Table S1

| **Bacterial strain or plasmid** | **Relevant features or genotype** | **Reference** |
|---|---|---|
| *Clostridioides difficile* UK1 | Clinical isolate | (Sorg and Sonenshein, 2010) |
| *Escherichia coli* DH5α | *endA1 recA1 deoR hsdR17 ($r_K^-$ $m_K^+$)* | NEB |
| *Escherichia coli* S17-1 | Strain with integrated RP4 conjugation transfer function; favors conjugation between *E. coli* and *C. difficile* | (Teng et al., 1998) |
| *Clostridioides difficile UK1::pdcB* | UK1 with intron insertion within *pdcB* (*CDR20291_0685*) | This study |
| *Clostridioides difficile* UK1::*codY* | UK1 with intron insertion within *codY* | (Nawrocki et al., 2016) |
| pMTL007-CE5 | ClosTron plasmid | (Heap et al., 2010) |
| pMTL007-CE5:Cdi-*pdcB*-840-841s | pMTL007-CE5 with group II intron targeted to *pdcB* | This study |
| pMTL007-CE5:Cdi-*recV*-143-144a | pMTL007-CE5 with group II intron targeted to *recV* | This study |
| pRPF185 | *E. coli/ C. difficile* shuttle plasmid | (Fagan and Fairweather, 2011) |
| pBA042 | pRPF185 containing 1.5 kb upstream region of *pdcB* in Translucent orientation. | This study |
| pBA043 | pRPF185 containing ~1.5 kb *pdcB* upstream region locked in Translucent orientation | This study |
| pBA045 | pRPF185 containing 1.5upstream region of *pdcB* in Opaque orientation. | This study |
| pBA046 | pRPF185 containing ~1.5 kb *pdcB* upstream region locked in Opaque orientation | This study |
| pBA048 | pRPF185 containing PdcB-EAL domain | This study |
| pBA050 | pRPF185 containing PdcA-EAL domain | This study |
| pBA051 | pRPF185 containing the *tet* promoter alone | This study |
| pBA052 | pRPF185 containing *dccA* under *tet* promoter | This study |

## References:


Fagan, R.P., Fairweather, N.F., 2011. *Clostridium difficile* Has Two Parallel and Essential Sec Secretion Systems. J. Biol. Chem. 286, 27483–27493. https://doi.org/10.1074/jbc.M111.263889

Heap, J.T., Kuehne, S.A., Ehsaan, M., Cartman, S.T., Cooksley, C.M., Scott, J.C., Minton, N.P., 2010. The ClosTron: Mutagenesis in Clostridium refined and streamlined. J. Microbiol. Methods 80, 49–55. https://doi.org/10.1016/j.mimet.2009.10.018

Nawrocki, K.L., Edwards, A.N., Daou, N., Bouillaut, L., McBride, S.M., 2016. CodY-Dependent Regulation of Sporulation in Clostridium difficile. J. Bacteriol. 198, 2113–2130. https://doi.org/10.1128/JB.00220-16

Sorg, J.A., Sonenshein, A.L., 2010. Inhibiting the initiation of Clostridium difficile spore germination using analogs of chenodeoxycholic acid, a bile acid. J. Bacteriol. 192, 4983–4990. https://doi.org/10.1128/JB.00610-10

Teng, F., Murray, B.E., Weinstock, G.M., 1998. Conjugal transfer of plasmid DNA from Escherichia coli to enterococci: a method to make insertion mutations. Plasmid 39, 182–186. https://doi.org/10.1006/plas.1998.1336

## Table S2 Oligonucleotides used for PCR reactions

| Name | Sequence (5' → 3') | Description |
|---|---|---|
| EBS-U | CGAAATTAGAAACTTGCGTTCAGT AAAC | Group II intron specific primer |
| ORG771 | GACTGAGCTCTGGGGAAATGTGTTTGATGA GATAAAAAAATTAGTAAAATA | Forward primer with with SacI to clone *CDR20291_0685* (*pdcB*) in pRPF185 |
| ORG772 | AACTGGATCCTCACTTATCGTCGTCATCCT TGTAATCCTTTGATAGTTGAAATTTATAAA ATTCAGAGGCAC | Reverse primer with with BamHI to clone *CDR20291_0685* (*pdcB*) in pRPF185 |
| ORG788 | GAGCTCAAGAGGAGTGGTTGAAAATGGCAA CAAGACCTATAGAAATAG | Forward primer with SacI to amplify *recV* |
| ORG789 | GGATCCTTAATGATGATGATGATGATGACC AATAAAGAAATTTTCACTAGCTTCATTAAT AGCTTGCTGAG | Reverse primer with BamHI and 6X His to amplify *recV* |
| ORG844 | GTTGTAAAAAAGTTACTATTTATTGAAAAT TTAGATACTTTTCTAAAATTATCTATG | Forward primer for CodY binding site in *pdcB* upstream region in Opaque orientation |
| ORG845 | CATAGATAATTTTAGAAAAGTATCTAAATT TTCAATAAATAGTAACTTTTTTACAAC | Reverse primer for CodY binding site in *pdcB* upstream region in Opaque orientation |
| ORG852 | GGTACCAGTTTAGGATAAAGTATTGCAAGA ACCAATCAG | Forward primer with KpnI site to clone ~1.5 kb upstream region of *pdcB* gene |
| ORG853 | GAGCTCCTTTTCCCCCTACAATATTACTAT TAGTGTAGTTTAATCAAC | Reverse primer with SacI site to clone ~1.5 kb upstream region of *pdcB* gene |
| ORG866 | ATGTATATTTTTATAGCTATTTTATTATAA AAAAAAAGAACCCTCGCATGTGTAAGGGTT ACTTTTTTTCTCTATTTTTTTTATTATATC ACTATTTTTTCCCTATTTCAATATTTTGAC ATTTTTTCCATTATACTGA | Forward primer to mutagenize inverted repeat from translucent orientation |
| ORG867 | TCAGTATAATGGAAAAAATGTCAAAATATT GAAATAGGGAAAAAATAGTGATATAATAAA AAAAATAGAGAAAAAAAGTAACCCTTACAC ATGCGAGGGTTCTTTTTTTTTATAATAAAA TAGCTATAAAAATATACAT | Reverse primer to mutagenize inverted repeat from translucent orientation |
| ORG868 | CATAAATTTAACTATTAACATAGATAATTT TAGAAAAGTATCTAAATTTTCAATAAATAG TAACTTTCGCATGTGTAAGGGTTACTTTTT TTCTCTATTTTTTTTTATTATATCACTATTT TTTCCCTATTTCAATATTTTGAC | Forward primer to mutagenize inverted repeat from opaque orientation |
| ORG869 | GTCAAAATATTGAAATAGGGAAAAAATAGT GATATAATAAAAAAAAATAGAGAAAAAAAGT AACCCTTACACATGCGAAAGTTACTATTTA TTGAAAATTTAGATACTTTTCTAAAATTAT CTATGTTAATAGTTAAATTTATG | Reverse primer to mutagenize inverted repeat from opaque orientation |
| ORG879 | GTATTATTTTGGTAAATATATTGTTACAAA AGGTTTATATTTTGC | Forward primer (*cmrRST* upstream): specific for orientation similar to published (410 bps) |

| | | |
|---|---|---|
| ORG880 | GGAAATATTGACAAAATAATATTACAATGTTAGAATAA | Reverse primer common for both orientation or *cmrRST.* |
| ORG881 | AGTATAATGCTATTATAATAAGAAAATAACTTTTTTATAAACATTGAGAT | Forward primer (*cmrRST* upstream): specific for flipped orientation (540 bps) |
| ORG882 | GAGCTCGGGGGAAAAGATGTATCCAGAAGATGGAGATAATTATTTAGATTTATTTAAACA | Forward primer with *SacI* to clone PdcB-EAL in pRPF185 |
| ORG883 | GGATCCTCACTTTGATAGTTGAAATTTATAAAATTCAGAGGCACTTACAGGTCTTCC | Reverse primer with *BamHI* to clone PdcB-EAL in pRPF185 |
| ORG884 | GAGCTCGGAGGAGATAAGATGCAAGAAATATTAAAAAATAAAATGTATTCAT | Forward primer with *SacI* to clone PdcA-EAL in pRPF185 |
| ORG885 | GGATCCTTAATTATCTAGCTTTAAAAGGTCAAAGATTTCTGTTGCTGTCTGCGGTTTTCC | Reverse primer with *BamHI* to clone PdcA-EAL in pRPF185 |
| ORG886 | TAATAAAATAGCTATAAAAATATACATATCAAATCAAATTAAG | Forward primer to amplify the flip orientation of *pdcB* upstream in Translucent strain. |
| ORG887 | CTTCTTAATTTGATTTGATATGTATATTTTTATAGC | Forward primer to amplify the flip orientation of *pdcB* upstream in Opaque strain. |
| ORG888 | CTGGATTTTTAAATTTATGTACTTTAAAATGGATATCC | Reverse primer to amplify the flip orientation both translucent and opaque of *pdcB* upstream. |
| ORG846 | GGTCTAAACTTAAAGAGTTCGATTCTTCTCTCATCTTTTCCCCCTACAATATTACTATTAGTG | SP1 primer for 5' RACE |
| ORG847 | CTCTAAGTATTTCATTAAAAAGATGTTAGTAATGACCACTAAATAAAAGTGGACGATTTTGTGG | SP2 primer for 5' RACE |
| ORG848 | CAATTATTTTATCCATGTAAAAATCATGTACTTTTATCTCAACGAATGTCCC | SP3 primer for 5' RACE |
| ORG921 | ATGGAAACTATGTACGTAATAATTAAAATAAAA | Forward primer to amplify *pdcB* promoter_1 |
| ORG922 | ACATTGACATTCTAATTCTGTAGTGTAATCGTA | Forward primer to amplify *pdcB* promoter_2 |
| ORG925 | ACTATTTATTGAAAATTTAGATACTTTTCTAAAATTATCTATG | Forward primer to amplify *pdcB* promoter_3 |
| ORG926 | ATACTTTTCTAAAATTATCTATGTTAATAGTTAAAT | Forward primer to amplify *pdcB* promoter_3 |

## Supplementary Methods

### Toxin ELISA

*C. difficile* cytosolic toxins were measured as described previously (46,47). In brief, one ml of *C. difficile* 16 h cultures were harvested. The cells were centrifuged at 17,000 g for 1 min and washed with sterile 1X PBS. The resulting pellets were resuspended in 200 µl of sterile 1X PBS, sonicated and centrifuged to harvest the cytosolic protein. 100µg of cytosolic proteins was used to measure the relative toxin levels using *C. difficile* premier Toxin A &B ELISA kit from Meridian Diagnostics Inc. (Cincinnati, OH).

### Surface Motility assay

*C. difficile* cultures were grown until mid-exponential phase in TY broth at 37°C. After adjusting their OD600 to 0.5, 10µl of each strain was spotted in TY media plates with 0.5% w/v agar for swimming motility and 1.8% w/v agar for swarming motility. The plates were incubated at 37°C for 72 hours and the motility was quantified by measuring the diameter of the spotted cultures. Since the spotted cultures were not a perfect circle, multiple diameters were taken for a single spotted culture and calculated the average. Motility assay was independently repeated at least three times.

### Biofilm Assay

Biofilm assay was carried out as described previously ( Purcell *et al.*, 2017) with slight modification. *C. difficile* cultures were grown until mid-exponential phase in TY broth at 37°C. After adjusting their OD600 to 0.5, cultures were seeded at 1:100 dilution to an untreated 24 well tissue culture plates containing 0.8 ml of TY medium. The plates were incubated at 37°C in an anaerobic chamber for 72 hours. The culture medium was carefully removed and the adherent biofilm was washed once with 1XPBS. The biofilm was stained for 30 min at room temperature with 0.1% (wt/vol) crystal violet in water. Excess crystal violet was carefully removed, and the biofilm were

washed twice with 1XPBS. To quantify the biofilms, the crystal violet was solubilized with ethanol and absorbance was read at 570 nm. Experiments were done at least three times.

**Genomic DNA extraction**

UK1_T and UK1_O cultures streaked from a single colony were allowed to grow overnight in TY agar at 37°C. Ten ml PBS was used to harvest the cells grown in the TY agar plate and was collected by centrifuging at 3,000g for 30 min at 4°C. Cell pellets were washed with 1 ml of TE (Tris-EDTA) buffer. After centrifugation, cell pellets were resuspended in 600 µl of genomic DNA solution. 60 µl of 50 mg/ml of lysozyme solution was added and the mixture was allowed to incubate for 2 hours at 37°C. 100 µl of 20% Sarkosyl, 15 µl of RNAase A (10mg/ml) and 15 µl of proteinase K (10 mg/ml) were added and incubated for additional 30 min at 37°C. 600 µl of 25:24:1 (v/v/v) phenol/chloroform/isoamyl alcohol was added and mixed by vortexing. The mixture was centrifuged at maximum speed for 10 min. The upper aqueous phase was transferred into a new clean tube. The process was repeated 3 times to collect as much of the upper aqueous phage. 200 µl of chloroform was added to the collected upper aqueous phage, mixed and centrifuged at maximum speed for 10 min. The resulting upper phase was transferred into a clean tube. DNA precipitation was carried out by adding 50 µl of 3M sodium acetate, pH 5.2 and 150 µl of 95% ethanol. The mixture was stored overnight at -20°C. DNA pellet was recovered by centrifuging at maximum speed for 5 min. The pellet was washed with 500 µl of 70% ethanol. The ethanol was allowed to dry at room temperature and DNA was dissolved in 50 µl of Nuclease Free Water (NFW).

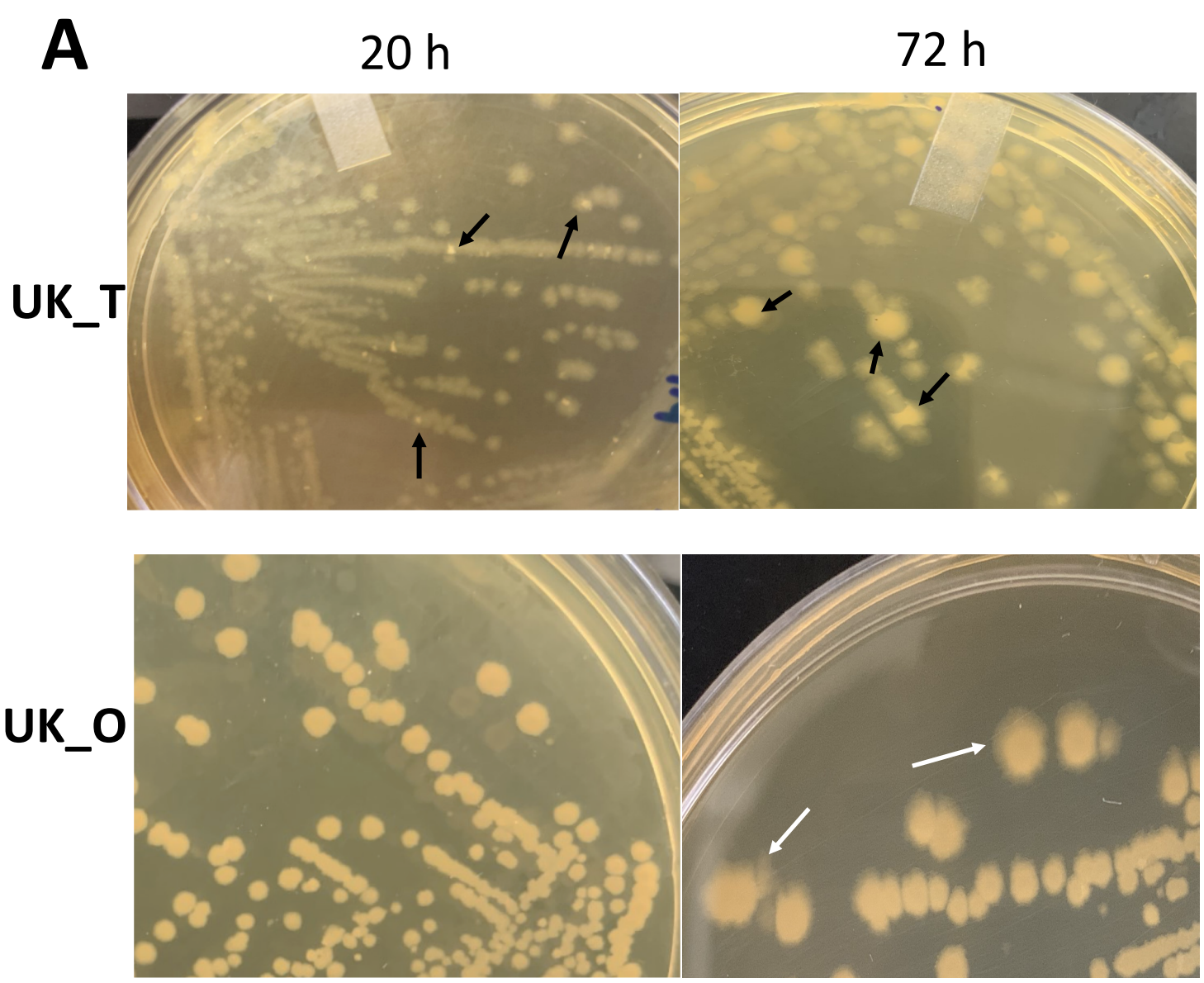
**General DNA techniques**

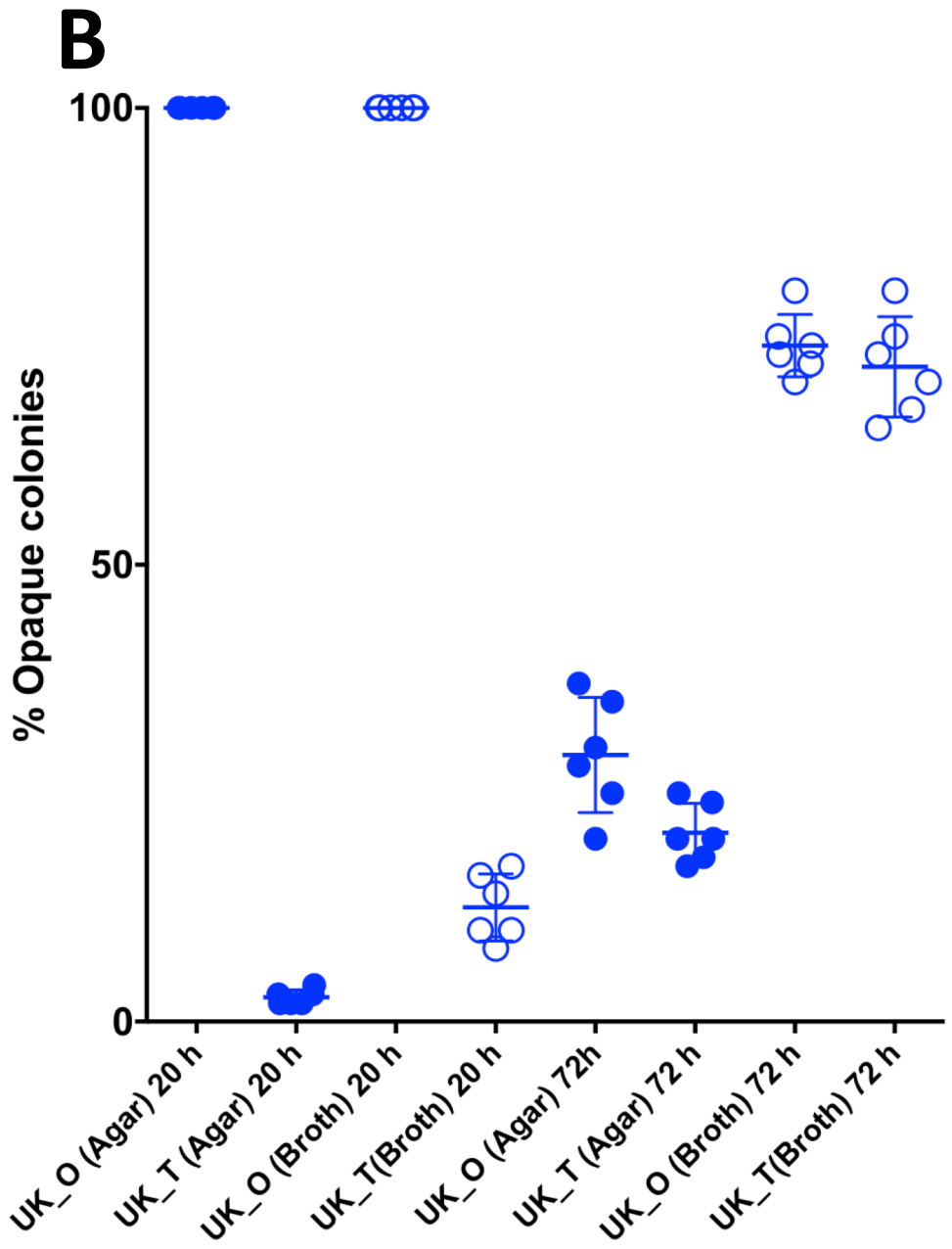
PCR reactions were carried out using gene specific primers (Table S 3.2). PCR products were extracted from the gel using Geneclean Kit (mpbio). Plasmid DNA was extracted using QIAprep Spin Miniprep Kit (Qiagen). Standard procedures were used to perform routine cloning.

**CRISPRi silencing of *pdcB***

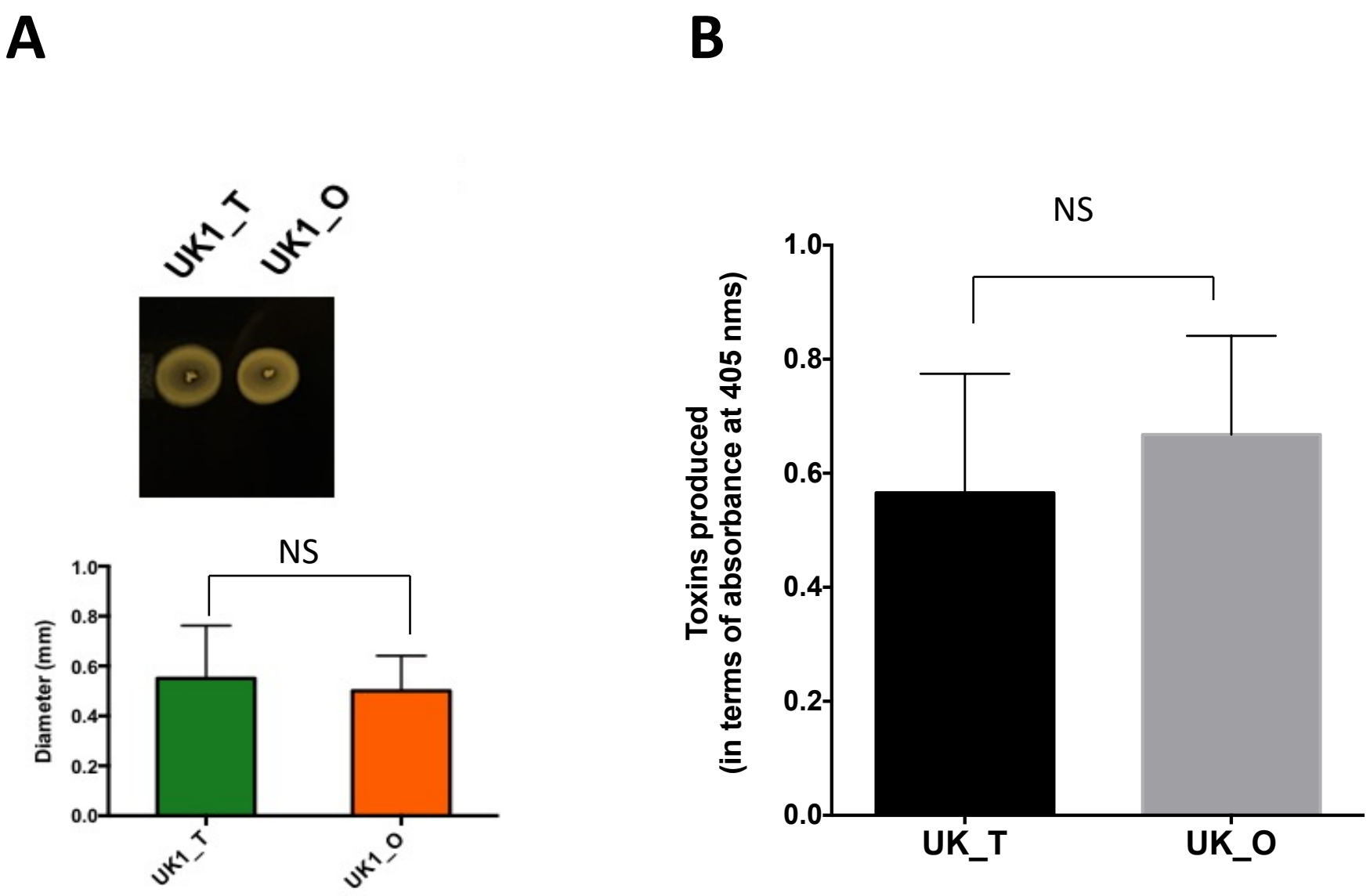
The single guide RNA (sgRNA) specific for *pdcB* was identified using CRISPRscan program and was synthesized by Genewiz. It was later cloned in between the MscI and NotI sites in CRISPRi vector pIA33. Transconjugants selected were streaked on TY+Thio plates with or without 1% xylose. The vector pIA33 expresses the defective Cas9 (dCas9) enzyme from a xylose inducible promoter. Thus, inducing the cultures with xylose will express the dCas9 enzyme which will pair with the sgRNA to block the transcription of *pdcB*.

Supplementary Figure 1



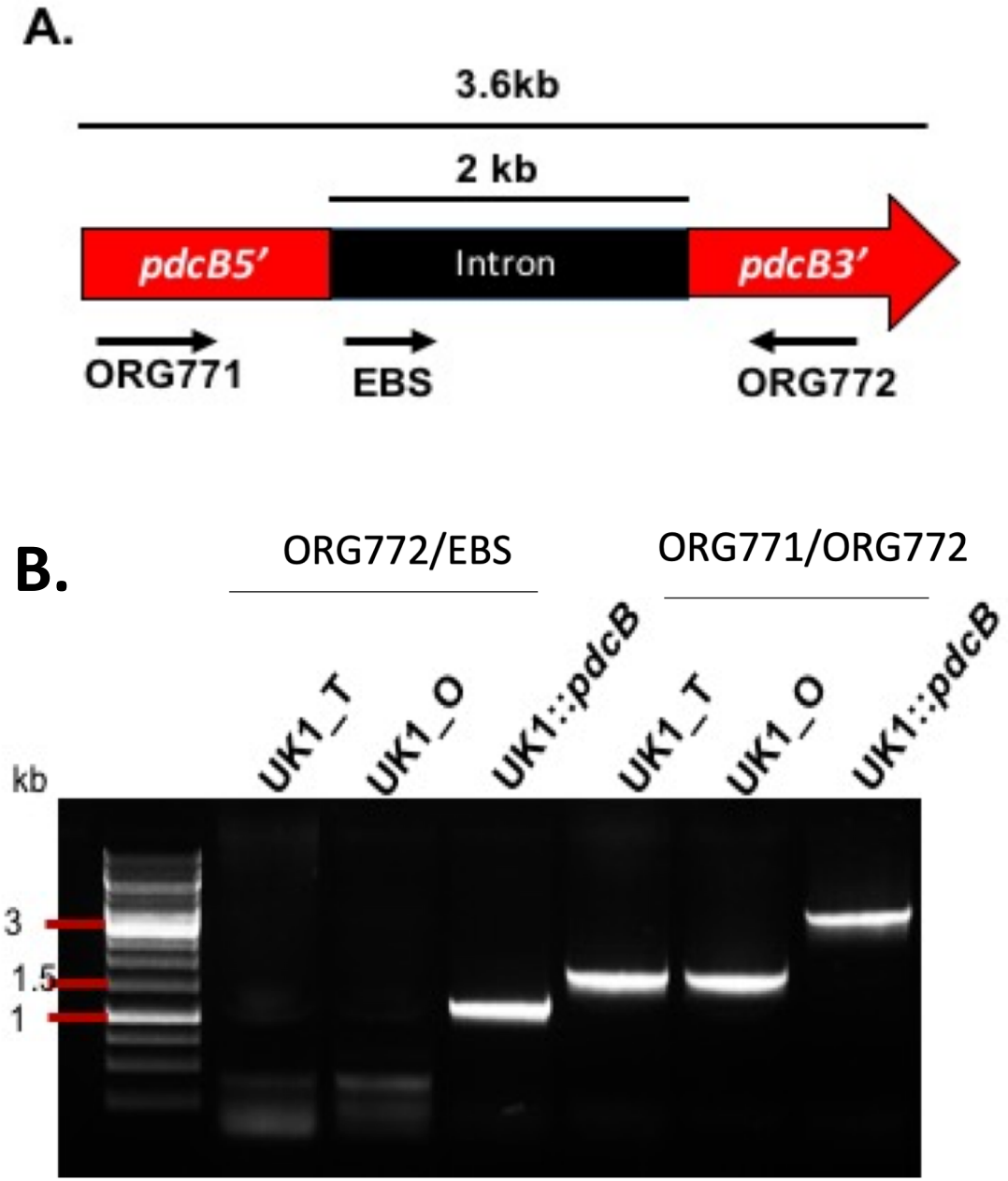
Supplementary Figure 2



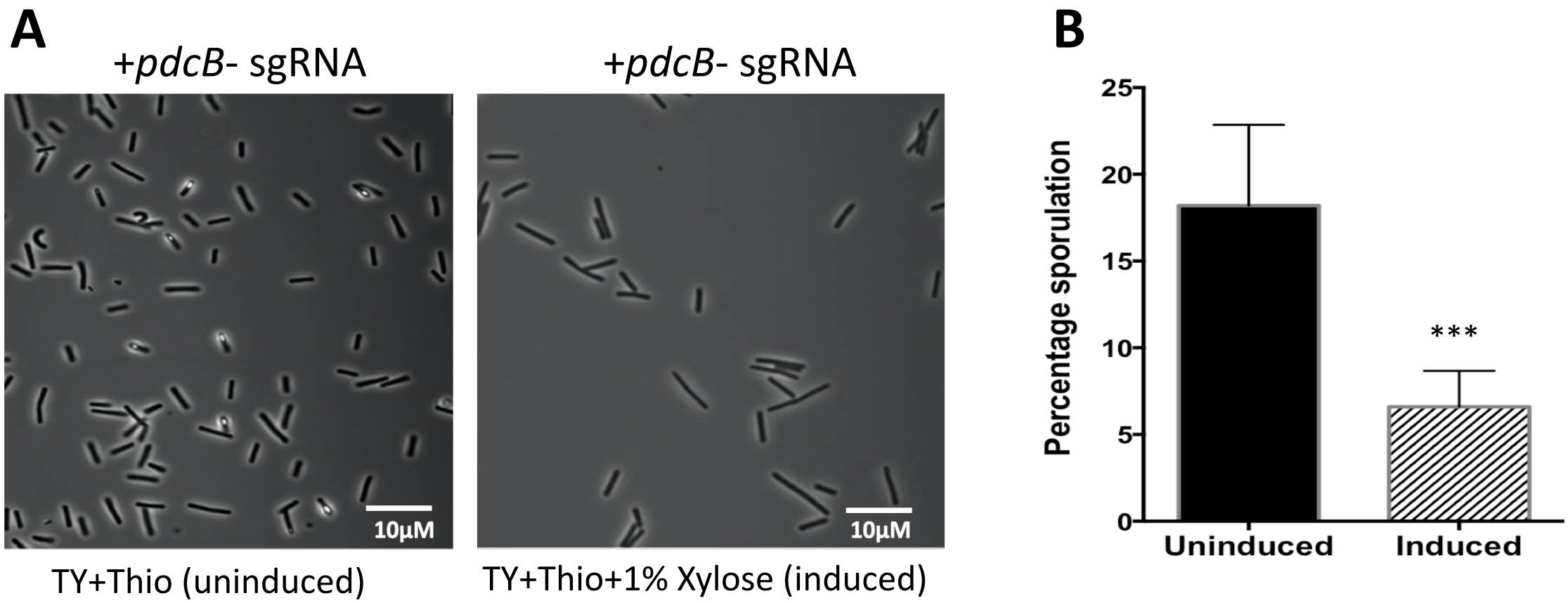
# Supplementary Figure 3

```
UK_O        GTTGTAAAAAAGTTACTATTTATTGAAAATTTAGATACTTTTCTAAAATTATCTATGTTAATAGTTAAAT
UK_T        GTTGTAAAAGGGTTCTTTTTTTTTTATAATAAAATAGCTATAAAAATATACATATCAAATCAAATTAAGAA
R20291      GTTGTAAAAGGGTTCTTTTTTTTTTATAATAAAATAGCTATAAAAATATACATATCAAATCAAATTAAGAA

UK_O        TTATGAACATTTTTTGTTTTTATGTTAGGATATTTCTTAGAAATGAAATACTTCTTAATTTGATTTGATAT
UK_T        GTATTTCATTTCTAAGAAATATCCTAACATAAAAACAAAAAATGTTCATAAATTTAACTATTAACATAGAT
R20291      GTATTTCATTTCTAAGAAATATCCTAACATAAAAACAAAAAATGTTCATAAATTTAACTATTAACATAGAT

UK_O        GTATATTTTTATAGCTATTTTATTATAAAAAAAAAGTAACCCTTTTACAAC
UK_T        AATTTTTAGAAAAGTATCTAAATTTTCAATAAATAGTAACTTTTTTACAAC
R20291      AATTTTTAGAAAAGTATCTAAATTTTCAATAAATAGTAACTTTTTTACAAC
```

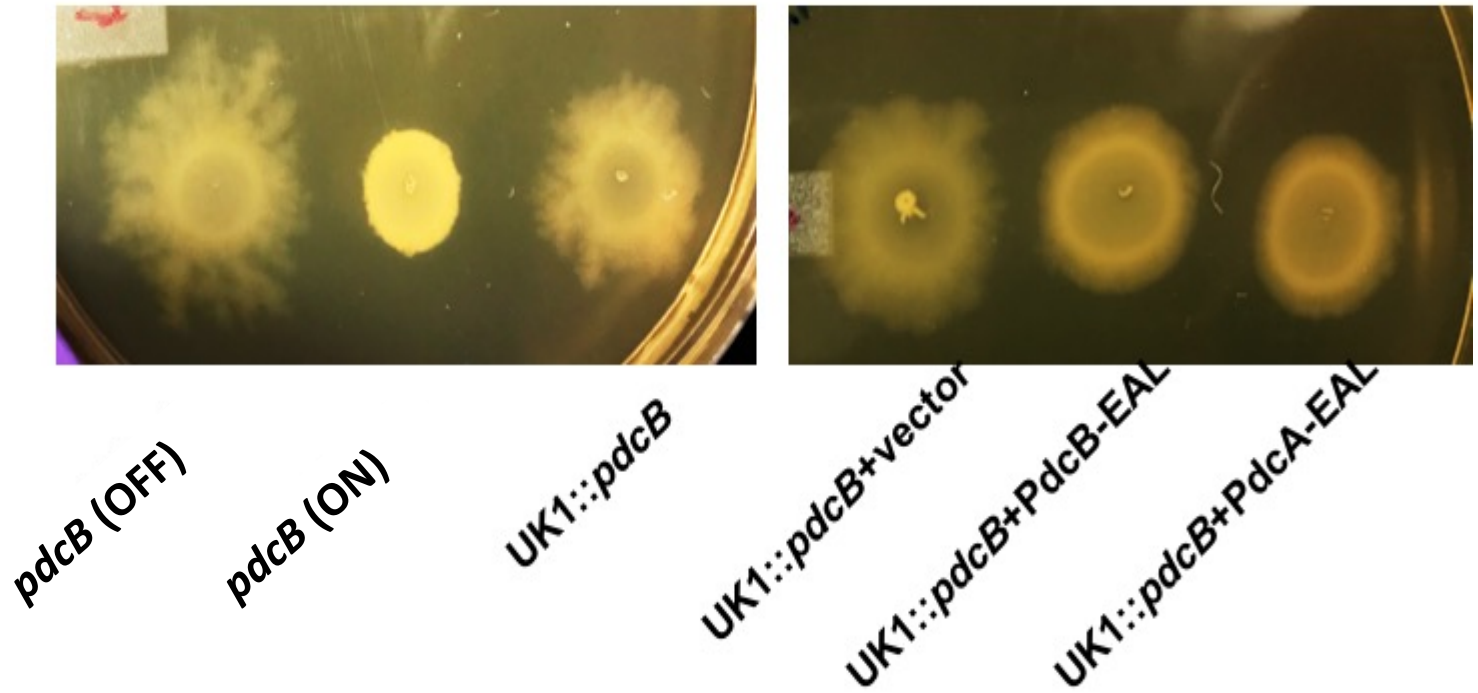
Supplementary Figure 4



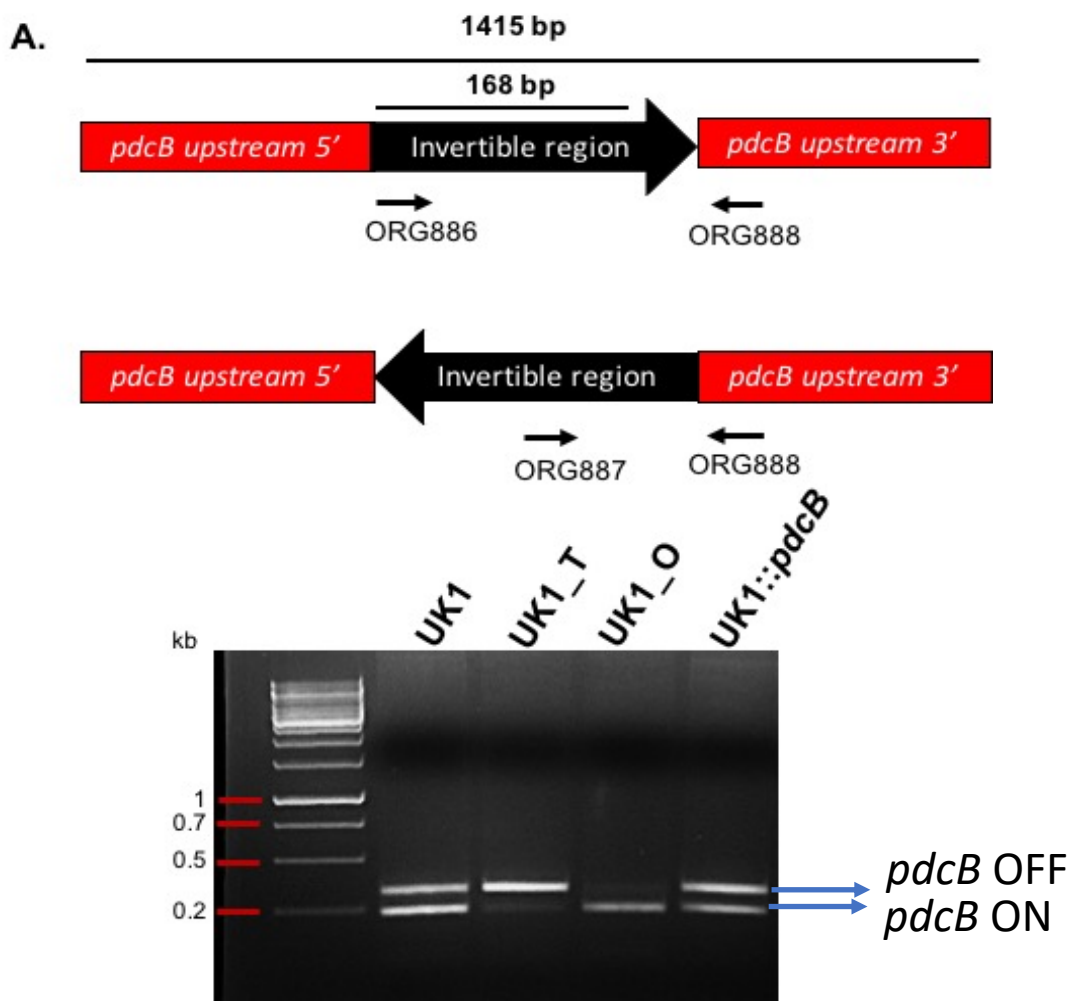
Supplementary Figure 5

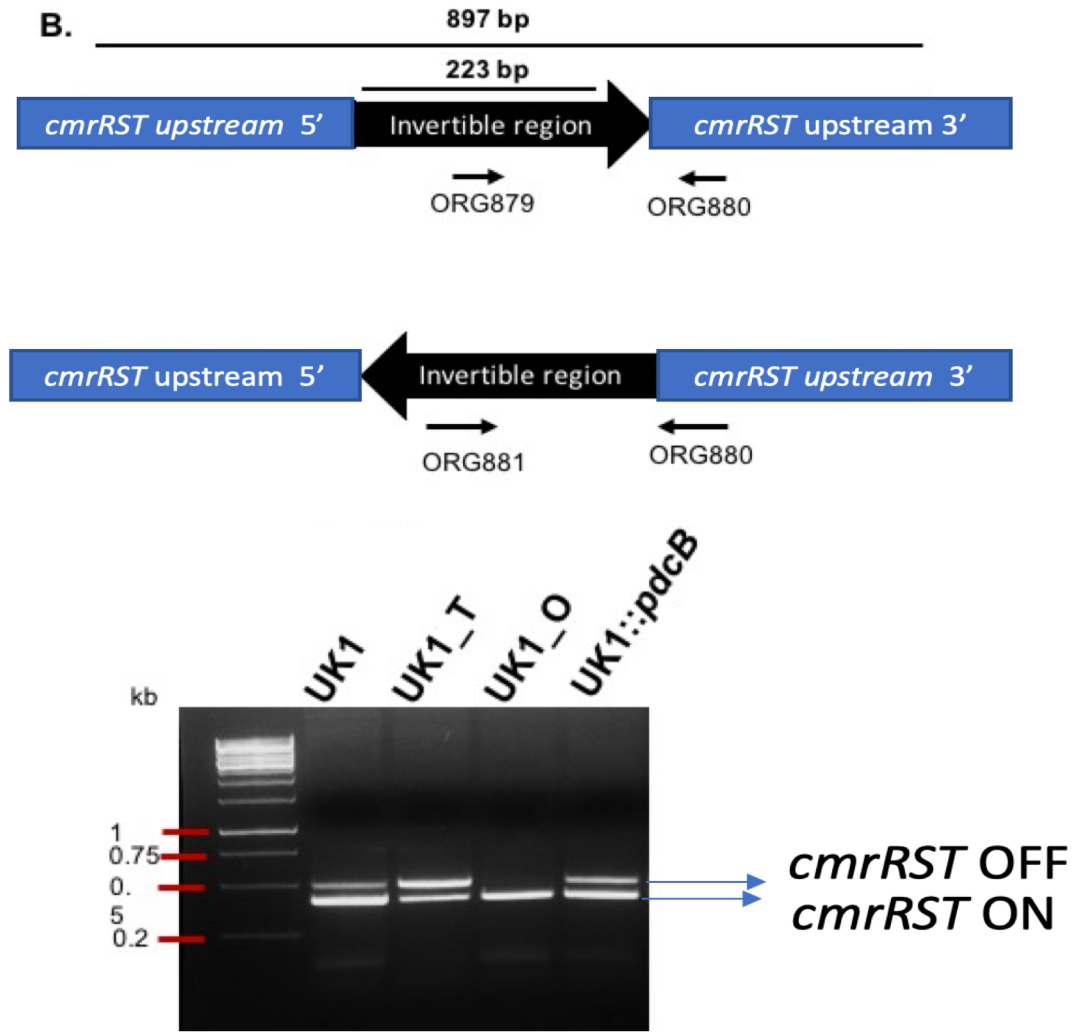


# Supplementary Figure 6



# Supplementary Figure 7



Supplementary Figure 8