVERVDM
MurA_2016-194   ELQGAEVYATDLRAAAALVLAGLKANGITRVRNLNLYLDRGYNFHIKLQQLGADVERVDM
MurA_consensus  ELQGAEVYATDLRAAAALVLAGLKANGITRVRNLNLYLDRGYNFHIKLQQLGADVERVDM
*****

MurA_2014-7      DQTSAEKTAQTIA 433
MurA_2014-195   DQTSAEKTAQTIA
MurA_2015-149   DQTSAEKTAQTIA
MurA_2016-78    DQTSAEKTAQTIA
MurA_2016-194   DQTSAEKTAQTIA
MurA_consensus  DQTSAEKTAQTIA
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Technical Appendix Figure. Alignment of the deduced amino acid sequences of wild type and C119D UDP-N-acetylglucosamine enolpyruvyl transferase. (MurA) of vancomycin-resistant *E. faecium* isolates. Strains 2014–7, 2014–195, 2015–149, and 2016–78 have fosfomycin MICs >1,024 mg/L. Strain 2016–194 has a fosfomycin MIC of 64 mg/L. The consensus sequence is derived from WP_002289003.1 and represents the MurA sequence of 450 *E. faecium* strains available in GenBank. Box indicates change from cysteine to aspartic acid at position 119. Asterisks indicate identity at each position.