Title

FLP-FRT-based method for unmarked deletions of *CHU_3237* (*porU*) and large genomic fragments of *Cytophaga hutchinsonii*

Running title

Deletions by FLP-FRT in *Cytophaga hutchinsonii*

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SUPPLEMENTAL MATERIAL

SUPPLEMENTAL METHODS

Construction of pSKSO8TG. A derivative of pSKSO8 (1), pSKSO83, whose the *oriC* region was replaced with PCR-amplified fragment with primers soriF and soriR6 at the restriction sites of *Pst*I and *Xba*I, was used as the vector backbone. A Rho-independent transcription terminator predicted by TransTermHP (2) between *gldJ* and *purF* was amplified by PCR with primers TermF and TermR, digested with *Xba*I and *Bgl*II, and inserted into the backbone of pSKSO8 digested with *Xba*I and *Bam*HI to generate pSKSO8T. The green fluorescent protein gene (*gfp*) was amplified from pSK1284gfp (1) with primers gfpF and gfpR (introducing a *Sal*I site next to *Bam*HI). The fragment was digested with *Xba*I and *Bam*HI and inserted into the corresponding site of pSKSO8T to generate pSKSO8TG. pSKSO8TG was designed as an expressing vector into which promoters and CTDs from *C. hutchinsonii* could be ligated upstream and downstream of *gfp*, respectively. Besides using pSKSO8TG as a fusion expression vector to secrete GFP into the medium (data not shown), it was used to construct the FLP recombinase expressing vector pCHF.

Measurement of growth rates in PY6 culture. Growth rates of cells in PY6 medium were measured using a Bioscreen C analyzer (Oy Growth Curves Ab Ltd, Helsinki, Finland) in a honeycomb plate. *C. hutchinsonii* cells were grown in PY6 medium to mid-exponential phase and harvested by centrifugation. Cells were resuspended in PY6 medium to an optical density at 600 nm of 0.7~0.8. Then they were inoculated into fresh

medium without or with antibiotics. The inoculum concentration was 3% (v/v) of 200 µl medium in each well. The measurement was carried out in triplicate. The cultures were grown at 30°C with continuously shaking (Amplitude: medium; Speed: normal) and OD_{600} measurements were taken every 90 min.

In-gel digestion and LC-ESI-MS/MS Analysis by Q Exactive. The gel slices were cut into small cubes of 1 mm^3 and then washed three times with ddH_2O. The gel pieces were destained with 50% ethanol at 37°C overnight and dehydrated with acetonitrile and dried in a speed-vacuum concentrator (Thermo Scientific, San Jose, CA). Disulfide bonds were cleaved by incubating with 200 µl of 10 mM DTT/100 mM ammonium bicarbonate for 1 h at 56°C. Alkylation of cysteines was performed by incubating with 200 µl of 55 mM iodoacetamide/100 mM ammonium bicarbonate for 45 min at room temperature in darkness. The gel pieces were dehydrated again and vacuum-dried. Gel pieces were rehydrated with trypsin solution (10 ng/µl in 100 mM ammonium bicarbonate). After a 15-min incubation at 4°C, the gel pieces were covered with 100 mM ammonium bicarbonate. Proteolysis was performed overnight at 37°C. The peptides in gel was extracted once with 100 µl 5% formic acid/50% acetonitrile, once with 75% acetonitrile, twice by 100% acetonitrile. All the supernatant was combined and vacuum-dried.

The peptide was resuspended in buffer A (2% ACN, 0.1% FA) and centrifuged at 20 000 g for 2min. The supernatant was transferred into sample tube and loaded onto an Acclaim PepMap 100 C18 trap column (Dionex, Sunnyvale, CA, 75 µm×2 cm) by EASY nLC1000 nanoUPLC (Thermo Scientific, San Jose, CA) and the peptide was eluted onto

an Acclaim PepMap RSLC C18 analytical column (Dionex, Sunnyvale, CA, 50 μm×15 cm). A 34 min-gradient program was run at 300 nl/min, starting from 5 to 30% B (80% ACN, 0.1% FA), followed by 2 min linear gradient to 40% B, then 2 min to 80% B, and maintenance at 80% B for 4 min.

The peptides were subjected to NSI source followed by tandem mass spectrometry (MS/MS) in Q Exactive (Thermo Scientific, San Jose, CA) coupled online to the UPLC. Intact peptides were detected in the Orbitrap at a resolution of 70 000. Peptides were selected for MS/MS using 25% NCE with 4% stepped NCE; ion fragments were detected in the Orbitrap at a resolution of 17 500. A data-dependent procedure that alternated between one MS scan followed by 15 MS/MS scans was applied for the top15 precursor ions above a threshold ion count of 3E4 in the MS survey scan with 5.0 s dynamic exclusion. The electrospray voltage applied was 1.8 kV. Automatic gain control (AGC) was used to prevent overfilling of the ion trap; 2E5 ions were accumulated for generation of MS/MS spectra. For MS scans, the m/z scan range was 350 to 1 800 Da.

Scanning electron microscopy. Samples for scanning electron microscopy were prepared as described by Ji (3). Cells were grown on Whatman No. 1 filter paper on Stanier agar and fixed with 2.5% (v/v) glutaraldehyde in 100 mM PBS buffer (NaH_2PO_4-Na_2HPO_4, pH 7.3) for 8 hours at 4°C. Fixed samples were washed twice in 100 mM PBS buffer, and dehydrated with 30%, 50%, 70%, 90% ethanol once and 100% ethanol for twice, then dried in a glass desiccator. Samples were processed according to

standard procedure, and viewed with a JEOL JSM-6700F field emission scanning electron microscope.

TABLE S1. Primers used in this study

Primers	Sequence [a]
blaF	CTGGTGAGTACTCAACCAAGTCA
blaR	TCCACGAATTCTTACCAGGTTCAA
isermF	TTAAAAGGATCCTACACTGCAGCAAAAGGTACCTCTTGACAACCACCCG
isermR	GAGCGAGCATGCGGAGCTGTCTAGATACATGAGCTCCGAGCAAGGCAAGACC
sermF	TATGGAGCTCTGACGCTCATCGGTATTTG
sermR	ACTAGGTACCTCTTGACAACCACCCGAC
frtSKF	CTTAGAGCTCATCAAGTCGACGTGTAGGCTGGAGCTGCTTCG
frtSKR	CGGAGGTACCGAATTAGCCATGGTCCATATG
KSfrtF	TTTAGGTACCGTGTAGGCTGGAGCTGCTTCG
KSfrtR	TACCGAGCTCTACGTGTCGACGAATTAGCCATGGTCCATATG
BssHII-ermF	ACATGCGCGCTGACGCTCATCGGTATTTGC
BssHII-ermR	TATTGCGCGCTCTTGACAACCACCCGAC
cfxA-F	ATTGGCGCGCAATCAGTTCTTTAGCGATTAC
cfxA-R	CACTGCGCGCTTTAAGATTTTACTGAAGTTTGC
soriR6	GATATACTGCAGATATTTTAAACAGC
soriF	CTATATCTAGAACTAGTGGATCCTATATATCATTTCTTC
TermF	CCATCTAGACAGAGGATCCTCAGATAGACTTTCATTTC
TermR	TCTGAGATCTGAGCTGCAAAAGTGAG
gfpF	CCGTTCTAGATATGGTAAGCAAAGGAGAAGAAC
gfpR	ATCGGATCCCAGAGTCGACTTTGTATAGTTCATC
ompAF	AGCCGAGCTCTTGCCACATTTGGTGTTTTTTTG
ompAR	CCAGTGATTTTTTTCTCCATACTTAATTTTTTTAATTAC
catF	GTAATTAAAAAAATTAAGTATGGAGAAAAAAATCACTGG
catR	AATGGGTACCGTTTAAGGGCACCAATAAC
cat-PstIF	AGCCCTGCAGTTGCCACATTTGGTGTTTTTTTG
cat-SDR	AACGGTACCCACTTGCATGCGATAAACTTTTAATTACGCCCCGCCCTGCCACTCATC
P1284galK-F	CTTGGAGCTCGCCACTGTTTGATGTAGTTAG
P1284galK-R	AAGCTCTAGATTAGCACTGTCCTGCTCCTTGTG
flpF	GGCCGCATGCCACAATTTGATATATTAT
flpR	ACGTGGTACCTGCGTACTTATATGCGTCTAT
cat-KpnIR	AATCGGTACCCAACTGCATGCGTTTAAGGGCACCAATAAC
C3237F	GTCTGAGCTCTTTCTGTTTCCGTTGGTTAC
C3237R	AAGTGTCGACTGCAGATTGACCAAACGTGTG
3237H1F	GTAAGGATCCAATATTCTGCTCGGGTTCTGC

3237H1R	GCACGGTACCAAATACCTAATAGGAAGATGC
3237H2F	ACCTGTCGACGTTTTTGAGAAACTGGTGCTC
3237H2R	ACTTGAGCTCTTGTTGGTTTTAACAGGCTCGG
3237UF	CCATACGGAAACCAGATATAC
3237UR	TGAACGCTTACGGAAGAAAG
3237DR	TACAGAAGACTGTACGCTTCC
3237Test	TGTTTTGCTGGTCATTC
0344H1F	GCCGTCTAGATCCGAAAAGCAGAAACATAGAG
0344H1R	CACCGAGCTCAACATAAAACCACCATAAGCAGCTC
0344H2F	CCGAGGTACCATTAAACTAACTATAAGGCAG
0344H2R	ATGTGGATCCTTACCTGTACCGCAACTGTTC
0344UF	GGCAAAGGTTTAACACATTCAGAG
0344UR	TCATTTGCTGATTGGTATAGGG
0344DR	GTGGCAATCATACAATCATTCAGG
3202H1F	TGGTGGATCCTTTAGGTGTGAAGAACTTTGC
3202H1R	CGTCGGTACCTTTCATGTTGTTCATCGTC
3202H2F	GCCAGTCGACAACATTTTGTAGTACCCGATC
3202H2R	TTAAGAGCTCAACTCCTACCACCAGACCCAG
3202UF	TCCGAAATGGATGCTAAGAC
3202UR	GGATAATAAAGCATGACTGGC
3202DR	AAATGAATTGCCTAGCTGG
3202Test	AGGAGTATTATTGGTCGTC
3190H1F	CGGCGAGCTCATTTAATTGTTGTAGTTACAC
3190H1R	CTGCGTCGACTAAAGTACTATAGAGTATG
3190H2F	AATTGGTACCGGAGGAATCGGAGCCTGAC
3190H2R	CTTAGGATCCAGCAGCGCTTACCAAATCTG
3190UF	TCATTTTGATACACAGACGC
3190UR	CGTCTTCGCCGTAATATAC
3190DR	CTATACCCATGATTGTCATC
0804H1F	TGGAGGATCCATGCCTTTTATCTGGATATTG
0804H1R	GAGTGGTACCTAGTGTTGATCTGGTAACTTC
0804H2F	GTCAGTCGACAATGGTACATTGGGCGTTAG
0804H2R	TTATGCATGCGAACTGATACAGCAACCGTAC
0804UF	GATTTTGGATTCGAACGCAAG
0804UR	CTCCAACAGACAACTCAATC
0804DR	GTATTTCTTTCGTCTCCTGG
0804Test	CATGGATTGAGCTTTACACC
0819H1F	TGCTGGATCCTTTGACATTAACGGGATTCAC
0819H1R	CGCAGGTACCATGTATTTTGTATGCAGTATC
0819H2F	GTTCGTCGACACCATGTATAACAGGTTCAGC
0819H2R	TACAGCATGCACAGGCAATGATCTCCTTCAG

0819UF	TCGATCTCTTATCTGGTGAC
0819UR	CGTATACCAGCACAAGTATGC
0819DR	TTCCCGATTCTTTGATTGCTC
0834H1F	TCTGGAGCTCTTTCAGCCTTTAGTATTAGGC
0834H1R	GTGCGTCGACTTTATAGAATTGCATAGTCTG
0834H2F	CGACGGTACCTTAAGTACGCAGAATCCTTC
0834H2R	TGTCGGATCCATATAGCAGATAACGCATCTC
0834UF	CTGTCATTTCTTCTACAAACATCCG
0834UR	TCACATTCGTAAAGTCCATCAC
0834DR	ACTTCCACAGGATGTATAGGTGC
0834Test	CGCAAAAGCACATTGG
0841H1F	ACTAGAGCTCACCTTTCAATGGCGTAACTTC
0841H1R	CGTTGTCGACGCCTGTATAGGTATAAGTAG
0841H2F	ATTGCCATGGTAGAAGGGTTAGCATCAGGAG
0841H2R	GTTCGGATCCTTCCATCTTCTAACGTTCG
0841UF	CAAATTCAAACCTTACGGTC
0841UR	GTTGTCATTCGGTACACTAC
0841DR	AGGAAGAATAATCTGTCAGG
0428H1F	TGCTGAGCTCTACTTGTAACTTCCCACTTTG
0428H1R	CTCAGTCGACATTCCCCTGTAAATCGTAATG
0428H2F	ATGACCATGGAACACAGATTGCACCCAAG
0428H2R	TCAAGGATCCAACAGCGATGGTATGGTCAG
0428UF	GTAGATTGAGTAGCAGTCTG
0428UR	TCAAAAAGCGTGTTGTACTC
0428DR	TCCAGAACCATCTCCTAACTG
0428Test	GCAGGAAAACTTTAACAGTG
0449H1F	CAGCGCATGCAGAACATAAAAGATCTGTAC
0449H1R	TAACGTCGACTGACAAGAATACAACGACCTG
0449H2F	GTTAGGTACCATCATAGAGCTCAGGCGTTAG
0449H2R	ATCTGGATCCTGCACCTATTCAGAATGGAG
0449UF	ACAGACTGATCGAAATGAAC
0449UR	TGTTCAGGAGCAAGTATTAG
0449DR	CTGTTTGTAATGCACCTACC
1075H1F	TAACGCATGCTGCTGCAGGGAATTCACCGTAC
1075H1R	ATTCGTCGACAGCTCCGGATGGTGTATTGATC
1075H2F	GCACGGTACCATATCGTAGAGGTATCTACAGC
1075H2R	TCTCGGATCCTTGTCGTTCGCGTTTGTCATC
1075UF	GTTTGCCTTAGGTGATTTCAG
1075UR	GGCATTGTTGTTCCAGATTAC
1075DR	AAGGTTTCCTGTGTTTCAAG
1075Test	TTGATTGCCTGTCTGTAC

1107H1F	CTTAGCATGCATTAAGCGAAAATGGTGCTGCTG
1107H1R	GCCGGTCGACGAACGTATATTCATATGTATAG
1107H2F	CCACGGTACCAGCATTCCGGGTTTATTATAAC
1107H2R	CGCAGGATCCATGTGTCAATAGAATACCGATC
1107UF	CGTAGTTATAACTGCTGACG
1107UR	TGGTCTGTATCAACTCATTG
1107DR	ATCATCCAGAGATTGACTGC

[a] Restriction sites on the primers are underlined

TABLE S2. Putative protein substrates of the T9SS absent from the culture fluid of the Δ*3237* mutant identified by LC-MS/MS

Locus tag	Protein description
CHU_0220	
CHU_0268	
CHU_0344	
CHU_0353	β-mannanase
CHU_0361	
CHU_0420	
CHU_0521	
CHU_0530	
CHU_0531	
CHU_0804	
CHU_0834	
CHU_0835	
CHU_0938	
CHU_0939	
CHU_0961	Candidate β-glycosidase
CHU_1075	Candidate β-glycosidase
CHU_1105	
CHU_1107	Endoglucanase
CHU_1155	Candidate xyloglucanase
CHU_1221	
CHU_1251	
CHU_1335	Endoglucanase-related protein
CHU_1336	Endoglucanase-related protein
CHU_1557	
CHU_1634	
CHU_1655	Non-processive endocellulase
CHU_1858	
CHU_1906	
CHU_2149	Candidate retaining β-glycosidase
CHU_2225	Gliding motility-related protein
CHU_2243	
CHU_2304	
CHU_2437	
CHU_2463	
CHU_2524	Subtilisin-like serine protease

CHU_2603	
CHU_2674	
CHU_2755	Concanavalin A-like lectin/glucanases superfamily
CHU_2780	
CHU_2818	
CHU_2850	
CHU_2852	Candidate β-glycosidase
CHU_2922	
CHU_3021	
CHU_3251	
CHU_3267	
CHU_3435	
CHU_3439	
CHU_3440	β-glycosidase related protein
CHU_3441	β-glycosidase related protein
CHU_3488	
CHU_3587	
CHU_3618	
CHU_3654	
CHU_3715	
CHU_3802	Possible protease

Fig. S1

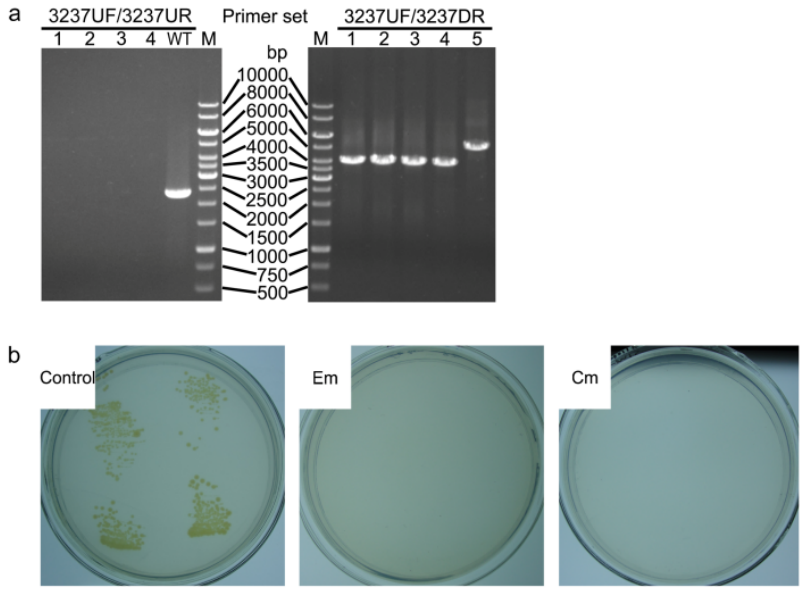

Fig. S1. PCR verification and plate tests of the unmarked deletion of *CHU_3237*. (a) PCR verification of the unmarked deletion of *CHU_3237*. *1-4*, four transformants of the Δ*3237* mutant; *5*, disruption mutant *3237*::*erm*; *WT*, wild-type strain; *M*, DNA marker. (b) plate tests to confirm the loss or existence of the resistance gene and pCHF in the Δ*3237* mutant. *Control*, PY6 agar without any antibiotics; *Em*, PY6 agar containing erythromycin; *Cm*, PY6 agar containing chloramphenicol.

Fig. S2

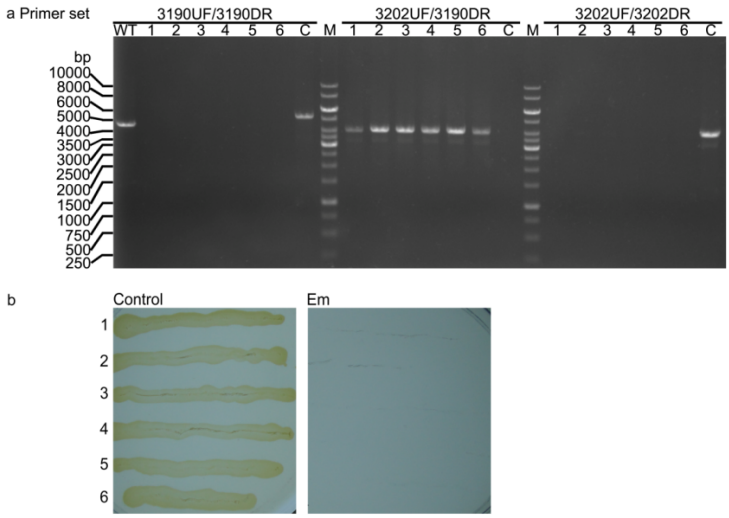

Fig. S2. PCR verification and plate tests of mutant Δ*3202-3190*. (a) PCR verification of mutant Δ*3202-3190*. *1-6*, six transformants of the Δ*3202-3190* mutant; *C*, mutant Δ*3202-3190*::*erm*; *WT*, wild-type strain; *M*, DNA marker. (b) plate tests to confirm the loss of the selectable marker in the Δ*3202-3190* mutant. *Control*, PY6 agar without any antibiotics; *Em*, PY6 agar containing erythromycin.

Fig. S3

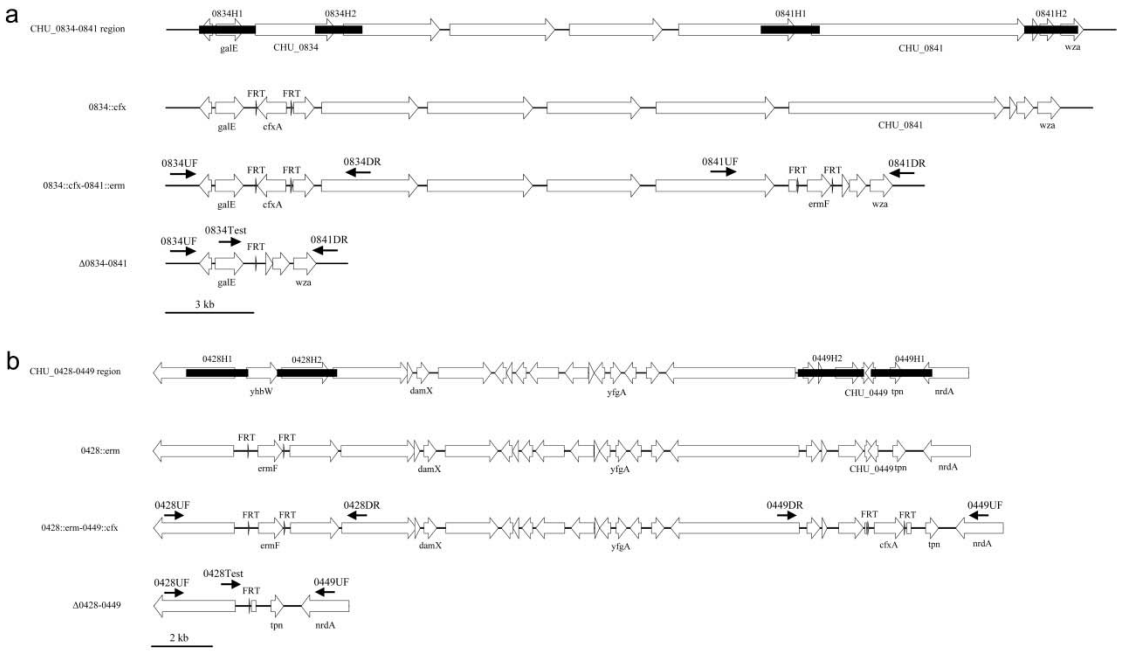

Fig. S3. Deletions of the fragments between *CHU_0834* and *CHU_0841* (a), *CHU_0428* and *CHU_0449* (b). Black arrows show approximate locations and orientations of primers; black filled boxes indicate homologous arms; open arrowheads show arrangements and orientations of genes; open boxes indicate residual genes; grey arrowheads indicate FRT sites and their relative orientations.

Fig. S4

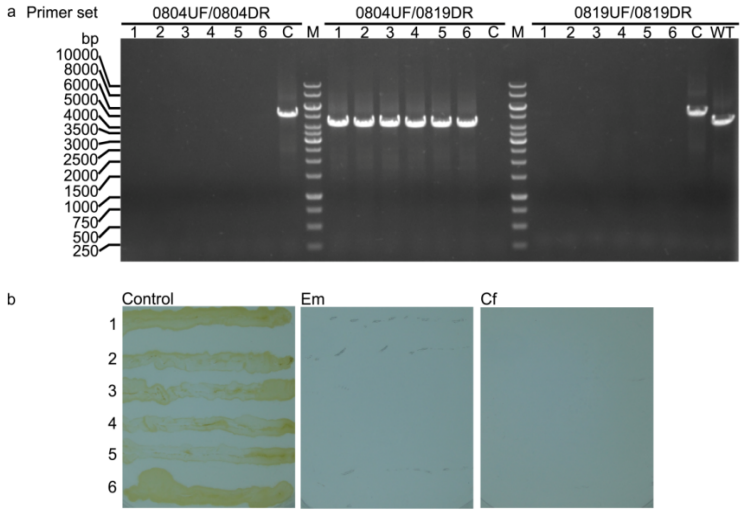

Fig. S4. PCR verification and plate tests of mutant Δ*0804-0819*. (a) PCR verification of mutant Δ*0804-0819*. *1-6*, six transformants of the Δ*0804-0819* mutant; *C*, mutant *0804*::*erm-0819*::*cfx*; *WT*, wild-type strain; *M*, DNA marker. (b) plate tests to confirm the loss of the selectable markers in the Δ*0804-0819* mutant. *Control*, PY6 agar without any antibiotics; *Em*, PY6 agar containing erythromycin; *Cf*, PY6 agar containing cefoxitin.

Fig. S5

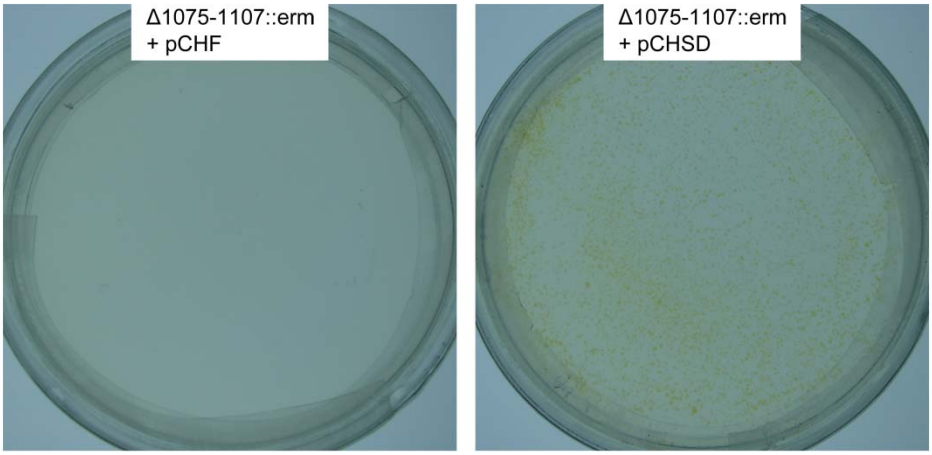

Fig. S5. Transformation efficiency of mutant Δ*1075-1107*::*erm*. *Left*, Δ*1075-1107*::*erm* with pCHF; *right*, Δ*1075-1107*::*erm* with control plasmid pCHSD.

Fig. S6

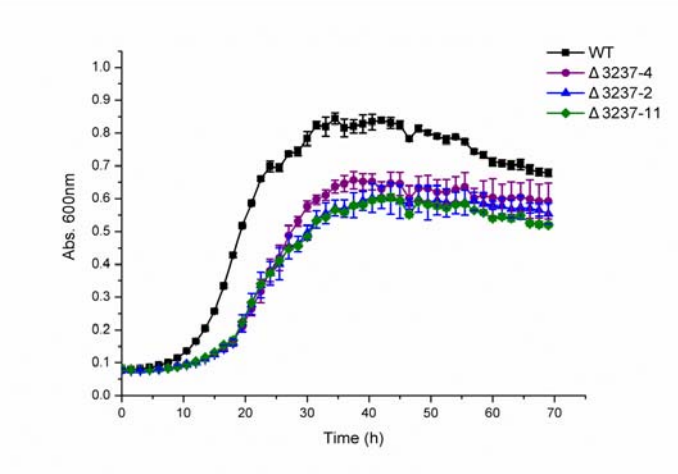

Fig. S6. Growth curves of the wild-type strain and the Δ*3237* mutant in PY6 medium measured by a Bioscreen C analyzer. The cells were grown at 30°C with continuously shaking (Amplitude: medium; Speed: normal) and OD_{600} measurements were taken every 90 min. *Black square*, wild-type strain; *Purple circle*, *blue triangle*, *green diamond*, three strains of the Δ*3237* mutant. All measurements were carried out in triplicate.

Fig. S7

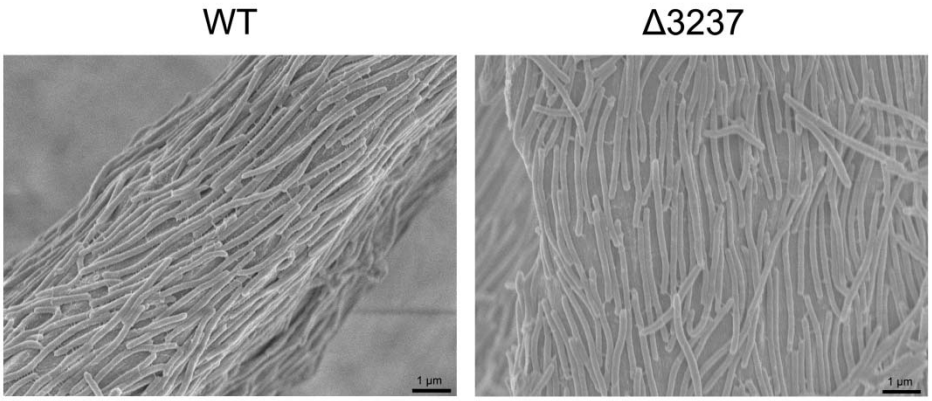

Fig. S7. Scanning electron microscopy of the arrangement of cells on filter paper fiber. *Left*, wild-type strain; *right*, mutant Δ*3237*. *Bars* indicate 1 μm.

REFERENCE

1. **Xu Y, Ji X, Chen N, Li P, Liu W, Lu X**. 2012. Development of replicative *oriC* plasmids and their versatile use in genetic manipulation of *Cytophaga hutchinsonii*. Appl. Microbiol. Biotechnol. **93**:697–705.

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