Supplementary Information

Applied Microbiology and Biotechnology

Improved site-specific mutagenesis in *Rhodococcus opacus* using a novel conditional suicide plasmid

Authors

Garima Jain¹, Helga Ertesvåg¹*

¹Department of Biotechnology and Food science, Norwegian University of Science and Technology (NTNU), Trondheim, NO-7491, Norway

*Correspondence to: helga.ertesvag@ntnu.no

### Supplementary Table S1: Plasmids used in the study.

| Name               | Description                                                                 | Source                        |
|--------------------|-----------------------------------------------------------------------------|-------------------------------|
| pEC18Kmob2         | pGA1-based expression vector. *P*lac, Km'<br>                               | (Tauch et al. 2002)           |
| pUV15tetORm::lucferase | pAL5000 based vector. Luciferase gene (*luc*) controlled by |
|                    | *P~myc1tetO*, TetR controlled by *P~myc*, Km'<br>                            | (Grant et al. 2013)           |
| pMDXluc            | pAL5000 based vector. *xylS* controlled by *P~myc1/TetO*, TetR reverse    | (Dragset et al. 2015)         |
|                    | repressor variant #28 (TetR#28) controlled by *P~myc*, *lac* controlled  |                                |
|                    | by *Pm*, Km'.                                                               |                                |
| pXMIJ19            | *E. coli-C. glutamicum* shuttle vector. *P*lac, *lacI*, Cm'.               | (Jakoby et al. 1999)          |
| pDD120             | Derivative of pB264. *P~const*, Km'<br>                                      | (DeLorenzo et al. 2018)       |
| pCR™Blunt II-TOPO® | CoIE1-derivative, *ccdB*, Zeocin', Km'.<br>                                 | Life Technologies             |
| pJQ200mp18         | P15-based suicide vector. SacB. Gm'<br>                                     | (Quandt and Hynes 1993)       |
| pHE95              | Conditional suicide plasmid for Gram-negative bacteria. Km', Ap'<br>       | (Gimmestad et al. 2009)       |
| pRMG2              | Derivative of pEC18Kmob2 containing *luc* from pUV15tetORm::controlled by | This study                    |
|                    | *P~lac*, Km'<br>                                                           |                               |
| pRMG3              | Derivative of pRMG2 with a NotI-linker (New English Biolabs Inc) replaced  | This study                    |
|                    | a small *Asel*-DNA fragment, Km'<br>                                       |                               |
| pRMG4              | Derivative of pRMG3 where a NotI-Ndel fragment from pMDX luc containing   | This study                    |
|                    | *Pm* and encoding the regulatory proteins *XylS* and TetR#28 replaced *P*lac.<br>Km'<br> |                               |
| pRMG5              | Derivative of pRMG4 where a NotI-EcoRV fragment encoding *XylS* was removed. Km'<br> | This study                    |
| pHE511             | A 1.2 kb PCR fragment (primer pair 1/2) from pXMJ19 encoding *lacI* was    | This study                    |
|                    | inserted (SLIC) into NotI-restricted pRMG3. Km'<br>                        |                               |
| pHE513             | Derivative of SacI-Ndel digested pRMG5 where a SacI-Ndel fragment from pDD120 containing *P~const* was inserted. Km'<br> | This study                    |
| pHE518             | Derivative of BsiWI-Ndel digested pRMG5 where a BsiWI-Ndel fragment from pUV15tetORm::luciferase containing *P~myc1/TetO* was inserted. Km'<br> | This study                    |
| pHE523             | Two PCR fragments from pRMG4 generated using primer pairs 3/4 and 5/6, were combined. Contains the control elements from pMDXluc including the 5' region between *P*~m~ and Ndel, but *P*~m~ is controlling rep. Km'<br> | This study                    |
| **pHE524** | Two PCR fragments from pRMG4 generated using primer pairs 4/7 and 6/8, were combined. Contains the control elements from pMDXluc but \( P_m \) is controlling \( rep \). The UTR of \( rep \) with the cRNA is retained. Km' | This study |
| **pMV10** | Derivative of pCR™Blunt II-TOPO® where three PCR products were combined. Contains 1.1 kb including the 5' part of \( PD630\_RS00415 \) (primers 9/10) and 1.2 kb including the 3' part of this gene (primers 11/12) separated by \( cat \) from pXMJ19 (primers 13/14) | This study |
| **pMV11** | Derivative of pQ200mp18 in which a 3.5 DNA fragment from pMV10 was inserted. Gm' Cm'. Km' | This study |
| **pMW3** | A 1.2 PCR fragment (primers 15/16) from \( R. \) opacus containing \( accD3 \) inserted in pCR™Blunt II-TOPO®, Km' | This study |
| **pMW4** | A 4.2 kb PCR fragment (primers 17/18) from \( R. \) opacus encoding \( fad32 \) and the 5' part of \( pks \). Km' | This study |
| **pDD112** | Derived from pNG2, chloramphenicol resistance gene (CM) optimized for \( R. \) opacus, \( P_{const} \), Gmr. Km' | This study |
| **pGW3** | A 1.2 PCR fragment (primers 19/20) from pDD112 containing \( cat \), \( P_{const} \) and \( rrnB \) was inserted, Km', Cm' | This study |
| **pGW4** | Ligation of a BamHI/XbaI digested DNA fragment from pGW2 containing \( cat \), \( P_{const} \) and \( rrnB \) ligated with a 4.8 kb SpeI/BglII digested fragment of pMW4 containing the 5' 1.3 kb of \( fad32 \), Km', Cm' | This study |
| **pGW5** | A PsblAI/XbaI digested DNA fragment encoding \( sacB \) from pGW1 was ligated to PsblAI/XbaI digested fragment of pHE524. Km' | This study |
| **pGW6** | A BsiWI cut pGW5 where a 4.1Kb BrsrGI/Acc651 restriction fragment from pGW4 containing ~1 kb homologous construct flanking each side of CM was inserted. Km', Cm', Fig. S5 | This study |
| **pGW7A** | A 1.2 kb PCR fragment (primers 23/24) containing 3' part of \( pks \) (1107 bp) and some 5' part of \( accD3 \) (69 bp) from \( R. \) opacus total DNA, cloned in pCR™Blunt II-TOPO®, Km' | This study |
| **pGW8A** | Complete \( accD3 \) excised on 1.56 kb NdeI/EcoRI restriction fragment from pMW3 and ligated into corresponding sites of pHE513. Km' | This study |
| **pGW7** | A 1.2 kb NsiI/AgeI restricted DNA fragment from pGW7A containing part of \( pks \) and the 5' part of \( accD3 \) replaced the \( fad32-CM \) encoding parts of pGW6. Km', Fig. S5 | This study |
| **pGW8** | PCR fragment (primers 25/26) from pGW8A carrying \( accD3 \) and UTR+RBS, SLIC cloned into Sphl/DraII cut pDD112 \( P_{const} \), Gm' | This study |

References to Table S1

DeLorenzo DM, Rottinghaus AG, Henson WR, Moon TS (2018) Molecular toolkit for gene expression control and genome modification in \( Rhodococcus \) opacus PD630. ACS Synth Biol 7:727-738 doi:10.1021/acssynbio.7b00416

Dragset MS, Barczak AK, Kannan N, Merk M, Flo TH, Valla S, Rubin EJ, Steigedal M (2015) Benzoic acid-inducible gene expression in \( Mycobacteria \). PLoS One 10:e0134544 doi:10.1371/journal.pone.0134544
Gimmestad M, Ertesvåg H, Heggeset TMB, Aarstad O, Svanem BIG, Valla S (2009) Characterization of three new *Azotobacter vinelandii* alginate lyases, one of which is involved in cyst germination. J Bacteriol 191:4845-53

Grant SS, Kawate T, Nag PP, Silvis MR, Gordon K, Stanley SA, Kazyanskaya E, Nietupski R, Golas A, Fitzgerald M, Cho S, Franzblau SG, Hung DT (2013) Identification of novel inhibitors of nonreplicating *Mycobacterium tuberculosis* using a carbon starvation model. Acs Chem Biol 8:2224-2234 doi:10.1021/cb4004817

Jakoby M, Ngouoto-Nkili CE, Burkovski A (1999) Construction and application of new *Corynebacterium glutamicum* vectors. Biotechnol Tech 13:437-441 doi:10.1023/A:1008968419217

Quandt J, Hynes MF (1993) Versatile suicide vectors which allow direct selection for gene replacement in gram-negative bacteria. Gene 127:15-21 doi:0378-1119(93)90611-6

Tauch A, Kirchner O, Löffler B, Götker S, Pühler A, Kalinowski J (2002) Efficient electrotoration of *Corynebacterium diphtheriae* with a mini-replicon derived from the *Corynebacterium glutamicum* plasmid pGA1. Curr Microbiol 45:362-7 doi:10.1007/s00284-002-3728-3
**Supplementary Table S2:** Oligonucleotide primers used in the study.

| S.No. | PCR Primers | Sequence (5’ to 3’)                        |
|-------|-------------|---------------------------------------------|
| 1     | p456F       | CGTGGCCGATTCATTATTTGCAACAGCTGATTGCCCTTCACC |
| 2     | p456R       | AGTGAAGCTACACTACATTATTGCGGGCGGATCAGCTTGCAATTC |
| 3     | VpA         | GCTAGAGTCATATGTGACTCCATTATTAG               |
| 4     | VpB         | CTCCTAGCTCTCGAAGCGAAAGGAAAGCAG             |
| 5     | REPpA       | GAGTCATGAAACATATGACTCTAGCCGGATCCG          |
| 6     | REPPpB      | CTCTTTGGGCTCGAGAGCTAGGAGCGAGACGAC          |
| 7     | VRNAA       | AGGTATTTTGTCATAAAGCCCTAAGGGGTAG            |
| 8     | REPRNAA     | TTAGGCTTTATGCAACAAATACCTGAAAAGTTG          |
| 9     | Oppgen1F    | GCCGGTACGTTGCCCATCTG                      |
| 10    | Oppgen1R    | GATTTGAGTTCATGCTCGTCGCTCCGACTGGACT        |
| 11    | Nedgen1F    | ACGACGACATGCTGAATCAACGCCGGACTGAA          |
| 12    | Nedgen1R    | GACGACGGGAAGCATGAAAC                      |
| 13    | CmF         | TACGCTAGGAGTTGGGTCGCTTTGGT                |
| 14    | CmR         | TACGCTAGGAGTTGGGTCGCTTTGGT                |
| 15    | AccD3F      | CATATGACACGACCGACGACGAG                   |
| 16    | AccD3R      | CGGCGGGTGGGCCAAGCTTTAC                   |
| 17    | UpperF      | TTCGCGGAAAGGAAGTCTG                       |
| 18    | UpperR      | GGATGATGGAAGATGCGACCTG                    |
| 19    | PMV10F      | CCAATATTTTCTTGTAGCTAATCGATAGCTGACGACGGAAC |
| 20    | PMV10R      | CTTTTTTTTTTTTGGAGCTAGTGATCGCTCCTTGCGGTACGTTTC |
| 21    | RoCMF       | CCTAGGTTGCCTTTGCCTTTGCCTAC               |
| 22    | RoCMR       | CCTAGGTTGCCTTTGCCTTTGCCTAC               |
| 23    | UppaccD3F   | ATGCCATTCTCGCGCGACGCGAAAGGTCTCC          |
| 24    | UppaccD3R   | ACCGGTTCGCGGTTATTTGGCCGTTCG             |
| 25    | dd112F      | GCCCGAAATGAGCGACGATCC                     |
| 26    | dd112R      | CCTTACTCTCGTTTTCTACGTCGACGACGCAAG         |
| 27    | testGJ7f1strecombF3 | AAACGACGGCGGATGAAATTG        |
| 28    | testGJ7f1strecombR3  | CCGACCTCGATGAAACCTTC            |
| 29    | testGJ7f1strecombR2  | CCGAGGAGTCTACATGTTC            |
| 30    | testGJ7f1strecombR2  | CCGAGGAGTCTACATGTTC            |
| 31    | delaccD3F   | CGTGGCAGGTCCTCCTACCAAGAAAG           |
| 32    | delaccD3R   | TGTGCGACCTGTCCTTTCCC               |
| 33    | Aegl2sacBF  | GGGTGTCGCTCCTAGTCTACCTGCGGCCCTCCC       |
|   |   |   |
|---|---|---|
| 34 | Agl2sacBR | TACCTGCTTTCTTTTGCGCTACGCGTGCACGGTATCGATAAG |
| 35 | testwt3F | GACGGCCGCAACCATTAC |
| 36 | testwt3R | CTTCCCACGTCAGTTCCATC |
| 37 | testmutF | GCAAGTTCAACGAGGCAATTC |
| 38 | testmutR | AGGCCAGATTCTCACCATAG |
| 39 | testvecF2 | CCAGCTCATCTGGCTCATATG |
| 40 | testvecR2 | CATCGTCGCTAGAGCCTTTCC |

**Fig. S1**: Mycolic acid gene cluster in *R. opacus*. *fad32* and *accD3* are present upstream and downstream of *pks*, respectively in the *R. opacus* genome.

**Fig. S2**: Standard suicide recombination vector pMV11 based on pJQ200mp18. The elements shown are P15Aori for replication in *E. coli*, oriT for conjugative transfer, cat encodes resistance to chloramphenicol, Gm' for gentamycin resistance and homologous arms flanking cat.
Fig. S3: Conjugation frequencies obtained using conditional suicide plasmid pGJ1 and standard suicide plasmid pMV11. Conjugation frequencies were defined as the number of colonies obtained on selective plates divided by the number of cells found on LA Nal. No colonies were obtained in the experiments using pMV11.

Fig S4: Homologous recombination with pGJ1. R1-R10 (Cm\(^r\), Km\(^r\), sucrose\(^r\) colonies), S1- Cm\(^s\), Km\(^s\) sucrose\(^s\) colony, C-control (pGJ1) Primers- CmF/R, Expected band- 0.94 kb. R4, R6, R8 and R9 shows the expected band as control.
Fig. S5: Conjugative, conditional suicide plasmids pGJ6 and pGJ7 used to construct mycolic acid negative *R. opacus* mutants.

Fig. S6: Colony PCR to check *R. opacus::pGJ6* mutant with primers testwt3F/R (1 kb). All Km<sup>+</sup> colonies tested (1-17) after the second recombination showed the band indicative of the wild type strain. R: wild type *R. opacus* (Control)
Fig. S7: Image showing part of the accD3 partially deleted in plasmid pGJ7 and the location of primers used for testing putative mutants.

Fig. S8: Mutant construction steps for R. opacus ΔaccD3 mutant strain via homologous recombination
**Fig. S9:** PCR testing *R. opacus ΔaccD3* mutant strain without complementation with primers delaccD3F/R. R: *R. opacus* wild type, GJ7(7): the strain selected after first recombination step. Colonies 1-10 were picked after sucrose selection. Expected bands for wild type: 0.719 kb, mutant: 0.192 kb and first recombinants: 12.4, 0.719 and 0.192 kb. All colonies (1-10) are either wild type or first recombinants.