SMC is recruited to oriC by ParB and promotes chromosome segregation in Streptococcus pneumoniae
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Supplementary information, Minnen et al. SMC is recruited to oriC by ParB and promotes chromosome segregation in Streptococcus pneumoniae.

Supporting Experimental Procedures:

Strains and growth conditions
Bacterial strains and plasmids used in this study are listed in Table 1. S. pneumoniae strains were grown as standing cultures in M17 broth (Terzaghi and Sandine, 1975) containing 0.5% (w/v) glucose (GM17) (for ChIP analysis) or in complex C+ Y medium (Martin et al., 1995) at 30°C or 37°C (for microscopy). For growth on plates 1% (v/v) of defibrinated sheep blood was added to GM17 agar. When applicable, ZnSO₄ was added to liquid medium at a final concentration ranging between 0.05 mM and 0.20 mM. E. coli EC1000 was grown at 37°C in a shaking incubator in TY broth (Bacto-Tryptone (1%), Bacto-yeast extract (0.5%) and 1% NaCl). When appropriate, antibiotics were added in the following concentrations for E. coli: ampicillin (amp) at 100 µg/ml and spectinomycin (spec) at 50 µg/ml. For S. pneumoniae the following concentrations were used: tetracycline (tet) 1 µg/ml, trimethoprim (trmp) 18 µg/ml, spectinomycin 100 µg/ml. For S. pneumoniae, storage of mid-exponential phase cultures was done by growing cells in GM17 or C+Y at 37°C to an OD₆₀₀nm of approximately 0.2 and 0.4, respectively. The cells were centrifuged for 2 min at 14000 rpm and the cell pellet was resuspended in a volume of fresh medium containing 14.5% glycerol (v/v) that would result in an OD₆₀₀nm of exactly 0.2 and 0.4, respectively. The cells were then aliquoted and stored at -80°C.

Construction of plasmids and strains
To construct plasmid pAM6, carrying gfp fused to smc under the control of the zinc-inducible czcD promoter (noted P_avg), a PCR with the primers 63 smc-F+XbaI and 64 smc-R+NotI (Table S1) was performed, using chromosomal DNA of strain D39 as a template. The amplified fragment was subsequently cleaved with XbaI and NotI and ligated into the SpeI and NotI sites of plasmid pJWV25 (Eberhardt et al., 2009) resulting in plasmid pAM6. The correct sequence of the plasmid was verified by sequencing using primers 103 pPP2-R, 71 gfp-F seq pJWV25, 20 smc-up-R+BamHI, 21 smc up F gfp, 22 smc gfp check F, 88 smc in seq F, 89 smc in seq2 F, 90 smc in seq R and 91 smc in seq2 R.

To construct plasmid pAM9, carrying gfp fused to scpB under the control of the P_avg, a PCR with the primers 69 scpB-F+SpeI and 70 scpB-R+NotI (Table S1) was performed, using chromosomal DNA of strain D39 as a template. The amplified fragment was subsequently cleaved with SpeI and NotI and ligated into the corresponding sites of plasmid pJWV25 (Eberhardt et al., 2009) resulting in plasmid pAM9. The correct sequence of the plasmid was verified by sequencing using primers 103 pPP2-R and 71 gfp-F seq pJWV25.

Strains AM10 (P_avg-gfp), AM13 (P_avg-gfp-scpB) and AM14 (P_avg-gfp-smc)
S. pneumoniae strains AM10, AM13 and AM14 were obtained by a double crossover recombination event between the bgaA regions located on plasmids pJWV25, pAM9 and pAM6, respectively, and the chromosomal bgaA locus. Transformants were selected on GM17 blood agar plates containing tetracycline (1 µg/ml) and correct integration was verified by PCR using primers 112 BgaA_down check R and 113 TetM check-F.
Strain AM39 (Δsmc::tmp)
The gene that encodes SMC was deleted from strain D39 by replacement with a trimethoprim resistance cassette. To prevent any polar effects of the gene replacement, the trimethoprim resistance gene was inserted without its own promoter and terminator using three-way long flanking PCR. This was done by amplifying the region downstream of smc with primers 18 smc-down-F+KpnI and 10 smc-up-F and the region upstream of smc with primers 17 smc-R+KpnI KO and 16 smc-up-F KO using chromosomal DNA of strain D39 as a template. The trimethoprim resistance cassette, without its promoter and terminator, was amplified from plasmid pKOT (Hendriksen et al., 2008) with primers 83 trmp-F + KpnI and 84 trmp-R + KpnI. The 3 PCR products were digested with KpnI and ligated together. The ligation mixture was subsequently transformed to strain D39. Transformants were selected on GM17 blood agar plates containing trimethoprim (18 μg/ml), after overnight incubation at 37 °C. Correct integration was verified by PCR using primer pairs 60 trmp F seq and 14 smc check F, 19 smc-check-F KO and 45 trmp R + NotI, 89 smc in seq2 F and 90 smc in seq R and with 60 trmp F seq and 14 smc check F. The construct was sequence verified. Chromosomal DNA of PCR verified mutants was backcrossed to D39 to check for normal transformation efficiencies and lack of second site suppressor mutants, basically as described (Caymaris et al., 2010). Southern blots were performed on chromosomal DNA of three mutants that appeared correct by PCR to independently verify the replacement and deletion of smc on the S. pneumoniae genome, using a probe upstream of smc and a probe inside the coding region of smc and using EcoRV as restriction enzyme, as shown in Figure S6.

Strain MT1 (parB-spec)
A PCR using primers 1 parB-up-F and 2 parB-up-R+Ascl and a PCR using primers 3 parB-down-F+NotI and 4 parB-down-R was performed on D39 chromosomal DNA. Another PCR using primers 38 spec-F-Ascl and 39 spec-R-NotI was performed using plasmid pORI38 (Leenhouts et al., 1996) as a template. PCR product parB-up was digested with Ascl, PCR product parB-down was digested with NotI and PCR product spec was digested with Ascl/NotI. The 3 PCR products were ligated together. The ligation was then amplified with PCR using the outside primers (1 and 4). The obtained PCR product was transformed to D39 competent cells. Transformants were selected on GM17 blood agar plates containing spectinomycin (100 μg/ml), after overnight incubation at 37 °C. Correct integration of the spectinomycin resistance cassette just after the parB locus was verified by PCR using primers 8 parB-check-F and 9 parB-check-R. The construct was sequence verified.

Strain MT2 (parB-gfp-spec)
A PCR using primers 1 parB-up-F and 6 parB-R+linker+BamHI and a PCR using primers 5 parB-F-down+EcoRI and 4 parB-down-R was performed using chromosomal DNA of strain MT1 as a template. The parB reverse primer contained a sequence which encodes for the following flexible domain breaking linker: GSGGEAAAKGS (Arai et al., 2001). Another PCR using
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primers 46 gfp_sf-F+BamHI and 47 gfp_sf-R+EcoRI was performed using plasmid pUC57-gfp as a template (Veening, unpublished). PCR product parB-up was digested with BamHI, PCR product parB-down was digested with EcoRI and PCR product gfp was digested with BamHI/EcoRI. The 3 PCR products were ligated together. The ligation product was transformed to D39 competent cells and transformants were selected on GM17 blood agar plates containing spectinomycin (100 μg/ml), after overnight incubation at 37 °C. Correct integration of the parB-gfp fusion was verified by PCR using primers 8 parB-check-F and 9 parB-check-R. The construct was sequence verified.

Strain MT3 (Δ(parB)-spec)
A PCR using primers 1 parB-up-F and 7 parB-R+EcoRI-KO and a PCR using primers 5 parB-down+F+EcoRI and 4 parB-down-R was performed using chromosomal DNA of strain MT1 as a template. PCR products parB-up and parB-down were digested with EcoRI. The 2 PCR products were ligated to each other. The ligation product was transformed to D39 competent cells. Transformants were selected on GM17 blood agar plates containing spectinomycin (100 μg/ml), after overnight incubation at 37 °C. Correct deletion of parB was verified by PCR using primers 8 parB-check-F and 9 parB-check-R and the locus was verified by sequencing.

JWV415 (Δ(parB)-spec, Δsmc::tmp)
Chromosomal DNA of strain MT3 was transformed to strain AM39 and transformants were selected on GM17 blood agar plates containing spectinomycin (100 μg/ml), after overnight incubation at 37 °C. Transformants were checked for the presence of trimethoprim resistance and both the smc and the parB deletions were verified by PCR.

AM15 (PZn-gfp-scpB, Δ(parB)-spec) and AM16 (PZn-gfp-smc, Δ(parB)-spec)
Chromosomal DNA of strain MT3 was transformed to strains AM13 and AM14, respectively, and transformants were selected on GM17 blood agar plates containing spectinomycin (100 μg/ml), after overnight incubation at 37 °C. Transformants were checked for the presence of tetracyclin resistance and both the presence of the gfp fusion and the parB deletion were verified by PCR.

**Chromatin Immuno-Precipitation (ChIP)**
Cells were grown to mid-exponential phase (OD600nm ~0.2) in GM17 medium at 37°C (with 0.15 mM ZnSO₄ where relevant) and 84 ml of culture was mixed by inverting with 8.4 ml of fixing solution (50 mM Tris pH 8.0, 100 mM NaCl, 0.5 mM EGTA, 1 mM EDTA, 30% (v/v) formaldehyde) and incubated at room temperature for 30 min. Cells were centrifuged for 3 min at 4°C at 6000 rpm and resuspended in 30 ml ice cold PBS. Cells were washed again with PBS and the pellet was resuspended in 1.5ml of TSEMS (50 mM Tris/HCl pH 7.4, 50 mM NaCl, 10 mM EDTA, 0.5 M Sucrose and 1 mM PMSF). Cells were washed twice more with TSEMS and the pellets aliquoted in three portions and were snap frozen in liquid nitrogen and subsequently stored at -80°C. One aliquot of each strain was resuspended in 2 ml ice cold lysis buffer (50 mM Heps-KOH, pH 7.55, 140 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.1% (w/v) sodium deoxycholate and RNase A (100 μg/ml))
and transferred to a 5 ml plastic tube. Cells were sonicated on ice using a Sonics vibra cell vcx 130. Pulse was on for 30 sec, then off for 30 sec and this was repeated for a total time of 3 min with the amplitude set to 75%. The lysates were transferred to 2 ml tubes and centrifuged for 10 min at 4°C at 13200 rpm. The supernatant was transferred to a clean 2 ml tube and centrifuged for 10 min 4°C 13200 rpm. The supernatant was pre-cleared with 100 µl proteinG coupled Dyna Beads (Invitrogen) and incubated on a turning wheel with powersetting 18 for 1 hour at 4°C (cold room). Beads were pelleted using a magnet and the pre-cleared supernatant was transferred to a new 2 ml eppendorf tube. 200 µl of the supernatant was kept at -20°C as the Whole Cell Extract (WCE) control. To 1500 µl of the pre-cleared supernatant, 10 µl of anti-GFP antibodies (rabbit serum, polyclonal, Invitrogen A-6455) was added and incubated on a turning wheel for 2 hrs at 4°C. 100 µl of proteinG coupled Dyna Beads (invitrogen) were added to the samples and incubated for 12 hours or more (overnight) on the turning wheel at 4°C. The beads were collected using a magnet and the supernatant was discarded. The beads were washed once for 5 min (vortex approximately 800 rpm) at room temperature with 1 ml lysis buffer containing 500 mM NaCl. The beads were pelleted with a magnet and subsequently washed with 1 ml wash buffer. Beads were pelleted and resuspended in 520 µl TES buffer. The WCE samples were thawed and combined with 300 µl TES and 20 µl 10% SDS. The samples were kept shaking at 65°C (little oven) for 6 hours (to overnight). This eluted the DNA from the beads and reversed cross links. Beads were collected using a magnet and 500 µl of the supernatants was transferred to a fresh 2 ml eppendorf cup. 12.5 µl Proteinase K (20 mg/ml in TE) was added to the samples and shaken for 2 hours at 37°C. 520 µl phenol was added and the solution was mixed by inverting and centrifuged for 2 min at max. speed. 450 µl of the top layer was pipetted off and transferred to a clean 1.5 ml eppendorf cup. 450 µl chloroform was added and the solution was mixed by inverting, spinned for 2 min at max. speed, 400 µl of the top layer was collected and transferred to a clean 1.5 ml eppendorf cup. 1 µl glycogen (20 mg/ml from Roche) was added, mixed by inverting and 40 µl sodium acetate, pH 5.3, was added and mixed by inverting. 1 ml of pure ethanol was added, mixed by inverting and incubated for 20 min at 20°C. The mixture was centrifuged for 15 min at 4°C max speed, the supernatant was carefully removed. 200 µl of 70% ethanol was added and the cup was inverted several times, subsequently centrifuged for 15 min at 4°C and the supernatant was removed. The pellet was air dried at 45°C and resuspended in 50 µl TE, pH 8 for 15 min at 45°C. The ChIP samples were stored in -20°C, 5 µl of the WCE was diluted tenfold and also stored in -20°C. To the remaining 45 µl, RNaseA (1 µl of 10 mg/ml stock) was added and incubated for 30 min at 37°C. The sample was run on a 2% agarose gel to check the sheering of DNA. 1 µl of the ChIP samples (1:25) and the diluted WCE (1:250) were used for qPCR in a 25 µl reaction.

Fluorescence Microscopy

Cells were grown at 30°C (or at 37°C for strain AM13) in plastic 5 ml capped tubes, filled to a maximum of 3 ml to allow for enough air for proper GFP folding, basically as described previously (Eberhardt et al., 2009). When appropriate, ZnSO₄ was added prior to imaging (0.15 mM for GFP-SMC and...
0.05 mM for GFP-ScpB). Strain AM13 (GFP-ScpB) and AM15 (GFP-ScpB, ΔparB) were induced with 0.05 mM ZnSO₄ and grown at 37°C to prevent the formation of inclusion bodies (not shown). 1 ml of mid-exponential growing cells (OD600nm between 0.30 and 0.45) were washed in resuspended in 100 µl of PBS (when applicable, 2 µg/ml of DAPI (Invitrogen) was added) of which 0.4 µl was subsequently spotted onto an agarose containing microscope slide.

Microscopy pictures were taken with a Deltavision (Applied Precision) IX71 Microscope (Olympus), using a CoolSNAP HQ2 camera (Princeton Instruments) and a 300W Xenon light source through a 100x oil immersion objective (phase contrast). For GFP signals, a Croma filterset was used with excitation wavelengths between 450 and 490 nm and emission at 500 and 550 nm. For DAPI, excitation wavelengths were between 340 and 380 nm and emission between 432 and 482 nm. For phase contrast images, the exposure time was 0.1 sec with 32% APLLC White LED light, for GFP and DAPI the exposure time was between 1 and 2 sec with 100% Xenon light. Images were deconvolved using softWoRx 3.6.0 (Applied Precision) and modified for publication using ImageJ (http://rsb.info.nih.gov/ij/) and CorelDRAW X3 (Corel Corporation).
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**Supporting Figures:**

![Graph](Minnen_et_al_Figure_S1)

**Fig S1: The ParB-GFP fusion is functional.** The strains used in this study were D39 (WT, black diamond) and its derivatives: MT1 (parB-spec, blue square), MT2 (parB-gfp-spec, green triangle) and MT3 (Δ(parB)-spec, red circle). For inoculation, stocks of bacteria grown in C+Y medium to an OD\(_{550nm}\) of 0.4 were diluted 10-fold in C+Y medium (pH 6.8-7.0) and incubated at 37°C in a 96 wells plate, OD\(_{595nm}\) measurements were taken every 15 minutes using a microtiterplate reader. Error bars indicate the standard deviation of the mean, calculated from three independent replicates. For improved visualisation of the lag phase, the Y-axis is in a linear scale.
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**Fig. S2. The GFP-SMC fusion.** (A) Schematic representation of the genetic layout of strain AM14 (P<sub>Zn</sub>-gfp-smc). Strain AM14 was generated by a double crossover integration event of plasmid pAM6 into the *bgaA* locus of strain D39. (B) Western blot analysis: Exponentially growing cells of strains AM14 (GFP-SMC) and AM16 (GFP-SMC, ΔparB) were harvested for Western blot analysis using antibodies against GFP, after 1h of induction with 0.15 mM ZnSO<sub>4</sub>. (C-D) Growth curves: The strains used in this study were D39 (WT; black / grey) and its derivatives: AM14 (P<sub>Zn</sub>-gfp-smc; red / orange) and AM38 (∆smc::trmp, P<sub>Zn</sub>-gfp-smc; violet / pink). For inoculation, stocks of bacteria grown in C+Y medium, or in C+Y medium +75 μM ZnSO<sub>4</sub>, to an OD<sub>550</sub> of 0.4 were diluted 100-fold in C+Y medium or in C+Y medium +75 μM ZnSO<sub>4</sub> (pH 6.8-7.0) and incubated at 37°C in a 96 wells plate, OD<sub>595nm</sub> measurements were taken every 10 minutes. Error bars indicate the standard deviation of the mean, calculated from six independent replicates. The difference between the C and D experiment is that cells for panel D were first precultured with ZnSO<sub>4</sub> whereas this was not the case for cells of panel C. For improved visualisation of the lag phase, the Y-axis is in a linear scale.
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**Fig. S3. Localization of ScpB depends on ParB.** Cells were analyzed by fluorescence microscopy as described in the Experimental Procedures. Micrographs of strains AM13 (P\textsubscript{Zn}\textsuperscript{-}gfp-scpB) (A) and AM15 (P\textsubscript{Zn}\textsuperscript{-}gfp-scpB, ΔparB) (B) are shown. Arrows indicate GFP-ScpB foci.
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**Minnen et al. Figure S4**

ChIP-PCR (GFP-SMC)

![Graph showing enrichment of SMC at oriC](image)

**Fig. S4. Enrichment of SMC at oriC depends on ParB.** Exponentially growing cells of strains AM10 (P_{Zn-gfp}), AM14 (P_{Zn-gfp-smc}) and AM16 (P_{Zn-gfp-smc}, ΔparB) were collected for ChIP analysis using anti-GFP antibodies. Input and eluate DNA samples were analyzed by real-time PCR. Pull-down efficiency (ChIP-DNA/input-DNA*100) is plotted for each primer pair. A typical outcome of a ChIP-PCR experiment is shown.
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**Figure S5. Viability assay on the parB and smc mutants.** Cells of strains D39 (wild type), MT3 (Δ(parB)-spec), AM39 (Δ smc::trmp) and JWV415 (Δ(parB)-spec, Δ smc::trmp) were grown to an optical density (600nm) of 0.2 and plated in Colombia blood agar. After overnight incubation at 37°C, the number of colony forming units (CFU) were counted and plotted. Error bars indicate the standard deviation of the mean, calculated from three independent replicates.
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**Minnen et al. Figure S6**

(A) Schematic representation of the *smc* locus in D39 and of strain AM39. SPD numbers are shown for genes lacking annotation. (B) Genomic DNA of the wild type (D39) and three individual transformants in which *smc* was successfully replaced by *trmp* as indicated by PCR was digested with EcoRV and hybridized using two separate probes as indicated in the figure. Each transformant lacked the *smc* gene (probe 2) and displayed the anticipated restriction pattern for probe 1.

**Fig. S6.** Southern blot analysis verified the successful construction of a *smc* replacement mutant. (A) Schematic representation of the *smc* locus in D39 and of strain AM39. SPD numbers are shown for genes lacking annotation. (B) Genomic DNA of the wild type (D39) and three individual transformants in which *smc* was successfully replaced by *trmp* as indicated by PCR was digested with EcoRV and hybridized using two separate probes as indicated in the figure. Each transformant lacked the *smc* gene (probe 2) and displayed the anticipated restriction pattern for probe 1.
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**Minnen et al._Figure_S7**

**Fig. S7.** *parB* and *smc* mutants are not hypersensitive to antibiotics or heat stress. Cells were grown in C+Y medium at 37°C or 40°C in microtiterplates in the presence of the indicated concentration of antibiotic and the OD600nm was measured every 10 minutes. Error bars show the standard deviation between at least 3 independent growth curves. For improved visualisation of the lag phase, the Y-axis is in a linear scale.
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**References**


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**Table S1. Oligonucleotides**

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<th>Primer</th>
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<td>1 parB-up-F</td>
<td>TGCCATCGGTAGCCCGTTAG</td>
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<tr>
<td>2 parB-up-R+Ascl</td>
<td>CGTATTGGCGCGCCGAAATAGTGGATAACCTTGATGAGA</td>
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<tr>
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<td>GCTAGGGGCGCGTTCTCAAGGTTATCCACTATGTTTTTCG</td>
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<tr>
<td>4 parB-down-R</td>
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</tr>
<tr>
<td>5 parB-F-down+EcoRI</td>
<td>GGGCGGAATTCCGGTGAATAAGGCTTGTCTTTTA</td>
<td>EcoRI</td>
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<tr>
<td>6 parB-R+linker+BamHI</td>
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95 parS 2025200 Sp forward CTTCAACAGTCGCTCCAACA
96 parS 2025200 Sp reverse AGCAGAATCCGGAGAGATTG
97 parS 2036431 Sp forward CAGACACATGGGGAAAATTTG
98 parS 2036431 Sp reverse GATGCGCCCATATCTTGTTG
99 parS 1936956 Sp forward TATTTCTTCGGCGACGTTC
100 parS 1936956 Sp reverse GTAGTAGATGGCGTTTGGAAA
101 ter Sp forward TTTTCACTGGGTTTTGGAGG
102 ter Sp reverse ATTTGCAAGGACCTTCCAC
103 pPP2-R Sp forward CGAAATACGGGACAGCATTG
112 BgaA_down check R AGCTAGAGTTCCGCAATTGG
113 TetM_check F CATATGTCCTGGCGGATCTATG
114 SPD_1620 F AAATTTCATCCGACGTGTCC
115 SPD_1620 R AGCTAGAGTTCCGCAATTGG
116 ter Sp forward 1056kb GAAAAATACCATCCCCAGCA
117 ter Sp reverse 1056kb AGCCTTGTTGCCTATCATG
118 pepS forward 250kb CCTTTGGGACCAAATCTTCA
119 pepS reverse 250kb TAAAGCATATCGGCCTTGCTC
120 valS forward 500kb GGATGGAAAATGTCCACGAC
121 valS reverse 500kb AGCCTTGTTGCCTATCATG
122 deco forward 750kb TTTTGCAGAGTTGGTTG
123 deco reverse 750kb TTTTGCAGAGTTGGTTG
124 ter Sp forward 1030kb rexB AATTTGCAAGGACCTTCCAA
125 ter Sp reverse 1030kb rexB TCCATCTGACATGGTCTGTA
126 def forward 1250kb CACGATCTGTCACTGCTGTA
127 def reverse 1250kb TCACATGACGGGGCTCTC
128 sun forward 1500kb TGAGAGAAGACCGAATGG
129 sun reverse 1500kb ATTGGTCAACGCTTGGCTTC
130 wrbA forward 1750kb TTGCTGAGAGTTACGGCTCTG
131 wrbA reverse 1750kb CATCAACTGAGCTCTGCAGCA
132 parS 1936956 Sp forward TCCATGAAAAATGCAATGAGAA
133 parS 1936956 Sp reverse AGCTTTGATCATTTGGCTTA
134 ter Sp forward 1048kb AAGAATCCAGAAGATTCAGA
135 ter Sp reverse AAAGAGCTATGGAGTTACAAACAC
136 comCDE promoter reverse GAAGCCCAATGCTCTATCCA
137 comCDE promoter forward TATCGCGGTTTTACGATTCA
138 parB prote forward ACGGTCTATCCAGCTGTGG
139 parB prote reverse ATAGGCGCGTCTCTTCTTCA
140 comE end reverse CCAAAATAGACTTTTGAGGAGGA
141 comE end forward TTGAAACAAGAGGGATCTCCA
142 rplB forward TTCTGCAACATCGCTTGG
143 rplB reverse AAAGACGGTTCGCGACTTTGA
144 rpoB forward TTCAAGGACATTACTCCTC
145 rpoB reverse TTCTACAACCTGGGCTGAAG
146 ftsH near rrlA forward ACGACTTTGAACAAGCGACA
147 ftsH near rrlA reverse CTCGTCGCAACAGCATAGC
148 CDS1192 TTTAATAGCGCGTCGAGA
149 CDS1192 reverse ATAGCAATGGCCAGCTTCT

*Relevant restriction sites are underlined.*