Supplemental Material for:

Use of an inter-species chimeric receptor for inducible gene expression reveals that metabolic flux through the peptidoglycan biosynthesis pathway is an important driver of cephalosporin resistance in *Enterococcus faecalis*

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Running title: Peptidoglycan synthesis drives enterococcal cephalosporin resistance

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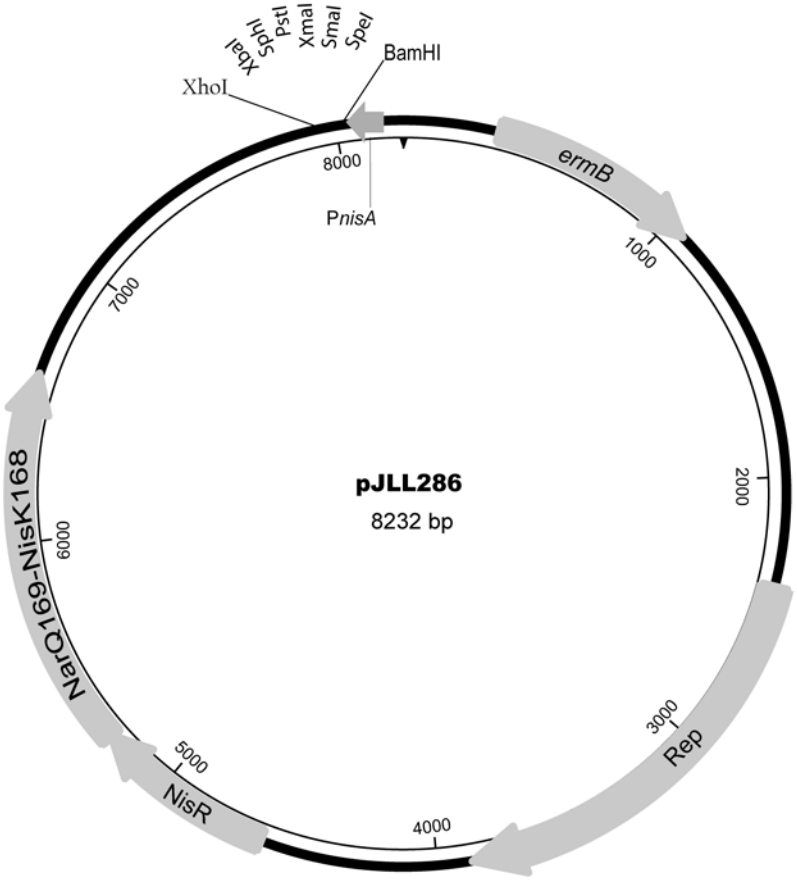
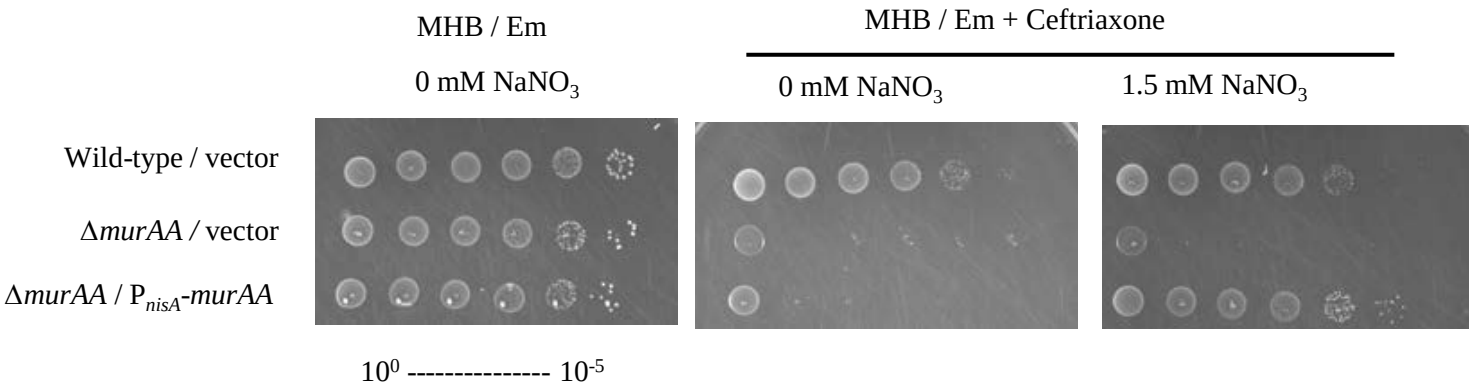
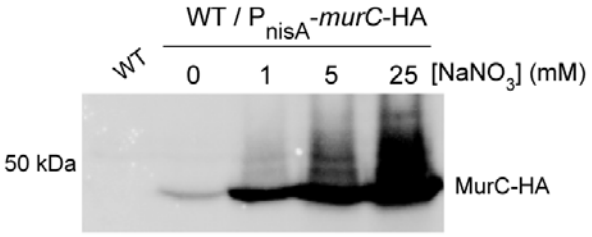


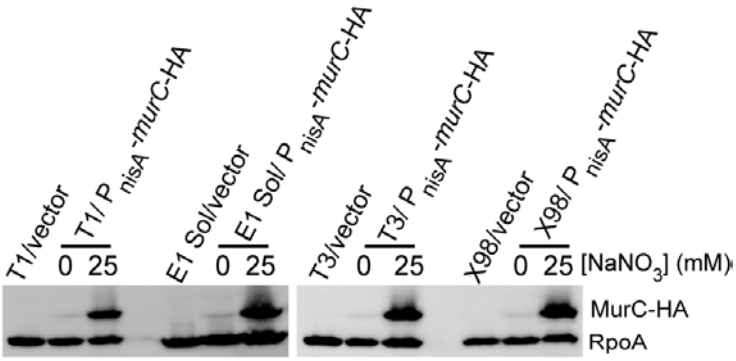
Supplemental figure 1. Map of the nitrate-inducible expression plasmid pJLL286.



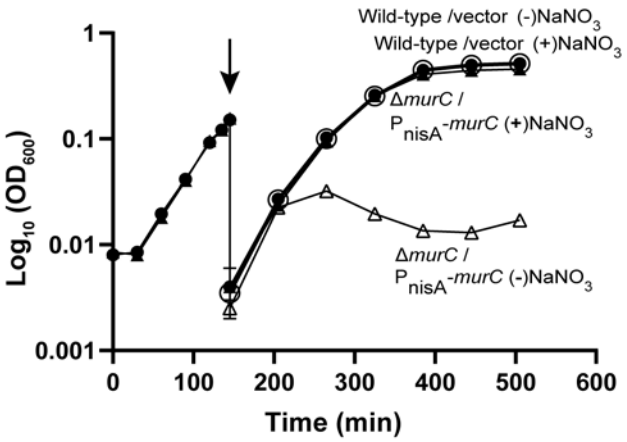
Supplemental figure 2. Nitrate-inducible expression of MurAA rescues the ceftriaxone resistant defect of the Δ*murAA* mutant on agar plates. Cultures were subjected to 10-fold serial dilutions and spotted on MHB supplemented as indicated and incubated at 37 ℃ for 24 h. Results are representative of a minimum of 2 independent biological replicates. Strains were Wild-type/vector, OG1/pJLL286; Δ*murAA*/vector, JL626/pJLL286; and Δ*murAA*/P_{nisA}-*murAA*, JL626/pJLL288.



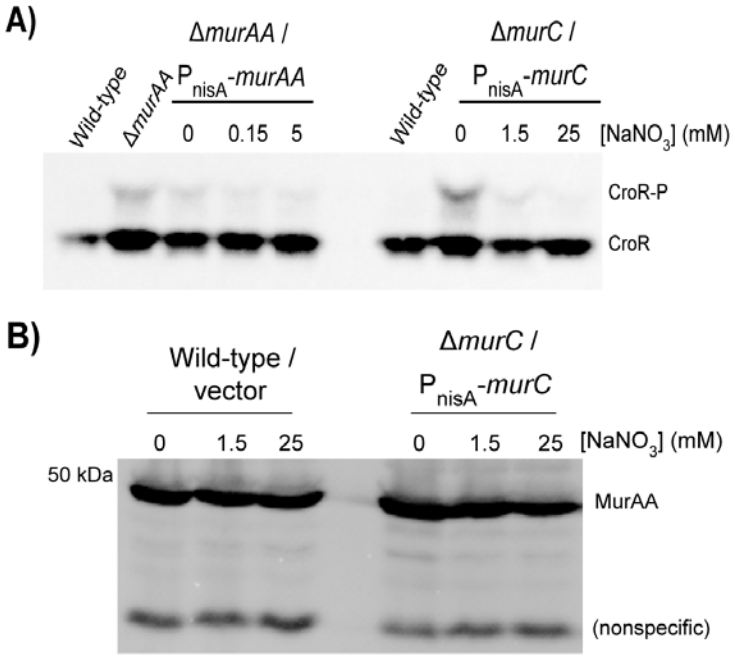
Supplemental figure 3. NarQ169-NisK168 mediates nitrate-responsive expression in *E. faecalis*. Immunoblot analysis with anti-HA antibody for MurC-HA in total protein lysates from exponentially growing *E. faecalis* cells with varying $NaNO_3$ concentrations as indicated. Results are representative of a minimum of 2 independent biological replicates. Strains were WT, OG1/pJLL286; WT/P_{nisA}-*murC-HA*, OG1/pJLL294.



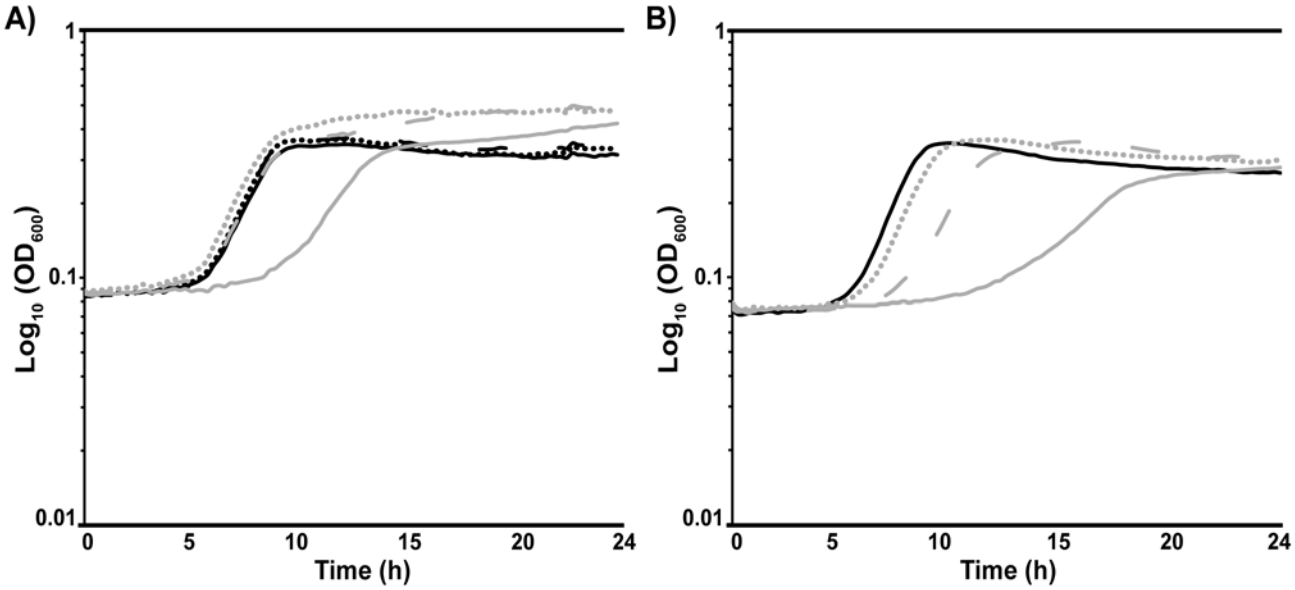
Supplemental figure 4. NarQ169-NisK168 mediates nitrate-responsive expression in multiple evolutionarily diverse lineages of *E. faecalis*. Immunoblot analysis with anti-HA antibody for MurC-HA in total protein lysates from exponentially growing *E. faecalis* cells with varying $NaNO_3$ concentrations as indicated. RpoA was used as a loading control. Results are representative of a minimum of 2 independent biological replicates. Strains were *E. faecalis* T1, E1 Sol, T3, or X98 carrying either pJLL286 (vector) or pJLL294 (P_{nisA}-*murC-HA)*.



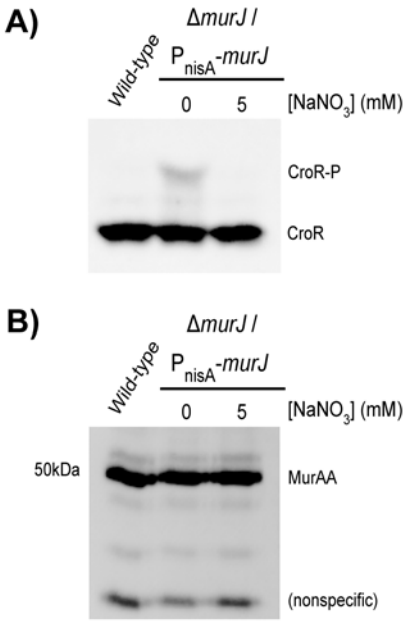
Supplemental figure 5. MurC is essential for growth. Cultures were grown to exponential phase in the presence of 5 mM $NaNO_3$, then washed (arrow) and suspended in media with or without 5 mM $NaNO_3$. Strains were, Wild-type/vector (circles), OG1 /pJLL286; Δ*murC* / P_{nisA}-*murC* (triangles), JL657/pJLL297. Open symbols, no $NaNO_3$, closed symbols, 5 mM $NaNO_3$. Data represent two biological replicates for each strain. Error bars are present but too small to be seen.



Supplemental figure 6. Immunoblot analysis of the MurC depletion strain. (A) Phos-tag SDS-PAGE and immunoblot analysis of total protein lysates from exponentially growing *E. faecalis* cells in the presence of varying $NaNO_3$ concentrations as indicated reveals activation of CroS/R signaling upon depletion of MurC, reflected by the presence of phosphorylated CroR (CroR-P) that migrates more slowly than unphosphorylated CroR during Phos-tag SDS-PAGE. Activation of CroS/R signaling is also observed upon deletion of MurAA, indicating that impairment of PG synthesis at either step triggers CroS/R activation. **(B)** Immunoblot analysis for MurAA reveals that MurAA is present upon depletion of MurC. Results are representative of a minimum of 2 independent biological replicates. Strains were Wild-type (OG1), Δ*murAA* (JL626), or Δ*murC* (JL657) carrying empty vector (pJLL286), pJLL288 (P_{nisA}-*murAA*), or pJLL297 (P_{nisA}-*murC*) as indicated.



Supplemental figure 7. Growth of the MurC (A) and MurJ (B) depletion strains at inducer concentrations used for MIC analysis. Strains were (A) wild-type/vector, OG1/pJLL286 (black lines); Δ*murC*/P_{nisA}-*murC*, JL657/pJLL297 (gray lines) at 2 (solid lines), 5 (dashed lines), and 12 (dotted lines) mM $NaNO_3$; and (B) wild-type/vector, OG1/pJLL286 (black line); Δ*murJ*/P_{nisA}-*murJ*, JL656/pJLL296 (gray lines) at 0 (solid lines), 0.1 (dashed lines), and 5 (dotted lines) mM $NaNO_3$.



Supplemental figure 8. Immunoblot analysis of the MurJ depletion strain. (A) Phos-tag SDS-PAGE and immunoblot analysis of total protein lysates from exponentially growing *E. faecalis* cells in the presence or absence of 5mM $NaNO_3$ reveals activation of CroS/R signaling upon depletion of MurJ, reflected by the presence of phosphorylated CroR (CroR-P) that migrates more slowly than unphosphorylated CroR during Phos-tag SDS-PAGE. **(B)** Immunoblot analysis for MurAA reveals that MurAA is present upon depletion of MurJ. Results are representative of a minimum of 2 independent biological replicates. Strains were Wild-type/vector, OG1/pJLL286; and Δ*murJ/* P_{nisA}*-murJ,* JL656/pJLL296 as indicated.