A central role for PBP2 in the activation of peptidoglycan polymerization by the bacterial cell elongation machinery

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PCR was performed using Q5 polymerase (NEB) according to the manufacturer’s instructions. Plasmid DNA and PCR fragments were purified using the Zyppy plasmid miniprep kit (Zymo Research) or the Qiaquick PCR purification kit (Qiagen), respectively. Sequencing reactions were carried out with an ABI3730xl DNA analyzer at the DNA Resource Core of Dana-Farber/Harvard Cancer Center (funded in part by NCI Cancer Center support grant 2P30CA006516-48).

Strain Constructions

A complete list of strains can be found in S3 Table.

HC555 [MG1655 yrdE-kan]: A KanR cassette was inserted in the intergenic space downstream of yrdE (genotype designated yrdE-kan in this paper) for use in transducing the mre locus with phage P1. The KanR cassette was amplified from pKD13 [1] using primers o1141 (TGGCGCTAATTTCTGAATTGTGCGGCTTGTTGCAAATTAATTCCGGGGATCCGTC GACC) and o1142 (ATAATCAACAGCTAACATGTAATAAACCTTCAACACCGTG TGTAGGCTGGAGCTGCT TCG). The resulting PCR product was purified and electroporated into recombineering strain TB10 (using the same protocol as described for recombineering with DY330 [2]), and recombinants were selected at 30°C on LB agar supplemented with 25 μg/mL kanamycin. The yrdE-kan allele was moved from this strain into MG1655 by P1-
mediated transduction, generating strain HC555. The growth rate and cell dimensions of this strain are indistinguishable from wild type.

PR5 [MG1655 mreC(R292H) yrdE-kan]: A strain harboring the chromosomal mreC(R292H) mutation was constructed by allelic exchange, using a previously described protocol [3]. The pir-dependent suicide plasmid pPR84 [sacB CmR] was introduced into the recipient strain HC555/pTB63 [yrdE-kan TetR] by conjugal transfer from the donor strain SM10(λpir). Briefly, 5 mL of exponential-phase cultures (OD_{600} ≈ 0.3) of the donor and recipient strains were filtered onto the same 0.2 μm PES filter. This filter was placed cell-side-up on an LB agar plate and incubated for four hours at 37°C. Cells from the filter were then resuspended in 1 mL of LB, then plated on LB agar supplemented with chloramphenicol and tetracycline, and incubated at 30°C for 24 hours to select for exconjugants that contain pPR84 integrated into the chromosome via a single cross-over. Exconjugants were streaked on the same medium, and screened to identify isolates with spherical cell shape (indicating that the cross-over had occurred at the mre locus, resulting in mreC(R292H) expression). An exconjugant colony was resuspended in LB, serially diluted, plated on LB agar lacking NaCl and supplemented with 6% sucrose, and incubated at 30°C for 24 hours to select for recombinants that have lost the sacB-containing plasmid via a single cross-over. Sucrose-resistant colonies were replica-streaked on LB agar with and without chloramphenicol. Sucrose-resistant, chloramphenicol-sensitive isolates were screened for spherical cell morphology, indicating that mreC(R292H) had replaced the wild-type allele of mreC at the native chromosomal locus. This was confirmed by PCR followed by Sanger
sequencing. Strain PR5 was obtained by P1-mediated transduction of the genomic region near \textit{yrdE-kan} (including \textit{mre(R292H)}) from the primary isolate into MG1655. Transductants were selected on M9 agar supplemented with casamino acids, glucose, and kanamycin, screened for spherical cell shape, and confirmed by PCR and sequencing of \textit{mreC}.

PR30 [MG1655 \textit{mreC(G156D) yrdE-kan}]: A strain harboring the chromosomal \textit{mreC(G156D)} mutation was constructed by allelic exchange using the suicide vector pPR93, following the same protocol as described above for PR5.

PR55 [MG1655 \textit{\Delta ybeM1::kan}]: A Kan$^R$ cassette was used to replace the \textit{ybeM} pseudogene, so that this marker could be used to co-transduce the \textit{mrd} locus. The Kan$^R$ cassette was amplified from pKD13 [1] using primers o1237 (TCGTTGGCGAATTTTACGACTCTGACAGGAGGTGGCAATGATTCCGGGGATCCGT CGACC) and o1238 (AGCGCCGAGTAAAAAAACATCATAAAATTGCGGCGGCCGCGCTGTAGGCTGGAGCT GCTTCG). The resulting PCR product was purified and electroporated into recombineering strain TB10 (using the same protocol as described for recombineering with DY330 [2]), and recombinants were selected at 30°C on LB agar supplemented with 25 \(\mu\)g/mL kanamycin. The \textit{\Delta ybeM::kan} allele was moved from this strain into MG1655 by P1-mediated transduction, generating strain PR55. The growth rate and cell dimensions of this strain are indistinguishable from wild type.
PR68 [MG1655 ΔybeM1::kan pbpA(L61R)]: A strain harboring the chromosomal pbpA(L61R) mutation was constructed by allelic exchange, using a previously described protocol [3]. The pir-dependent suicide plasmid pPR101 [sacB CmR] was introduced into the recipient strain PR55/pTB63 [ΔybeM1::kan / Pnative::ftsQAZ TetR] by conjugative transfer from the donor strain SM10(λpir). Exconjugants that had integrated the plasmid into the genome via a single cross-over were selected on medium containing chloramphenicol and tetracycline. Exconjugants were then plated on sucrose to select for loss of the plasmid via a second recombination event. SucR CmS colonies were screened by PCR and sequencing for the presence of the pbpA(L61R) mutation. Strain PR68 was obtained by P1-mediated transduction of the genomic region near ΔybeM::kan (including pbpA(L61R)) from the primary isolate into MG1655.

PR101 [MG1655 ΔybeM1::cat]: A CmR cassette was used to replace the ybeM pseudogene, so that this marker could be used to co-transduce the mrd locus. The CmR cassette was amplified from pKD3 [1] using primers o1415 (TCGTTGGCGAATTTTACGACTCTGACAGGAGGTGGCAATG CATATGAATATCCTCCT TAG) and o1416 (AGCGCCGAGTAAAAAAACATCATAATAATTGCAGGCGGCGGCGCGCGTGTAGGCTGGAGCT GCTTC). These primers are designed such that the ΔybeM1::cat lesion is identical to the ΔybeM1::kan lesion in PR55, the only difference being the antibiotic resistance cassette. The resulting PCR product was purified and electroporated into recombineering strain TB10 (using the same protocol as described for recombineering with DY330 [2]), and recombinants were selected at 30°C on LB agar supplemented
with 25 μg/mL chloramphenicol. The Δ*ybeM::cat* allele was moved from this strain into MG1655 by P1-mediated transduction, generating strain PR101. The growth rate and cell dimensions of this strain are indistinguishable from wild type (*S2 Table*).

**PR93** [MG1655 Δ*ybeM1::cat pbpA(L61R)]: The Δ*ybeM1::cat* cassette was transferred from donor strain PR101 to recipient strain PR68 [MG1655 Δ*ybeM1::kan pbpA(L61R)] by P1-mediated transduction. Since *ybeM* and *pbpA* are closely linked, most transductants contained the wild-type *pbpA* sequence from donor strain PR101. PCR and sequencing were used to identify a rare Kan<sup>S</sup> Cm<sup>R</sup> transductant that retained the *pbpA(L61R)* sequence.

**PR115** [MG1655 Δ*ybeM1::cat pbpA(T52A)]: This strain was constructed in a two-step procedure. First, the Δ*ybeM1::cat* cassette from PR101 was transduced into a suppressor strain derived from PR30 [mreC(G156D)] that contains the spontaneous mutation *pbpA(T52A)*. Although *ybeM* and *pbpA* are closely linked, all transductants retained the *pbpA(T52A)* mutation, because this mutation permits survival on LB. P1 lysates were prepared on this intermediate strain, and the Δ*ybeM1::cat pbpA(T52A)* locus was co-transduced into MG1655, generating strain PR115. The presence of the *pbpA(T52A)* mutation was confirmed by PCR and sequencing.

**PM7** [MG1655 Δ*ybeM2::kan*] was a gift from Dr. Piet de Boer. This strain contains a kanamycin resistance cassette in the *ybeM* locus. Since the exact junction points are different from those in PR55 [Δ*ybeM1::kan*], the allele is designated Δ*ybeM2::kan*. 
PM11 [MG1655 ΔybeM2::kan rodA(A234T)] contains a rodA(A234T) mutation in the PM7 genetic background. This strain was a gift from Dr. Piet de Boer.

PR134 [MG1655 ΔrodZ::cat]: A Cm\textsuperscript{R} cassette was used to replace the region between the 2nd codon and 7th codon from the stop codon of rodZ, as described previously [2,4]. The Cm\textsuperscript{R} cassette was amplified from pKD3 [1] using primers o1953 (CTCCCGCGTTACCCGTCTGTTACTGCGCCGGTGATTGTTCGTGTAGGCTGGAGC TGCTTC) and o1954 (CGGCATCTCAATTCTCATTAAACGTACCTGCAGCGAATGCATATGAATATCCTCCTT AG). The resulting PCR product was purified and electroporated into MG1655/pKD46 as described previously [1], and recombinants were selected at 42\textdegree C on M9 agar supplemented with casamino acids, glucose, and 25 \(\mu\)g/mL chloramphenicol. PR134 was made by P1 transduction of ΔrodZ::cat from this intermediate strain into an MG1655 recipient strain.

HC558 [MG1655 ΔpbpArodA::kan]: A Kan\textsuperscript{R} cassette was used to replace the region between the 2nd codon of pbpA and 5th codon from the stop codon of rodA, as described previously [2]. The Kan\textsuperscript{R} cassette was amplified from pKD13 [1] using primers o1094 (TGAGTGATAAGGGAGCTTTGAGTAGAAAACGCAGCGGATGATTCCGGGGATCCGCGACC) and o1095 (CCACTGCTTACGCAGTACCTTTGAGTAGAAGAAAACGCAGCGGATGATTCCGGGGATCCGCGACC)
TTCG). The resulting PCR product was purified and electroporated into TB10/pCX16, using the same protocol as described for recombineering with DY330 [2]), and recombinants were selected at 30°C on LB agar supplemented with 25 μg/mL kanamycin. HC558/pRY47, HC558/pHC857, and HC558/pSS43 were made by P1-mediated transduction of ΔpbpArodA::kan from this intermediate strain into MG1655 containing the corresponding plasmid.

**Plasmid constructions**

A complete list of plasmids can be found in S4 Table.  

**pMS5 [colE1 cat lacI Plac::nativeRBS-mreCD]:** The insert (XbaI-nativeRBS-mreC-mreD-HindIII) was amplified using MG1655 genomic template DNA and primers o882 (GTCA TCTAGA CTGCCTGGTCTGATACGAGAATACGCATAACTTATG) and o905 (GTCA AAGCTT TTATTGCACTGCAAACTGCTGACGG). This insert was cloned into pRY47 [5] using the XbaI and HindIII restriction sites.

**pPR49 [colE1 cat lacI Ptac::nativeRBS-mreC(R292H)mreD]:** Primers o882, o918 (CTGCATCAGATGTTCTAGCTAGCTGCCTGGTCTGATACGAGAATACGCATAACTTATG), and o905 (GCTAATGAACTGCACTGCAAACTGCTGACGG). were used to amplify MG1655 genomic DNA and introduce the R292H mutation into mreC using overlap-extension PCR. The product was PCR purified, digested with XbaI/HindIII, and cloned into similarly digested pHC800 [5].
pPR50 [colE1 cat lacI Ptac::nativeRBS-mreC(G156D)mreD]: Primers o882, o914 (GACCAACAACATCTTTGTCGCTGATGACCGGC), o915 (GCGACAAAAGATGTGTTGGTCAGGTGGTGG), and o905 were used to amplify MG1655 genomic DNA and introduce the G156D mutation into mreC using overlap-extension PCR. The product was PCR purified, digested with XbaI/HindIII, and cloned into similarly digested pHC800 [5].

pPR57 [colE1 bla Pt7::His6-SUMO-mreC(45-367)]: Primers o883 (GTCA AAGCTT CTATTGCCCTCCCGGCAC) and o920 (ATTGGT GGATCC GCCGTCAGTCCTTTCTACTTTGTTC) were used to amplify the insert (BamHI-mreC(45-367)-HindIII) from MG1655 genomic DNA. This insert was cut with BamHI/HindIII and ligated into similarly digested pTD68 [6].

pPR84 [cat mobRP4 sacB mreC(R292H)mreD]: Primers o1157 (GTCAGAAGCTGCTGCCTGGTCTACGAG) and o1158 (GTCA TCTAGATTTATGGCACTGCAAATGCTGACG) were used to amplify the insert (Sacl-mreC(R292H)-mreD-XbaI) from pPR49. This insert was cut with Sacl/XbaI and ligated into similarly digested pDS132 [3].

pPR93 [cat mobRP4 sacB mreC(G156D)mreD]: Primers o1157 and o1158 were used to amplify the insert (Sacl-mreC(G156D)-mreD-XbaI) from pPR50. This insert was cut with Sacl/XbaI and ligated into similarly digested pDS132 [3].
pPR101 [cat mobRP4 sacB rlmH pbpA(L61R)]: Primers o1285 (GTCAGAGCTCACATCGCTGGTTCCGTGCTGG) and o1286 (GTCATCTAGATCCCCCATCTCGTAGGCACCTG) were used to amplify the insert (a segment of genomic DNA encompassing a 3’ fragment of rlmH and the 5’ half of pbpA(L61R), flanked by SacI and XbaI restriction sites) from a suppressor mutant derived from PR5, containing the spontaneous mutation pbpA(L61R). This insert was cut with SacI/XbaI and ligated into similarly digested pDS132 [3].

pPR128 [attHK022 tetAR lacIq Plac::msfgp-GS-pbpA(L61R)]: pbpA(L61R) was PCR amplified from PR68 gDNA using primers o264 (GCTAAAGCTTTTTATTCGGATTATCCGTCATG) and o1041 (GCTAGGATCCAAACTACGAACTCTTTTCGCGACTATACG). The resulting PCR product was digested with BamHI and HindIII restriction enzymes and cloned into pHC943, which was pre-digested with the same enzymes [8].

pSS43 [colE1 cat lacIq Plac::RodA'-GGGSx3'-PBP2] was generated in two steps. First, the insert containing RodA was amplified from MG1655 genomic DNA as a template with primers oSS37 (TCGACAAGCTTTTACACGCTTTTCGACAACATTTTCCTGTGG) and oSS59 (GTTAATCTTTTAAGGAGATATACCATGACGGATAATCCGAATAAAAAACATTCTGG). The resulting PCR product was then assembled with XbaI/HindIII-digested pRY47 [colE1 cat lacIq Plac::empty] using the isothermal assembly procedure [7]. This intermediate plasmid was amplified using primers oSS62
(CCGCAGCGGAGGACCATTAGCTTGTACCCGATACGCGAGCGAAGCTGAGCGTACCGATACGCGAGCGAACGTGAAGCGACTGCTG) and oSS75

(AGAACCACCACCACCGGAGCCACCGCCTACCCGCTACCACCACGCCTTTTGCGACAACATT TTCCT) to create the vector for isothermal assembly with an insert containing GGGsx3-'PBP2, amplified from MG1655 genomic DNA with primers oSS61

(CTCGCGTATCGGTGACAAGCTTAATGGTCTCCGCTGCCGCAACCCGCTGGATTTTTCCGCA) and oSS74

(GGTGGCGGTAGCCGGCGGTGGCTCCGGTGGCGGTCTAACTACAGAACACTCTTTTC GCGAC).

pSS50 \([colE1\ bla\ P_{T7}\cdot His6-SUMO-FLAG-RodA'\cdot GGGsx3\cdot PBP2]\) was generated in a two-piece isothermal assembly reaction with an insert containing RodA' GGGsx3-'PBP2, which was amplified from pSS43 \([colE1\ cat\ lacl\ q\ P_{lac}\cdot RodA']\ GGGsx3\cdot PBP2]\) with oligonucleotide primers oSS82

(GGGTCATCCACGGATAATCCGAAATAAAAAACATTCTGGGATAAGTCCATCTCGAT CCC) and oSS84

(GCAGCCGGATCCCCTTCCTGAGTCACCCGCGGGCTTATGGTCTCCGCTGCCGAC AACCGC), and pAM172 \([colE1\ bla\ P_{T7}\cdot His6-SUMO-FLAG-RodA}\) [4], which was amplified with oligonucleotide primers oSS83

(TCCAGAAATGTTTTTTTATTCCGATTATCCGATGGATGACCCCCGCCAGGGCTTTGAAAC AAC) and oSS85

(AATCCAGCGGTGGCCGCAGCGGAGGACCATTAGCCCGGGTGGACTGAGCGAAGG GGATCC).
pSS51 \( [\text{colE1 bla } P_{T7}:\text{His6-SUMO-FLAG-RodA}'-\text{GGGSx3-}'\text{PBP2(L61R)}] \) was generated in a two-piece isothermal assembly reaction with an insert containing \( \text{PBP2(L61R)} \), which was amplified from PR68 gDNA with oligonucleotide primers oSS74 and oSS84, and pSS50, which was amplified with oligonucleotide primers oSS75 and oSS85.

pSS52 \( [\text{colE1 bla } P_{T7}:\text{His6-SUMO-FLAG-RodA(A234T)'}-\text{GGGSx3-}'\text{PBP2}] \) was generated in a two-piece isothermal assembly reaction with an insert containing RodA(A234T), which was amplified from PR151 with oligonucleotide primers oSS75 and oSS82, and pSS50, which was amplified with oligonucleotide primers oSS74 and oSS83.

pSS60 \( [\text{colE1 bla } P_{T7}:\text{His6-SUMO-FLAG-RodA(D262A)'}-\text{GGGSx3-}'\text{PBP2}] \) was generated in a two-piece isothermal assembly reaction with an insert containing RodA(D262A), which was generated by overlap extension PCR using oligonucleotide primers oSS36 (TTGGTGATCCCATGACGGATAATCCGAATAAAAAAACATTCTGGG), oSS37, oSS96 (ACGCCATACTGCCTTTATCTTCGCGGTACTGGC), and oSS97 (CGAAGATAAAGGCCAGTATGGCGTGCTGGGGAGAA), and pSS50, which was amplified with oligonucleotide primers oSS74 and oSS83.

pSS62 \( [\text{colE1 bla } P_{T7}:\text{His6-SUMO-FLAG-RodA(D262A)'}-\text{GGGSx3-}'\text{PBP2(L61R)}] \) was generated in a two-piece isothermal assembly reaction with an insert containing RodA(D262A), which was generated by overlapping PCR using oligonucleotide primers.
oSS36, oSS37, oSS96, and oSS97, and pSS51, which was amplified with oligonucleotide primers oSS74 and oSS83.

**pAAY71** [**aacC1 P<sub>Syn135</sub>::mCherry**]: To a vector for expressing cytoplasmic mCherry, the mCherry gene was PCR-amplified from pAAY65 [**aacC1 P<sub>Syn135</sub>::ssdsbA-mCherry**] [53] template using primers oAAY1 (TTTTTCATATGTCCAAGGGCGAGGAGGATAACCTG) and oAAY2 (TTTTGTCGACTTATTAGGATCCGCAGCACCCTTTGTAC). The resulting PCR product was digested with NdeI and Sall restriction enzymes and cloned into pAAY65, which was pre-digested with the same enzymes.

**References**


