Heteroresistance to fosfomycin in *S. pneumoniae* - Supplementary Material

Methods

Knock-out mutants. For the construction of murA1 knock-out mutants, *murA1* upstream and downstream flanking regions were amplified and fused to the bicistronic cassette Janus. Fast Start Taq DNA polymerase (Roche Molecular Biochemicals, Rotkreuz, Switzerland) was used for amplification if not stated otherwise. For primers and PCR conditions see Table S1. The resulting fragment was introduced into the recipient strains 106.44, Taiwan^{23F}, Hungary^{19A} and D39 as described before (34) using Columbia sheep blood agar (CSBA) plates containing 500µg/ml kanamycin to select transformants (if not stated otherwise all chemicals from Sigma, USA). Insertion was confirmed by PCR.

Hungary::*murA1*$_{D39}$ and D39::*murA1*$_{D39}$ back-transformant. For the construction of mutants D39::*murA1*$_{D39}$ back-transformant and Hungary::*murA1*$_{D39}$ the *murA1* of D39 and its up- and downstream flanking regions were amplified and fused to a spectinomycin cassette obtained from pR412 plasmid. The obtained construct was used to transform D39*murA1*::Janus strains in order to obtain the D39::*murA1*$_{D39}$ back-transformant and Hungary*murA1*::Janus strain to produce strain Hungary::*murA1*$_{D39}$. Transformants were selected on CSBA plates containing 200µg/ml spectinomycin under anaerobic conditions.

Cloning *murA1*$_{D39}$-spectinomycin construct for mutagenesis. For the insertion of *murA1-spectinomycin* construct into pGEX-6p-1 plasmid the murA1-spectinomycin construct was amplified from D39::*murA1*$_{D39}$ back-transformant and ligated into pGEM-T Easy vector (pGEM-T Easy Vector System, Promega, Switzerland) according to the manufacturer's protocol. Inserts of plasmids obtained from minipreps (Wizard Plus SV Minipreps DNA purification System, Promega, USA) were

sequenced as described earlier (36). Deletion of guanine at position 1249 of the insert was detected.

The plasmid was digested with ScaI restriction enzyme (all restriction enzymes from New England Biolabs, USA), then NotI-HF. The *murA1*-spectinomycin-construct was isolated from a 1% agarose gel. In parallel pGEX-6p-1 plasmid (GE Healthcare, Sweden) was linearized with NotI-HF, then dephosphorylated with Shrimp Alkaline Phosphatase (SAP). The *murA1*-spectinomycin construct was inserted using T4 DNA ligase (New England Biolabs, USA) overnight at 16°C. Transformation of competent *E. coli* SURE cells with 10 µl of the ligation reaction, was performed at 42°C for 2 min. Transformants were selected on Luria-Bertani-Agar (LBA) plates containing 100 µg/ml ampicillin and 200 µg/ml spectinomycin. Plasmid pGEX::*murA1*$_{D39}$-spect-1 was isolated and insert checked by sequencing as described before (36).

Insert G_{1090}A into *murA1*$_{D39}$-spectinomycin construct for D39::*murA1*$_{Hungary19A}$ and Hungary::*murA1*$_{Hungary19A}$ back-transformant. For the construction of D39::*murA1*$_{Hungary19A}$ and Hungary::*murA1*$_{Hungary19A}$ back-transformant mutation G_{1090}A was introduced into pGEX::murA1$_{D39}$-spect-1 by QuikChange II Site-Directed Mutagenesis Kit (Stratagene, USA), using *E. coli* SURE cells for cloning thus obtaining pGEX::*murA1*$_{Hungary19A}$-spect. Deletion G_{1249} was cured by a second mutagenesis reaction using Phusion High Fidelity Polymerase (Fisher Scientific, Switzerland). The product obtained was purified and template plasmid was digested with DpnI restriction enzyme overnight at 37°C, then ligated at 4°C for 3 days with T4 DNA ligase. The construct was amplified with Phusion High-Fidelity polymerase. The amplified construct containing mutation G_{1090}A, characteristic of *murA1*$_{Hungary19A}$ and re-inserted G_{1249} was isolated from 1% agarose gel and used to transform D39*murA1*::Janus to get the *murA1* switch-mutant and Hungary*murA1*::Janus to

obtain the Hungary::*murA1*$_{Hungary19A}$ back-transformants as described before. The presence of inserted constructs was confirmed by sequencing.

Hungary::*murA1+murB*$_{D39}$ and D39::*murA1+murB*$_{Hungary19A}$ mutants. For the construction of Hungary::*murA1+murB*$_{D39}$ and D39::*murA1+murB*$_{Hungary19A}$ mutants in order to switch *murB* sequences between strains D39 and Hungary^{19A} the *murB*$_{D39}$ gene was amplified. It was then fused to the kanamycin resistance gene amplified from the Janus cassette, as was the downstream flanking region of *murB*$_{Hungary19A}$. Amplification was performed using iProof polymerase (Bio-Rad, Switzerland), fragments were fused as described elsewhere (35) with Phusion polymerase. The *murB*$_{D39}$-kanamycin construct was then transformed into Hungary::*murA*$_{D39}$ as described above and transformants selected on CSBA plates containing 200 µg/ml kanamycin. A second construct was put together with *murB*$_{Hungary19A}$. MurB$_{Hungary19A}$ lacks the last 15 amino acids (Ser$_{302}$ – Arg$_{316}$) compared to MurB$_{D39}$. The *murB*$_{Hungary19A}$-kanamycin construct was transformed into D39::*murA1*$_{Hungary19A}$. Replacement of genes was confirmed by sequencing.

Recombinant protein expression. The *murA1* sequences of strains D39 and Hungary^{19A} were inserted into pGEX-6p-1 plasmid at its XhoI and BamHI sites. SAP was used for dephosphorylation. Ligation was performed with T4 DNA ligase at 4°C for 3 days. *E. coli* SURE cells were transformed and transformants selected on LBA with 100 µg/ml ampicillin. Plasmids were isolated with Promega Wizard and used to transform competent *E. coli* BL21 cells for expression. The *murA1* sequence was confirmed in isolated plasmids by sequencing.

Single clones of *E. coli* BL21::pGEX-*murA1*$_{D39}$ and *E. coli* BL21::pGEX-*murA1*$_{Hungary19A}$ were grown in LB broth with 100 µg/ml ampicillin to OD_{600} 0.5 at 37°C at 150 rpm then protein expression was induced with 0.1 mM of Isopropyl β-D-1-thiogalactopyranoside (IPTG) overnight at 20°C and 150 rpm. Cells were pelleted (10

min, 4000 g) and lysed on ice under native conditions with 25 ml of lysis buffer (50mM NaH_2PO_4, 300mM NaCl, 10mM Imidazol, 1 mg/ml lysozyme, pH 8.0) (Roche, Switzerland) for 30 min. Proteases were inhibited by addition of 1 µg/ml aprotinine, 1 µg/ml leupeptine, 1 µg/ml pepstatine and 1 mM phenylmethanesulfonfluoride (PMSF). Protein purification was performed on Glutathione Sepharose 4 Fast Flow (GE Healthcare, Sweden) as suggested by the manufacturer using PreScission Protease (GE Healtcare, Sweden) to cleave recombinant proteins from the GST-Tag. Recombinant MurA1 was separated from the beads with Qiagen Polypropylene columns. The buffer was exchanged to protein stocking buffer (10mM Tris-HCl, pH 7.5) on Amicon Ultra-4 filter device (Millipore, Ireland). Protein samples were analyzed by SDS-PAGE and were >95 % of the expected protein. Identity of recombinant proteins was confirmed by MS peptide fingerprinting.

Table S1 –– Primers used in construction of mutants & probes

Primer	Sequence 5'-->3'	Purpose	PCR conditions: Annealing temperature / Extension time
***murA1*-Janus construct**			
KoMurA1_f2	5'-AAACTCTAAAGTCCAAAAAGGTAGTCGTAA-3'	Amplification upstream *murA1*	57 °C / 1 min 30 s
DAM406_rev+b3	5'-TCTGGAATAGGCATAGATCTAAGTTTTCAATACTCTTT-3'	Amplification upstream *murA1*, overlap Janus cassette	57 °C / 1 min 30 s
DAM351_rev+f4	5'-ATAAGGAAAGGGGCCCTAGACCGTAGAGGTGTTTATGA-3'	Amplification downstream *murA1*, overlap Janus cassette	60 °C / 1 min 30 s
KoMurA1_b5	5'-CCAGCCAGAACCTCAGAAGAAGTC-3'	Amplification downstream *murA1*	60 °C / 1 min 30 s
b3_rev+DAM406	5'-AAAGAGTATTGAAAACTTAGATCTATGCCTATTCCAGA-3'	Amplification Janus cassette, overlap upstream *murA1*	61 °C / 1 min 30 s
f4_rev+DAM351	TCATAAACACCTCTACGGTCTAGGGCCCCTTTCCTTAT	Amplification of Janus cassette, overlap downstream *murA1*	61 °C / 1 min 30 s
DAM351	5'-CTAGGGCCCCTTTCCTTATGCTTTTGGAC-3'	Fusion *murA1* upstream - Janus cassette	55.2 °C / 3 min
KoMurA1_f2A	5'-GATGTACGACTTATCCCGTGAGGT-3'	Fusion *murA1* downstream - *murA1* upstream + Janus cassette	55.7 °C / 3 min
KoMurA1_b5A	5'-CGAAATGCTACGAACACTGAC-3'	Fusion *murA1* downstream - *murA1* upstream + Janus cassette	55.7 °C / 3 min
rpsL_mitte_b1	5'-GAATTTACGAAGGGCTGAGTTAGGTTT-3'	Control insertion Janus cassette, with KoMurA1_f2A	55 °C / 2 min
Kan_start_f1	5'-AGGAAATAATAAATGGCTAAAATGAGAAT-3'	Control insertion Janus cassette, with KoMurA1_b5B	50 °C / 2 min
***murA1*-spectinomycin construct**			
MurA1_B8rev+spect_f1	5'-AGACTTGTTGAGGATTAAACTAGTGGATCCCCCGTT-3'	Amplification SPT cassette, overlap upstream *murA1*	58 °C / 1 min 30 s
spect_b1+KoMurA1_f4rev	5'-CATAAACACCTCTACGGTATAGTTCCCTTCAAGAGC-3'	Amplification SPT cassette, overlap *murA1* downstream flanking region	58 °C / 1 min 30 s
spect_b1rev+KoMurA1+KoMurA1_f4	5'-GCTCTTGAAGGGAACTATACCGTAGAGGTGTTTATG-3'	Amplification *murA1* downstream, overlap SPT casette	58 °C / 1 min 30 s
KoMurA1_b6	5'-ATCATGAACCTGAAATCCCCCTGTA-3'	Amplification *murA1* downstream, overlap SPT casette	58 °C / 1 min 30 s
KoMurA1_f1	5'-AGTATATCTGATGTCAAAGTTCTCGGCG-3'	Amplification *murA1* and upstream, overlap SPT cassette	59 °C / 2 min 10 s
MurA1_B8+spect_f1rev	5'-AACGGGGGATCCACTAGTTTAATCCTCAACAAGTCT-3'	Amplification *murA1* and upstream, overlap SPT cassette	59 °C / 2 min 10 s
KoMurA1_b5B	5'-CCGCATTTTTCTTGTGTAACGA-3'	Fusion *murA1* SPT construct, with KoMurA1_f2A	52.3 °C / 3 min
KoMurA1_f2B	5'-GGTATGTATAAAACGCTTCTGTGA-3'	Aplification whole *murA1* SPT construct, with KoMurA1_b5B	52 °C / 3 min
pGEX_sequencing_F	5'-GGGCTGGCAAGCCACGTTTGGTG-3'	Amplification *murA1*Hungary19A+G1249, seq. pGEX::*murA1*D39-spect-1	68 °C / 1 min
pGEX_sequencing_R	5'-CCGGGAGCTGCATGTGTCAGAGG-3'	Amplification *murA1*Hungary19A+G1249, seq. pGEX::*murA1*D39-spect-1	68 °C / 1 min
Mutagenesis primers			
SDMspC_G1090A_f	5'-GGTGGACGTGATTTACGTGGGACCAGTGTTAAAGCGACC-3'	Introduce G1090A in pGEX::*murA1*D39-spect-1	68 °C / 8 min 30 s
SDMspC_G1090A_r	5'-GGTCGCTTTAACACTGGTCCCACGTAAATCACGTCCACC-3'	Introduce G1090A in pGEX::*murA1*D39-spect-1	68 °C / 8 min 30 s
SDMexp_G1249_f	5'P-GGAGCGGATATTAGACTTGTTGAGGATTAAACTAGTGGATC-3'	Cure deletion G1249; Primer 5' phosphorylated	65° C / 4 min
SDMexp_G1249_r	5'P-GATCCACTAGTTTAATCCTCAACAAGTCTAATATCCGCTCC-3'	Cure deletion G1249; Primer 5' phosphorylated	65° C / 4 min
***murB*-kanamycin construct**			
KoMurB_f1	5'-CGTCAATACTTGTTTGCTAAGTTCAATGTTG-3'	Amplify *murB*D39 or *murB*Hungary19A+ upstream, sequencing	55° C / 40 s
murB_D39_PCR1_r	5'-GCCATTTATTATTTCCTTCCTCTCTACCTCTTGCAGGGAGTAAAACC-3'	Amplify *murB*D39 + upstream, overlap KAN cassette	55° C / 40 s
murB_D39_PCR2_f	5'-GGTTTTACTCCCTGCAAGAGGTAGAGAGGAAGGAAATAATAAATGGC-3'	Amplify KAN cassette, overlap *murB*D39	55 °C / 20 s
murB_H_PCR2_r	5'-GCAGCAGCTTTTCAAATACAGCTTCCTAAAACAATTCATCCAG-3'	Amplify KAN cassette, overlap *murB*D39	55 °C / 20 s
murB_H_PCR3_f	5'-CTGGATGAATTGTTTTAGGAAGCTGTATTTGAAAAGCTGCTGC-3'	Amplify downstream *murB*Hungary19A, overlap KAN cassette	55 °C / 20 s
Ko_murB_b6	5'-GGTTCATTTGGCTTCATCCCACC-3'	Amplify downstream *murB*Hungary19A, overlap KAN cassette	55 °C / 20 s
murB_H_PCR1_r	5'-GCCATTTATTATTTCCTTCCTCTCTACTTGCTTTCACCCAAGATCCGGAC-3'	Amplify *murB*Hungary19+ upstream, overlap KAN cassette	55° C / 40 s
murB_H_PCR2_f	5'-GTCCGGATCTTGGGTGAAAGCAAGTAGAGAGGAAGGAAATAATAAATGGC-3'	Amplify KAN cassette, overlap *murB*Hungary19A	55 °C / 20 s
murB_D_PCR2_r	5'-GGGCTCTGTCAGGTCCCCACTAAAACAATTCATCCAGTA-3'	Amplify KAN cassette, overlap *murB*Hungary19A	55 °C / 20 s
murB_D_PCR3_f	5'-CTGGATGAATTGTTTTAGTGGGGACCTGACAGAGCCC-3'	Amplify downstream *murB*D39, overlap KAN cassette	55 °C / 20 s
Ko_murB_us_F	5'-GGACATGTCATGGATTTTGTCATCAAG-3'	Amplify whole *murB* KAN construct for sequencing	55 °C / 1 min
Ko_murB_ds_R	5'-CCATTCATTTCCAAGTTCGTCATAGG-3'	Amplify whole *murB* KAN construct for sequencing	55 °C / 1 min
Recombinant proteins			
pGEX_murA1_For3	5'-GAGGATCCATGAGAAAAATTGTTATCAATGGTGGA-3'	Amplification *murA1* incl. restriction site BamHI	51 °C / 2 min
pGEX_murA1_Rev2	5'-AAACTCGAGTTATTAATCCTCAACAAGTCTAATATC-3	Amplification murA1 incl. restriction site XhoI	51 °C / 2 min

Primer	Sequence 5'-->3'	Purpose	PCR conditions: Annealing temperature / Extension time
Sequencing primers			
T7_FOR	5'-TAA TAC GAC TCA CTA TAG GG-3'	Seq. pGEM-T::*murA1*D39-SPT and pGEX::*murA1*D39-spect-1	-
MurA1_For	5'-ATGAGAAAAATTGTTATCAATGGTGGA-3'	Seq. pGEM-T::*murA1*D39-SPT and pGEX::*murA1*D39-SPT-1	-
SeqSpecC_1306_F	5'-AGGCCGTTTTGGTGAAGCGACAGT-3'	Seq. pGEM-T::*murA1*D39-SPT and pGEX::*murA1*D39-SPT-1	-
SpecC_1921_F	5'-GCAACAACCGCTTACCCCTCTTTT-3'	Seq. pGEM-T::*murA1*D39-SPT and pGEX::*murA1*D39-SPT-1	-
SeqSpecC_2938_F	5'-GCGGGAAATGCAGTGGCTGAATCT-3'	Seq. pGEM-T::*murA1*D39-SPT and pGEX::*murA1*D39-SPT-1	-
SeqSpecC_3292_F	5'-TTATTTTCCCAACGCAGGCAAGTC-3'	Seq. pGEM-T::*murA1*D39-SPT and pGEX::*murA1*D39-SPT-1	-
SeqSpecC_3943_R	5'-GCCCAGCCAGAACCTCAGAAGAAGT-3'	Seq. pGEM-T::*murA1*D39-SPT and pGEX::*murA1*D39-SPT-1	-
murA1_Rev	5'-TTAATCCTCAACAAGTCTAATATCCGC-3'	Seq. pGEM-T::*murA1*D39-SPT and pGEX::*murA1*D39-SPT-1	-
SP6_REV	5'-TATTTAGGTGAACTATAG-3'	Seq. pGEM-T::*murA1*D39-SPT and pGEX::*murA1*D39-SPT-1	-
MurB_for	5'-GTGACATGTCTGTAAGAGAAAAAATGCTTG-3'	Seq. *murB*-KAN construct	-
MurB_for2	5'-CTCACATCTTGCAGTCTTGTAAGGTCTTG-3'	Seq. *murB*-KAN construct	-
murB_Rev	5'-GTAAAACCACCTGCATACATCTTCGC-3'	Seq. *murB*-KAN construct	-
KoMurB_b5	5'-CACGGGGTTGGTTGATGATAGCACG-3'	Seq. *murB*-KAN construct	-
Gene expression studies			
MURA1_F	5'-CTCGTCCGATTGACTTACACCTTAA-3'	Measure gene expression *murA1*	-
MURA1_R	5'-ACTTCATGTTATCTCCCTCGTAGCT-3'	Measure gene expression *murA1*	-
MURA1_M	5'-CCATAGCTTCAAACGCC-3'	Measure gene expression *murA1* - FAM probe	-
murA2-tgt2F	5'-CCAGGTGGTTGTACGATTGGTA-3'	Measure gene expression *murA2*	-
murA2-tgt2R	5'-CCCATAGCTTCCAGACCTTTCAAA-3'	Measure gene expression *murA2*	-
murA2-tgt2M1	5'-CCGTCCTATTGATCTTCA-3'	Measure gene expression *murA2* - FAM probe	-
TaqMan 16S_For	5'-GACGATACATAGCCGACCTGAGA-3'	Measure gene expression *16S*	-
TaqMan 16S_Rev	5'-GTAGGAGTCTGGGCCGTGTCT-3'	Measure gene expression *16S*	-
16S-tgt1M2probe	5'-CCAGTGTGGCCGATC-3'	Measure gene expression 16S - FAM probe	-

Abbreviations: SPT (spectinomycin); KAN (kanamycin); incl. (including); seq. (sequencing)

Figure S1

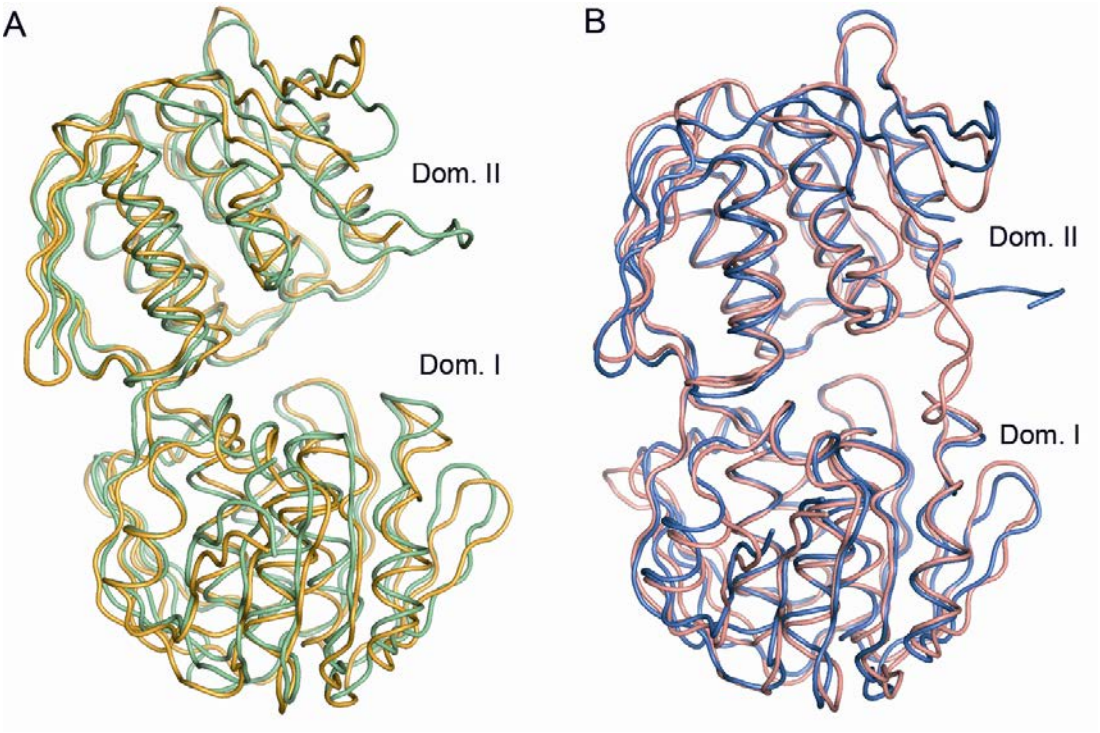

Figure S1 - Closest structural homologues for MurA1 $_{D39}$ and for MurA1 $_{Hungary19A}$ (A) Structural superimposition of MurA1$_{D39}$ (orange) and open conformation of the MurA from *E. cloacae* (green, PDB code 1EJD), rmsd = 1.8 Å. (B) Structural superimposition of MurA1Hungary19A (blue) and the closed conformation of MurA from *B. anthracis* (pink, PDB code 3SG1), rmsd = 1.3 Å.

Figure S2

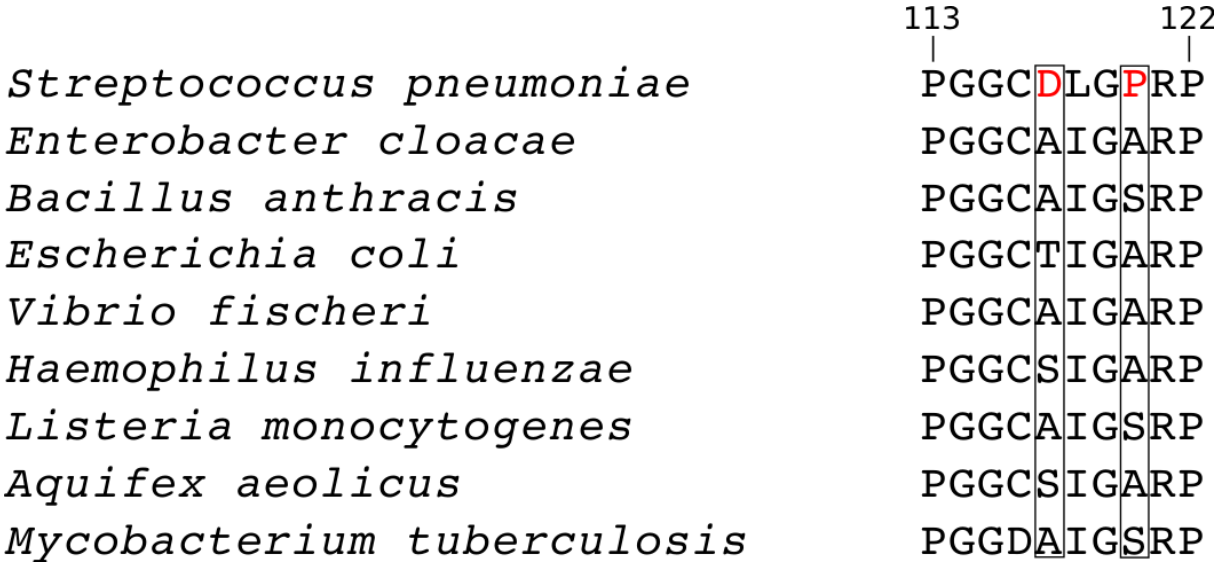

Figure S2 - Sequence alignment of the MurA1 flexible loop from several species. The loop containing catalytic Cys116 presents some differences compared to that of close homologues (highlighted in a box). In *S. pneumoniae* the loop differs in the presence of an Asp residue after Cys116 and one more Pro residue at position 120.