**Supplementary file 6.** **Plasmids used in this study.**

| **Plasmid** | **Description** | **Construction/Source** |
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| pAI039 | Integrative plasmid bearing *dipM*-*sfmTurquoise2ox* | Izquierdo-Martinez et al., 2023 |
| pBAD24 | Replicating plasmid for the expression of genes under the control of the arabinose-inducible PBAD promoter, AmpR | Guzman et al., 1995 |
| pET51b(+) | Plasmid for overexpressing proteins with a cleavable N-terminal Strep-II tag and a C-terminal 10xHis tag | Novagen |
| pLY009 | pTB146 bearing *bacA*F130R | (a) Amplification of *bacA*F130R from pLY154 by PCR with primers oLY001 and oLY002  (b) Insertion into *Sap*I/*BamH*I-treated pTB146 by Gibson assembly |
| pLY070 | pXCHYC-2 bearing *pbpC*1-39nt*-dipM*670-888nt*-pbpC*250-396nt | (a) Amplification of P*xyl*-*pbpC*1-39nt from pMT993 by PCR with primers CS008 and oLY158  (b) Amplification of *dipM*670-888nt from pAI039 by PCR with primers oLY159 and oLY160  (c) Amplification of *pbpC*250-396nt-*mCherry* from pMT993 by PCR with primers oLY161 and oLY162  (d) Gibson assembly of the three fragments  (e) Digestion of pXCHYC-2 and the assembly product with *Asc*I and *Nhe*I and subsequent ligation |
| pLY073 | pXmVENN-1 bearing *pbpC* | (a) Digestion of pMT906 with *Nhe*I and *Kpn*I  (b) Ligation of the released *pbpC* fragmentinto *Nhe*I/*Kpn*I-treated pXmVENN-1 |
| pLY074 | pXmVENN-1 bearing *pbpC*∆4-39nt | (a) Amplification of *pbpC*∆4-39nt from pMT906 by PCR with primers oLY169 and CC3277-rev2  (b) Restriction of the PCR product with *Nhe*I and *Kpn*I  (c) Ligation into *Nhe*I/*Kpn*I-treated pXmVENN-1 |
| pLY075 | pXmVENN-1 bearing *pbpC*1-39nt*-dipM*670-888nt*-pbpC*250-2202nt | (a) Amplification of *pbpC*1-39nt*-dipM*670-888ntfrom pLY070 by PCR with primers CC3277-for and oLY170  (b) Amplification of *pbpC*250-2202nt from pMT906 by PCR with primers oLY171 and CC3277-rev2  (c) Gibson assembly of the two fragments  (d) Digestion of pXmVENN-1 and the assembly product with *Nhe*I and *Kpn*I and subsequent ligation |
| pLY076 | pXmVENC-2 bearing *bacA*∆4-32nt | (a) Amplification of *bacA*∆4-32nt from pMT812 by PCR with primers oLY172 and CC1873-rev  (b) Digestion with *Nde*I and *Sac*I  (c) Ligation into *Nde*I/*Sac*I-treated pXmVENC-2 |
| pLY086 | pXmVENC-2 bearing *bacA* | (a) Digestion of pMT812 with *Nde*I and *Sac*I  (b) Ligation of the released *bacA* fragment into *Nde*I/*Sac*I-treated pXmVENC-2 |
| pLY087 | pXmVENC-2 bearing *bacA*K4S | Site-directed mutagenesis of pLY086 by PCR with primers oLY192 and oLY193 |
| pLY088 | pXmVENC-2 bearing *bacA*K4S/K7S | Site-directed mutagenesis of pLY087 by PCR with primers oLY194 and oLY195 |
| pLY099 | pXmVENC-2 bearing *bacA*S3A | (a) Amplification of *bacA*S3A from a custom-synthesized gene block by PCR with primers oLY217 and oLY218  (b) Amplification of *bacA*106-483nt from pLY086 by PCR with primers oLY219 and CC1873-rev  (c) Gibson assembly of the two fragments  (d) Digestion of pXmVENC-2 and the assembly product with *Nde*I and *Sac*I and subsequent ligation |
| pLY100 | pXmVENC-2 bearing *bacA*Q5A | (a) Amplification of *bacA*Q5A from a custom-synthesized gene block by PCR with primers oLY217 and oLY218  (b) Amplification of *bacA*106-483nt from pLY086 by PCR with primers oLY219 and CC1873-rev  (c) Gibson assembly of the two fragments  (d) Digestion of pXmVENC-2 and the assembly product with *Nde*I and *Sac*I and subsequent ligation |
| pLY101 | pXmVENC-2 bearing *bacA*A6S | (a) Amplification of *bacA*A6S from a custom-synthesized gene block by PCR with primers oLY217 and oLY218  (b) Amplification of *bacA*106-483nt from pLY086 by PCR with primers oLY219 and CC1873-rev  (c) Gibson assembly of the two fragments  (d) Digestion of pXmVENC-2 and the assembly product with *Nde*I and *Sac*I and subsequent ligation |

**Supplementary file 6.** **Plasmids used in this study (continued).**

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| **Plasmid** | **Description** | **Construction/Source** |
| pLY102 | pXmVENC-2 bearing *bacA*K7S | (a) Amplification of *bacA*K7S from a custom-synthesized gene block by PCR with primers oLY217 and oLY218  (b) Amplification of *bacA*106-483nt from pLY086 by PCR with primers oLY219 and CC1873-rev  (c) Gibson assembly of the two fragments  (d) Digestion of pXmVENC-2 and the assembly product with *Nde*I and *Sac*I and subsequent ligation |
| pLY104 | pXmVENC-2 bearing *bacA*F2Y | Site-directed mutagenesis of pLY086 by PCR with primers oLY222 and oLY223 |
| pLY107 | pET51b(+) bearing MCS-*mVenus* from pXmVENC-2 | (a) Amplification of MCS-*mVenus* from pXmVENC-2 by PCR with primers oLY227 and oLY228  (b) Insertion into *Nco*I/*Avr*II-treated pET51b(+) by Gibson assembly |
| pLY112 | pLY107 bearing 2x*mreB*EC 1-33nt | (a) Annealing of oligonucleotides oLY240 and oLY241  (b) Ligation into pLY107 cut with *Nde*I and *Kpn*I  (c) Annealing of oligonucleotides oLY242 and oLY243  (d) Ligation into the plasmid from step (b) cut with *Xho*I and *EcoR*I |
| pLY115 | pXmVENC-2 bearing 2x*mreB*EC 1-33nt-*bacA*Δ4-32nt-*mVenus* | (a) Amplification of 2x*mreB*EC 1-33nt from pLY112 by PCR with primers oLY249 and oLY250  (b) Insertion into *Nde*I-treated pLY076 by Gibson assembly |
| pLY116 | pTB146 bearing *bacA*∆4-32nt | (a) Amplification of *bacA*∆4-32nt from pLY076 by PCR with primers oLY251 and oLY002  (b) Insertion into *Sap*I/*BamH*I-treated pTB146 by Gibson assembly |
| pLY117 | pTB146 bearing *bacA*F2Y | (a) Amplification of *bacA*F2Y from pLY104 by PCR with primers oLY252 and oLY002  (b) Insertion into *Sap*I/*BamH*I-treated pTB146 by Gibson assembly |
| pLY118 | pTB146 bearing *bacA*K4SK7S | Amplification of *bacA*K4SK7S from pLY088 by PCR with primers oLY253 and oLY002.  (b) Insertion into *Sap*I/*BamH*I-treated pTB146 by Gibson assembly |
| pLY119 | pTB146 bearing *bacA* | (a) Amplification of *bacA* from pMT812 by PCR with primers oLY001 and oLY002  (b) Insertion into *Sap*I/*BamH*I-treated pTB146 by Gibson assembly |
| pLY131 | pXmVENC-2 bearing *bacA*F2E | Site-directed mutagenesis of pLY086 by PCR with primers oLY269 and oLY270 |
| pLY132 | pXmVENC-2 bearing *bacA*K4E/K7E | (a) Site-directed mutagenesis of in pLY086 by PCR with primers oLY277 and oLY278  (b) Site-directed mutagenesis of the resulting plasmid by PCR with primers oLY279 and oLY280 |
| pLY133 | pTB146 bearing *bacA*F2E | (a) Amplification of *bacA*F2E from pLY131 by PCR with primers oLY273 and oLY002  (b) Insertion into *Sap*I/*BamH*I-treated pTB146 by Gibson assembly. |
| pLY134 | pTB146 bearing *bacA*xE | (a) Amplification of *bacA*xE from pLY131 by PCR with primers oLY274 and oLY002  (b) Insertion into *Sap*I/*BamH*I-treated pTB146 by Gibson assembly |
| pLY135 | pTB146 bearing *bacA*∆M | (a) Amplification of *bacA*∆M from pLY086 by PCR with primers oLY275 and oLY002.  (b) Insertion into *Sap*I/*BamH*I-treated pTB146 by Gibson assembly |
| pLY136 | pTB146 bearing *bacA*K4E/K7E | (a) Amplification of *bacA*K4EK7E from pLY132 by PCR with primers oLY276 and oLY002  (b) Insertion into *Sap*I/*BamH*I-treated pTB146 by Gibson assembly |
| pLY138 | pXmVENC-2 bearing *bacA*F2E/K4E/K7E | (a) Site-directed mutagenesis of pLY131 by PCR with primers oLY281 and oLY278  (b) Site-directed mutagenesis of the resulting plasmid by PCR with primers oLY279 and oLY282 |
| pLY139 | pTB146 bearing *bacA*F2E/K4E/K7E | (a) Amplification of *bacA*F2EK4EK7E from pLY138 by PCR with primers oLY283 and oLY002  (b) Insertion into *Sap*I/*BamH*I-treated pTB146 by Gibson assembly |
| pLY144 | pXmNeonGreenC-4 bearing *creS*∆1-81nt | Deletion of nt 1-81 of *creS* in pLY149 by inverse PCR with primers oLY287 and oLY288 |
| pLY145 | pXmNeonGreenC-4 bearing *bacA*1-24nt*-creS*82-1371nt | Replacement of the first 81 nucleotides of *creS* with *bacA*1-24nt in pLY149 by inverse PCR with primers oLY289 and oLY290 |
| pLY149 | pXmNeonGreenC-4 bearing *creS* | (a) Amplification of *creS* from genomic DNA of CB15N by PCR with primers creS-F and oLY301  (b) restriction with *Nde*I and *Kpn*I  (c) Ligation with pXmNeonGreenC-4 cut with *Nde*I and *Kpn*I |
| pLY154 | pXmVENC-2 bearing *bacA*F130R | Site-directed mutagenesis of pLY086 by PCR with primers oLY004 and oLY005 |
| pLY155 | pXmVENC-2 bearing 2x*mreB*Ec1-33nt*-bacA* F130R/31-483nt | Site-directed mutagenesis of pLY115 by PCR with primers oLY004 and oLY005 |
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**Supplementary file 6.** **Plasmids used in this study (continued).**

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| **Plasmid** | **Description** | **Construction/Source** |
| pMAB234 | pVGFPC-4 bearing pbpC11-396nt-*mCherry* | a) Digestion of pMT993 with *Nde*I and *Nhe*I to isolate a fragment containing *pbpC1*1-396nt-*mCherry*  b) Ligation into pVGFPC-4 cut with *Nde*I and *Nhe*I |
| pMT812 | pXVENC-2 bearing *bacA* | Kühn et al., 2010 |
| pMT813 | pNPTS138 derivative used to generate an in-frame deletion in *bacA* | Kühn et al., 2010 |
| pMT815 | pNPTS138 derivative used to generate an in-frame deletion in *bacB* | Kühn et al., 2010 |
| pMT906 | pXVENN-1 bearing *pbpC* | Kühn et al., 2010 |
| pMT993 | pXCHYC-2 bearing *pbpC*1-396nt | Kühn et al., 2010 |
| pTB146 | Plasmid for overexpression of protein with N-terminal His6-SUMO fusion, AmpR | Bendezu et al., 2009 |
| pVGFPC-4 | Integrative vector for the production of fusion proteins carrying a C-terminal eGFP tag under the control of van, GentR | Thanbichler et al., 2007 |
| pXCHYC-2 | Integrative vector for the production of fusion proteins carrying a C-terminal mCherry tag under the control of P*xyl*, KanR | Thanbichler et al., 2007 |
| pXmVENC-2 | Integration plasmid for the production of fusion proteins carrying a C-terminal mVenus tag under the control of P*xyl*, KanR | (a) Site-directed mutagenesis of *venus* by inverse PCR using pXVENC-2 as template and primers venus-mut-for/-rev  (b) amplification of *venus*(A207K) from the mutagenized vector by PCR using primers Pxyl-GA-for and venus-GA-r2  (c) insertion of the PCR product into *Nde*I/*Nhe*I-treated pXVENC-2 by Gibson Assembly |
| pXmVENN-1 | Integration plasmid for the production of fusion proteins carrying an N-terminal mVenus tag under the control of P*xyl*, Strep/SpecR | (a) Amplification of *mVenus* from pXmVENC-2 by PCR with primers oLY167 and oLY168  (b) Digestions of pXVENN-1 and the PCR product with *Nde*I and *Bsr*GI and subsequent ligation |
| pXmNeonGreenC-4 | Integration plasmid for the production of fusion proteins carrying a C-terminal mNenoGreen tag under the control of P*xyl*, GentR | (a) Amplification of *mNeonGreen* from pmNeonGreen-N1 (Allele Biotechnology) by PCR with primers oLY299 and oLY300  (b) Digestion of pXGFPC-4 and the PCR product with *Age*I and *Nhe*I and subsequent ligation |
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