**Additional File 5: Plasmids, strains, and oligonucleotides used in the study**

**Table S1: List of vectors constructed during this study**

|  |  |  |  |
| --- | --- | --- | --- |
| Number | Plasmid | Source | Assembly |
|  | **Constructs ordered for synthesis** |  |  |
| 1 | pBMH\_CBD\_X\_C1 | Biomatik | Synthesis |
| 2 | pBMH\_C2 | Biomatik | Synthesis |
| 3 | pBMH\_C3 | Biomatik | Synthesis |
| 4 | pBMH\_ScarlessCipC3 | Biomatik | Synthesis |
| 5 | pBMH\_BB2thlOid\_noRBS | Biomatik | Synthesis |
| 6 | pBMH\_BB2fdxOid\_noRBS | Biomatik | Synthesis |
| 7 | pBMH\_BB2facOid\_noRBS | Biomatik | Synthesis |
| 8 | pBMH\_CA\_C0353ss | Biomatik | Synthesis |
| 9 | pBMH\_CA\_C0205ss | Biomatik | Synthesis |
| 10 | pBMH\_Tcpf\_noRBS | Biomatik | Synthesis |
| 11 | pBMH\_RBS\_CatP | Biomatik | Synthesis |
|  |  |  |  |
|  | **Assembly of scaffoldin constructs** |  |  |
| 12 | pJ202\_FLAG\_2xStop | This laboratory (unpublished) |  |
| 13 | pJ204\_2xStop | This laboratory (unpublished) |  |
| 14 | pJ201\_CipC\_CBD\_X\_C1\_C2 | This work | pJ201 + 1 + 2 |
| 15 | pJ202\_CipC\_CBD\_X\_C1\_C2\_C3 | This work | pJ202 + 14 + 3 |
| 16 | pJ201\_BBCipC3\_FLAG | This work | pJ201 + 15 + Annealed oligos |
| 17 | pJ202\_ScarlessCipC3\_FLAG | This work | pJ202 + 4 + Annealed oligos |
| 18 | pBMH\_NCipC\_CA\_C0353ss | Previous work (unpublished) | 1 + 15 + 8 + Annealed oligos |
| 19 | pBMH\_NCipC\_CA\_C0205ss | Previous work (unpublished) | 1 + 15 + 9 + Annealed oligos |
| 20 | pJ204\_NCipC\_stop | Previous work (unpublished) | 1 + 15 + 13 + Annealed oligos |
| 21 | pJ201\_CipC2F3\_CA\_C0353ss | This work | pJ201 + 14 + PCR product |
| 22 | pJ201\_CipC2F3\_CA\_C0205ss | This work | pJ201 + 14 + PCR product |
| 23 | pJ201\_CipC2F3\_stop | This work | pJ201 + 14 + PCR product |
| 24 | pJ202\_RBS\_SaSrtA\_nostop | This work | pJ202 + thlRBS + PCR product |
| 25 | pJ204\_RBS\_SaSrtA\_stop | This work | 13 + 24 |
| 26 | pJ204\_CipC2F3\_CA\_C0205ss\_SaSrtA | This work | pJ201 + 19 + PCR product |
|  |  |  |  |
|  | **Analysis of terminator efficacies** |  |  |
| 27 | pJ201\_lacIQ | Previous work [29] |  |
| 28 | pJ201\_CipA2\_FLAG | Previous work [29] |  |
| 29 | pJ201\_lacIQ\_TpepN | This work | 27 + annealed oligos |
| 30 | pJ201\_lacIQ\_TslpA\_LA | This work | 27 + annealed oligos |
| 31 | pJ201\_lacIQ\_TslpA\_CD | This work | 27 + annealed oligos |
| 32 | pJ201\_lacIQ\_EcoT1 | This work | 27 + annealed oligos |
| 33 | pJ201\_lacIQ\_phiTD1 | This work | 27 + annealed oligos |
| 34 | pJ201\_lacIQ\_TtyrS | This work | 27 + annealed oligos |
| 35 | pJ201\_lacIQ\_TgyrA | This work | 27 + annealed oligos |
| 36 | pJ201\_lacIQ\_TpepN\_CipA2\_FLAG | This work | 30 + 29 |
| 37 | pJ201\_lacIQ\_TslpA\_LA\_CipA2\_FLAG | This work | 31 + 29 |
| 38 | pJ201\_lacIQ\_TslpA\_CD\_CipA2\_FLAG | This work | 32 + 29 |
| 39 | pJ201\_lacIQ\_EcoT1\_CipA2\_FLAG | This work | 33 + 29 |
| 40 | pJ201\_lacIQ\_phiTD1\_CipA2\_FLAG | This work | 34 + 29 |
| 41 | pJ201\_lacIQ\_TtyrS\_CipA2\_FLAG | This work | 35 + 29 |
| 42 | pJ201\_lacIQ\_TgyrA\_CipA2\_FLAG | This work | 36 + 29 |
|  |  |  |  |
|  | **Assembly of enzyme constructs** |  |  |
| 43 | pJ201\_Cel9G\_wt | This laboratory (unpublished) |  |
| 44 | pJ202\_Cel48F\_wt | This laboratory (unpublished) |  |
| 45 | pJ202\_Xyn10A\_wt | This laboratory (unpublished) |  |
| 46 | pJ202\_Cel9G\_FLAG | This work | pJ202 + 43 + annealed oligos |
| 47 | pJ201\_Cel48F\_FLAG | This work | pJ201 + 44 + annealed oligos |
| 48 | pJ202\_Xyn10A\_FLAG | This work | 12 + 45 |
| 49 | pJ201\_lacIQ\_CatP | This work | 27 + 11 |
| 50 | pJ201\_lacIQ\_BB2thlOid\_CatP | This work | 49 + 5 |
| 51 | pJ201\_lacIQ\_BB2fdxOid\_CatP | This work | 49 + 6 |
| 52 | pJ201\_lacIQ\_BB2facOid\_CatP | This work | 49 + 7 |
| 53 | pJ201\_lacIQ\_BB2facOid\_Xyn10A\_FLAG | This work | 52 + 48 |
| 54 | pJ201\_lacIQ\_BB2thlOid\_Xyn10A\_FLAG | This work | 50 + 48 |
| 55 | pJ201\_lacIQ\_BB2thlOid\_Xyn10A\_FLAG\_TtyrS | This work | 34 + 54 |
| 56 | pJ201\_lacIQ\_BB2fdxOid\_Cel9G\_FLAG | This work | 51 + 46 |
| 57 | pJ201\_lacIQ\_BB2facOid\_Cel48F\_FLAG | This work | 52 + 47 |
| 58 | pJ201\_lacIQ\_BB2fdxOid\_Cel9G\_FLAG\_EcoT1 | This work | 32 + 56 |
| 59 | pJ201\_lacIQ\_BB2fdxOid\_Cel9G\_FLAG\_EcoT1\_BB2facOid\_Cel48F\_FLAG | This work | 57 + 58 |
|  |  |  |  |
|  | **Integration vectors** |  |  |
| 60 | pMTL-JH16 | This laboratory [42] |  |
| 61 | pMTL-JH16\_TpepN\_CipA2\_FLAG | This work | 60 + 36 |
| 62 | pMTL-JH16\_TslpA\_LA\_CipA2\_FLAG | This work | 60 + 37 |
| 63 | pMTL-JH16\_TslpA\_CD\_CipA2\_FLAG | This work | 60 + 38 |
| 64 | pMTL-JH16\_EcoT1\_CipA2\_FLAG | This work | 60 + 39 |
| 65 | pMTL-JH16\_phiTD1\_CipA2\_FLAG | This work | 60 + 40 |
| 66 | pMTL-JH16\_TtyrS\_CipA2\_FLAG | This work | 60 + 41 |
| 67 | pMTL-JH16\_TgyrA\_CipA2\_FLAG | This work | 60 + 42 |
| 68 | pMTL-JH16\_lacIQ | Previous work [29] |  |
| 69 | pMTL-JH16\_BBCipC3\_FLAG | This work | 60 + 16 |
| 70 | pMTL-JH16\_ScarlessCipC3\_FLAG | This work | 60 + 17 |
| 71 | pMTL-JH16\_CipC2F3\_CA\_C0353ss | This work | 60 + 21 |
| 72 | pMTL-JH16\_CipC2F3\_CA+C0205ss | This work | 60 + 22 |
| 73 | pMTL-JH16\_CipC2F3\_stop | This work | 60 + 23 |
| 74 | pMTL-JH16\_CipC2F3\_CA\_C0205ss\_SaSrtA | This work | 60 + 26 |
| 75 | pMTL-JH14 | This laboratory [42] |  |
| 76 | pMTL-JH14\_lacIQ | This work | 75 + 68 |
| 77 | pMTL82254\_facOid\_CatP | This laboratory (unpublished) |  |
| 78 | pMTL-JH14\_lacIQ\_facOid | This work | 76 + 77 |
| 79 | pMTL-JH14\_lacIQ\_facOid\_Cel9G-FLAG | This work | 78 + 46 |
| 80 | pMTL-JH14\_lacIQ\_facOid\_Cel48F\_FLAG | This work | 78 + 47 |
| 81 | pMTL-JH14\_lacIQ\_BB2facOid\_Xyn10A\_FLAG | This work | 78 + 53 |
| 82 | pMTL-JH12 | This laboratory [42] |  |
| 83 | pMTL-JH12\_lacIQ | This work | 82 + 68 |
| 84 | pMTL-JH12\_lacIQ\_GF | This work | 83 + 59 |
| 85 | pBW1 | This work | 76 + PCR fragment |
| 86 | pBW1\_BB2facOid\_Xyn10A-FLAG\_TtyrS | This work | 85 + 55 |
|  |  |  |  |
|  | **Sortase expression plasmids** |  |  |
| 87 | pJ201\_lacIQ\_Tcpf\_CatP | This work | 49 + 10 |
| 88 | pMTL82151 | This laboratory  | Synthesis |
| 89 | pMTL82151\_Tcpf\_CatP | This work | 88 + 87 |
| 90 | pMTL82151\_Tcpf\_CaSrtA | This work | 89 + PCR fragment |
| 91 | pMTL82151\_Tcpf\_LmSrtA | This work | 89 + PCR fragment |
| 92 | pMTL82151\_Tcpf\_BcSrtA | This work | 89 + PCR fragment |

This table contains a full list of plasmids used during the study. The source of each plasmid and the assembly strategy have been indicated; numbers in the ‘Assembly’ section refer to the relevant plasmids used for assembly. In the ‘Assembly’ section, plasmids labelled ‘synthesis’ were synthesised by the indicated company, while fragments described as ‘PCR product’ were generated by PCR, and those described as ‘annealed oligos’ were generated by the annealing of two complementary oligonucleotides.

**Table S2: List of bacterial strains used during the study**

|  |  |  |
| --- | --- | --- |
| Strain | Relevant genotype | Details |
| *E. coli*  |  |  |
|  Top10 |  | Cloning strain, ordered from ThermoFisher |
|  pAN-2 | Contains pAN-2 plasmid [84] | Methylation host for transformation into *C. acetobutylicum* |
| *C. acetobutylicum*  |  |  |
|  ATCC 824 |  | Deletion of genes from CA\_P0066 to CA\_P0071 [71] |
|  *pyrE*- |  | Disruption of *pyrE* gene by integration of pMTL-JH12 |
|  CEL01 | thl:BBCipC3\_FLAG | Integration of a gene encoding BBCipC3\_FLAG (BioBrick-2 assembled CipC3-FLAG) at the *thl* locus |
|  CEL02 | thl:ScarlessCipC3\_FLAG | Integration of a gene encoding ScarlessCipC3\_FLAG (single fragment CipC3-FLAG) at the *thl* locus |
|  CEL03 | thl:CipC2F3 | Integration of a gene encoding CipC2F3 (a CipC3 variant with FLAG-tag located between the second and third cohesins) into the *thl* locus |
|  CEL04 | thl:CipC2F3-CA\_C0353ss | Integration of a gene encoding CipC2F3-CA\_C0353ss (CipC2F3 fused to the cell wall sorting signal of the cyclic AMP phosphorylase CA\_C0353) into the *thl* locus |
|  CEL05 | thl:CipC2F3-CA\_C0205ss | Integration of a gene encoding CipC2F3-CA\_0205ss (CipC2F3 fused to the cell wall sorting signal of the Icc-family phosphohydrolase CA\_C0205) into the *thl* locus |
|  CEL06 | thl:CipC2F3-CA\_C0205ss\_SaSrtA | Integration of an operon consisting of CipC2F3-CA\_C0205ss and *S. aureus srtA* into the *thl* locus |
|  CEL07 | pMTL82151:CaSrtA | Introduction of the pMTL82151\_Tcpf\_CaSrtA vector, leading to expression of *C. acetobutylicum* *srtA* (CA\_C0204) from the PTcpf promoter (BB2-format *C. perfringens* thiolase promoter with *C. acetobutylicum* *thl* RBS) |
|  CEL08 | thl:CipC2F3-CA\_C0205ss pMTL82151:CaSrtA | Introduction of the pMTL82151\_Tcpf\_CaSrtA vector into strain CEL05 |
|  CEL09 | thl:CipC2F3-CA\_C0205ss pMTL82151:LmSrtA | Introduction into strain CEL05 of the pMTL82151\_Tcpf\_LmSrtA vector, containing the *L. monocytogenes* *srtA* gene under the control of the PTcpf promoter |
|  CEL10 | thl:CipC2F3-CA\_C0205ss pMTL82151:BcSrtA | Introduction into strain CEL05 of the pMTL82151\_Tcpf\_BcSrtA vector, containing the *B. cereus srtA* gene under the control of the PTcpf promoter |
|  CEL11 | pyrE:Cel9G\_FLAG | Integration of facOid\_Cel9G\_FLAG (a gene encoding Cel9G-FLAG under the transcriptional control of PfacOid, the *C. pasteurianum* ferredoxin promoter with introduced ideal *lac* operator) at the *pyrE* locus |
|  CEL12 | pyrE:Cel48F\_FLAG | Integration of facOid\_Cel9G\_FLAG (a gene encoding Cel48F-FLAG under the transcriptional control of PfacOid) at the *pyrE* locus |
|  CEL13 | pyrE:Xyn10A\_FLAG | Integration of BB2facOid\_Xyn10A\_Flag (a gene encoding Xyn10A-FLAG under the transcriptional control of PBB2facOid, the BB2-format *C. pasteurianum* ferredoxin promoter with introduced ideal *lac* operator and *C. acetobutylicum* *thl* RBS) at the *pyrE* locus |
|  CEL14 | pyrE-:GF | Truncation of *pyrE* and the integration of BB2fdxOid\_Cel9G\_Flag\_EcoT1\_BB2facOid\_Cel48F\_Flag (cassette consisting of a gene encoding Cel9G-FLAG under the control of PBB2fdxOid, the BB2-format *C. sporogenes fdx* promoter with introduced ideal *lac* operator, and *C. acetobutylicum thl* RBS; the EcoT1 terminator; and a gene encoding Cel48F-FLAG under the control of PBB2facOid) at the *pyrE* locus |
|  CEL15 | pyrE:XGF | Integration of the XGF gene cassette at the pyrE locus. The XGF gene cassette consists of a gene encoding Xyn10-FLAG under the control of PBB2thlOid, the BB2-format *thl* promoter with introduced ideal *lac* operator; the TtyrS terminator; a gene encoding Cel9G-FLAG under the control of PBB2fdxOid; the EcoT1 terminator; and a gene encoding Cel48F-FLAG under the control of PBB2facOid. |
|  CEL16 | pyrE:XGF pMTL82151:CaSrtA | Introduction of the pMTL82151\_Tcpf\_CaSrtA vector into strain CEL15 |
|  CEL17 | pyrE:XGF thl:CipC3 | Integration of a gene encoding BBCipC3-FLAG at the *thl* locus and the XGF gene cassette at the *pyrE* locus |
|  CEL18 | pyrE:XGF thl:CipC2F3  | Integration of a gene encoding CipC2F3 at the *thl* locus and the XGF gene cassette at the *pyrE* locus |
|  CEL19 | pyrE:XGF thl:CipC2F3 pMTL82151:CaSrtA | Introduction of the pMTL82151\_Tcpf\_CaSrtA vector into strain CEL18 |
|  CEL20 | pyrE:XGF thl:CipC2F3-CA\_C0205ss | Integration of a gene encoding CipC2F3-CA\_C0205ss at the *thl* locus and the XGF gene cassette at the *pyrE* locus |
|  CEL21 | pyrE:XGF thl:CipC2F3-CA\_C0205ss pMTL82151:CaSrtA | Introduction of the pMTL82151\_Tcpf\_CaSrtA vector into strain CEL20 |
|  A2 | thl:CipA2 | Integration of a gene encoding CipA2-Flag at the *thl* locus [29] |
|  T1 | thl:TpepN\_CipA2 | Integration of TpepN\_CipA2 (gene encoding CipA2 downstream of the *L. lactis pepN* terminator) at the *thl* locus |
|  T2 | thl:TslpA\_LA\_CipA2 | Integration of TslpA\_LA\_CipA2 (gene encoding CipA2 downstream of the *L. acidophilus slpA* terminator) at the *thl* locus |
|  T3 | thl:TslpA\_CD\_CipA2 | Integration of TslpA\_CD\_CipA2 (gene encoding CipA2 downstream of the *C. difficile slpA* terminator) at the *thl* locus |
|  T4 | thl:EcoT1\_CipA2 | Integration of EcoT1\_CipA2 (gene encoding CipA2 downstream of the *E. coli rrnB* T1 terminator) at the *thl* locus |
|  T5 | thl:phiTD1\_CipA2 | Integration of phiTD1\_CipA2 (gene encoding CipA2 downstream of the *B. subtilis* Φ29 phage late TD1 terminator) at the *thl* locus |
|  T6 | thl:TtyrS\_CipA2 | Integration of TtyrS\_CipA2 (gene encoding CipA2 downstream of the *B. subtilis tyrS* tRNA terminator) at the *thl* locus |
|  T7 | thl:TgyrA\_CipA2 | Integration of TgyrA\_CipA2 (gene encoding CipA2 downstream of the *B. subtilis gyrA* terminator) at the *thl* locus |

This table contains the details of the *E. coli* and *C. acetobutylicum* strains used during this study, and provides details of the modifications made to the recombinant *C. acetobutylicum* strains.

**Table S3: Oligonucleotides used during this study**

|  |  |
| --- | --- |
| Oligonucleotide | Sequence |
| Flag\_fw | GAATTCGCGGCCGCACTAGTGATTACAAGGATGACGACGATAAGTAATGAGCTAGCGCGGCCGCCTGCAG |
| Flag\_rev | CTGCAGGCGGCCGCGCTAGCTCATTACTTATCGTCGTCATCCTTGTAATCACTAGTGCGGCCGCGAATTC |
| TpepN\_fd | gcGAATTCGCGGCCGCACTAGTtaatttataaataaaaatcaccttttagaggtggtttttttatttataaattaGCTAGCGCGGCCGCCTGCAGcg |
| TpepN\_rev | cgCTGCAGGCGGCCGCGCTAGCTAATTTATAAATAAAAAAACCACCTCTAAAAGGTGATTTTTATTTATAAATTAACTAGTGCGGCCGCGAATTCgc |
| TslpA\_LA\_fd | gcGAATTCGCGGCCGCACTAGTtgaaaaaggcagagcgaaagctctgtcttttttGCTAGCGCGGCCGCCTGCAGcg |
| TslpA\_LA\_rev | cgCTGCAGGCGGCCGCGCTAGCAAAAAAGACAGAGCTTTCGCTCTGCCTTTTTCAACTAGTGCGGCCGCGAATTCgc |
| TslpA\_CD\_fd | gcGAATTCGCGGCCGCACTAGTaaatataaaaagacttctcagatgagaagtcttttttgtgaaaGCTAGCGCGGCCGCCTGCAGcg |
| TslpA\_CD\_rev | cgCTGCAGGCGGCCGCGCTAGCTTTCACAAAAAAGACTTCTCATCTGAGAAGTCTTTTTATATTTACTAGTGCGGCCGCGAATTCgc |
| EcoT1\_fd | gcGAATTCGCGGCCGCACTAGTccaggcatcaaataaaacgaaaggctcagtcgaaagactgggcctttcgttttatctgttgtttgtcggtgaacgctctcGCTAGCGCGGCCGCCTGCAGcg |
| EcoT1\_rev | cgCTGCAGGCGGCCGCGCTAGCGAGAGCGTTCACCGACAAACAACAGATAAAACGAAAGGCCCAGTCTTTCGACTGAGCCTTTCGTTTTATTTGATGCCTGGACTAGTGCGGCCGCGAATTCgc |
| phiTD1\_fd | gcGAATTCGCGGCCGCACTAGTaacaatcaaaagaaaagcctatcgtctgaggaacggtaggctcttttgtagcatatagttgGCTAGCGCGGCCGCCTGCAGcg |
| phiTD1\_rev | cgCTGCAGGCGGCCGCGCTAGCCAACTATATGCTACAAAAGAGCCTACCGTTCCTCAGACGATAGGCTTTTCTTTTGATTGTTACTAGTGCGGCCGCGAATTCgc |
| TtyrS\_fd | gcGAATTCGCGGCCGCACTAGTataatcaatcgtcccttcgtgtaaacgaaggggcgttttttatttGCTAGCGCGGCCGCCTGCAGcg |
| TtyrS\_rev | cgCTGCAGGCGGCCGCGCTAGCAAATAAAAAACGCCCCTTCGTTTACACGAAGGGACGATTGATTATACTAGTGCGGCCGCGAATTCgc |
| TgyrA\_fd | gcGAATTCGCGGCCGCACTAGTaagaagaagtgtgaaaaagcgcagctgaaatagctgcgcttttttgtgtcataaGCTAGCGCGGCCGCCTGCAGcg |
| TgyrA\_rev | cgCTGCAGGCGGCCGCGCTAGCTTATGACACAAAAAAGCGCAGCTATTTCAGCTGCGCTTTTTCACACTTCTTCTTACTAGTGCGGCCGCGAATTCgc |
| 9G\_arm\_fd | aattGCTAGCGTGTAGTAGCCTGTGAAATAAGTAAGG |
| 9G\_arm\_rev | aattGGCGCGCCATATACAGATACTTTGGATGGCGTACAGC |
| C2FC3FD | ttaaACTAGTGATTACAAGGATGACGACGATAAGCAACCTACAAAGGAACTTAAAGTAGC |
| C2FC3\_BMHrev | GAAACAGCTATGACCATGATTACG |
| C2FC3\_204rev | CTTAGGTACGAACTCGATTGACG |
| SrtA\_start\_fd | ttaaCATATGAAAAAATGGACAAATCGATTAATGACAATCG |
| SrtA\_nostop\_rev | ttaaGCTAGCTTTGACTTCTGTAGCTACAAAGATTTTACG |
| CaSrtA\_fd | aaaCATATGAAAAAGTTAAACATTATAGCAGCTACACTTATATCATCAGG |
| CaSrtA\_rev | aaaGCTAGCTTAAATTCTTTTAGCTTTTATAATAAGTCTATGAGTAGCTATTCTTATAGG |
| Bcer\_fw | cgcatAtgaataagcaaagaatttatagtatagtagc |
| Bcer\_rev | agcgctagcttacttcttcgccttcgttcttact |
| Gb\_Lm\_Fw | agtgtatcaaaatttaggaggttagttCatatgttaaagaaaacaattgcaataataattttaatcatcgggct |
| Gb\_Lm\_Rev | atacttttccttcatcaaaattcccccaaaatgctagcttatttactagggaaatatttattctctaattct |

This table contains a list of the oligonucleotides used during this study. Bases in lower case indicate those that are included solely for the purpose of facilitating subsequent restriction digests, and are thus not included in the final constructs. Oligonucleotides were ordered from Eurofins MWG Operon.