Supplemental Material for:

Introducing a new host for biotechnology: Genetic tool development for the thermotolerant *Bacillus coagulans*

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SUPPLEMENTARY MATERIALS AND METHODS

Bacterial strains and media. *B. coagulans* strains were routinely grown in rich BC medium (9) at 45°C (genetic engineering) or 50°C (sporulation tests; LacZ cultures), at 120 rpm (for aerated cultures). For anaerobic growth BC broth was supplemented with 1% glucose. Sporulation medium (SM) contains per liter: 8 g nutrient broth, 2 g $(NH_4)_2HPO_4$, 3.5 g $(NH_4)_2SO_4$, 10 g Bis-Tris, 0.12 g $MgSO_4 \cdot 7\ H_2O$, 1 g KCl, 0.164 g $Ca(NO_3)_2 \cdot 4\ H_2O$, 1.25 mg $MnCl_2 \cdot 4\ H_2O$, 0.15 mg $FeSO_4$, pH 6.7.

Transformation. The electroporation protocol for *B. coagulans* DSM 1 (for pNZ124, pNZ8048, and pNW33n derivatives) is a modified protocol from that described elsewhere (9). *B. coagulans* DSM 1 was grown overnight at 45°C in 10 ml BC medium in a 100 ml bottle, without shaking. 100 ml BC medium in a 1 l bottle was inoculated with the overnight culture to a turbidity at 600 nm of 0.05 and incubated at 45°C, 120 rpm, until a turbidity of 0.45-0.65 was reached (approximately 3 hours). Cells were pelleted at 4 °C, washed three times with ice-cold SG medium (50 ml, 25 ml, and 12.5 ml, respectively), resuspended in 200 µl ice-cold SG medium and used immediately for electroporation. Seventy-five microliters of cell suspension was mixed with 1 µg of plasmid DNA in an ice-cold electroporation cuvette (1 mm gap) and kept on ice for 2 min. The electroporation settings using a Bio-Rad electroporator were 1.5 kV, 25 µF and

600Ω. Immediately after electroporation, cells were transferred to 1.3 ml of pre-warmed (45°C) RG medium and incubated for 3 h in an Eppendorf Thermomixer at 600 rpm. During the last hour antibiotic resistance was induced by addition of sublethal concentrations of appropriate antibiotics (0.025 $mg \cdot l^{-1}$ tetracycline or 0.2 $mg \cdot l^{-1}$ chloramphenicol). Cells were spread on BC plates containing the appropriate antibiotic and incubated at 45°C for 1-3 days.

Construction of Δ*sigF* and Δ*lacZ* strains. For deletion of the *sigF* gene, the upstream and downstream regions required for double crossover recombination were cloned into the integration vector pMH77. Vector pMH77 is based on the lactococcal cloning vector pNZ124 (7) and has a thermosensitive replicon in *B. coagulans* (9). The *cat* gene of pNZ124 was modified to contain an NcoI site overlapping the *cat* start codon, resulting in pMH3 (9). The pMH3 SalI-NcoI fragment containing the *cat* promoter was replaced by a synthetic SalI-NcoI fragment containing a *B. coagulans* promoter (GU323910). The resulting plasmid was designated pMH71. To enable multiple use of the Cre-*lox* system, *lox66* and *lox71* sites (4,5) flanking the promoter-*cat* region were introduced by PCR using primers P1 and P2 (sequences of oligonucleotides are presented in Supplementary Table 1) with pMH71 as template. The resulting PCR product was digested with BglII-SalI and used to exchange with the BglII-SalI promoter-*cat* region of pMH71, resulting in plasmid pMH77. The *sigF* upstream and downstream regions were generated by PCR using primers P3 and P4 or P5 and P6, respectively, with DSM 1 chromosomal DNA as a template. The *sigF*-upstream PCR product was cloned as SalI-NheI fragment in pMH77. Subsequently, the *sigF*-downstream PCR product was cloned as EcoRI-XhoI fragment. The resulting integration plasmid was designated pMH79. The

integrity of the sequences of the upstream and downstream regions was confirmed by sequencing.

For the use of the Cre-*lox* system, the *cre* gene was cloned into a pNZ124 derivative containing *tetK*. First, the *tetK* gene was amplified by PCR using primers P7 and P8 and pGhost8:IS*S1* as template (6) and cloned as SalI-BglII fragment into pNZ124 replacing the *cat* gene. Then the *cre* gene was cloned on a HindIII-EcoRI fragment from pNZ5347 (4), resulting in plasmid pMH66.

For deletion of the *lacZ* gene, the flanking recombination regions were obtained by PCR using oligonucleotides P11 and P12 (upstream) and P13 and P14 (downstream), digested with SacI and PstI (blunted), and SnaBI and BamHI (blunted), respectively, and cloned into the SacI-PvuII sites of pMH77, resulting in integration plasmid pLAC.

For integration, a colony harboring the integration plasmid was cultured overnight at 45°C, after which the temperature was shifted to 60°C and incubation was continued for 1.5 hours. A dilution series was plated on BC plates containing chloramphenicol and incubated overnight at 60°C. Single colonies were tested for double crossover recombination by PCR analysis. For removal of the *cat* gene using the Cre-*lox* system, the double crossover strain was transformed with pMH66 using electroporation. Cells were plated on BC plates containing tetracycline. Transformants were tested for the absence of *cat* by PCR analysis, and the pMH66 plasmid was cured by incubation at 60°C. Deletions were confirmed by sequence analysis.

In the case of the *lacZ* mutant, single crossover recombinants were selected on BC plates containing chloramphenicol and incubated overnight at 60°C. After successive inoculation of single crossover recombinants in the absence of chloramphenicol, cells were plated on BC plates containing 60 $mg \cdot l^{-1}$ X-gal (5-bromo-4-chloro-3-indolyl-β-D-

galactopyranoside). A double-crossover white colony was selected that showed chloramphenicol sensitivity and deletion of *lacZ* gene was verified by sequence analysis.

Sporulation test. 100 µl of a culture grown for 18 hours in SM medium was incubated at 80°C for 30 min and plated on BC plates. Samples were also assayed for viable cell number using colony counts without heat treatment.

Construction of reporter plasmids. The *B. coagulans lacZ* gene was PCR-amplified with P15 and P16, digested with NcoI and BamHI and cloned into the NcoI-BglII sites of pNZ8048 (3), resulting in pNZlac. Amplifications of promoter regions were done with oligonucleotides P17 and P18 (*spoIIAA*), P19 and P20 (*dacF*), P21 and P22 (*cotE*), P23 and P24 (*spoIID*), P25 and P26 (*pta*), P27 and P28 (*ldhL*), P29 and P30 (*pgi*) PCR fragments were digested with NcoI and XbaI enzymes and inserted of corresponding sites of pNZlac (see Table 1). In all cases where PCR was involved, the sequences were checked by sequencing.

Construction of D-lactate dehydrogenase plasmid. A *B. coagulans* ATCC 23498 genomic fragment encoding amylase gene promoter (P_{amy}) activity (8) was produced as a synthetic DNA fragment introducing an NcoI site overlapping the *amy* start codon and flanking BglII and BamHI sites (see Figure S1). The fragment was cloned in pGEM-T Easy (Promega) and sequenced. *Lactobacillus delbrueckii* subsp. *bulgaricus* LMG 6901 (BCCM/LMG, Gent, Belgium) was grown at 37°C in MRS broth (Difco). The *L. bulgaricus* LMG 6901 *ldhA* gene encoding an D-lactate dehydrogenase (1) was

generated by PCR using primers P9 and P10. The PCR product was cloned as a blunt-XbaI fragment into pUC18 digested with XbaI-SmaI and its integrity was confirmed by nucleotide sequence analysis. Subsequently, P_{amy}, as BglII-NcoI fragment, and the *ldhA* gene, as RcaI-PstI fragment, were cloned into pNW33n digested with BglII-PstI. The resulting plasmid, pJS27, has the *ldhA* gene of *L. bulgaricus* translationally fused to the *B. coagulans* ATCC 23498 P_{amy}.

Fermentations and analytical procedures. Batch fermentations were performed in a bioreactor (3 l Applikon ®) with 1 l of BC broth without Bis-Tris buffer and supplemented with 30 or 50 $g \cdot l^{-1}$ glucose as described previously (9). Organic acids (formic acid, acetic acid, propionic acid, butyric acid, pyruvic acid, lactic acid, 2-hydroxy butyric acid, glycolic acid, oxalic acid, sorbic acid, fumaric acid, succinic acid, benzoic acid, maleic acid, malic acid, citric acid) were measured after (trans)-esterification to their corresponding methyl-ester using a proton donor. Due to this esterification to the methyl-esters, the results are the sum of the concentrations of the acids present in the free-, ester- and salt-form. Hexanoic acid was added as an internal standard and the samples were assayed using a GLC equipped with two capillary columns with a different polarity coupled to two flame ionization detectors using similar settings as described elsewhere (2). Identification of the components was based on the two retention times obtained. D- and L-lactates were methylated to methyl-lactate and measured by head-space analysis on a chiral column. Peak areas of the D-lactate and L-lactate peaks were used to calculate the relative contribution of each enantiomer to the chiral purity.

FIG. S1. Nucleotide sequence of synthetic DNA fragment containing the *B. coagulans* ATCC 23498 P_{amy} (position -289 to -3, (8)) flanked by BglII and NcoI-BamHI sites (underlined). The ATG start codon of the *amy* gene in the NcoI site is printed boldface.

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  1  AGATCTTGGT TCCCCACCTT TTTTACAGAC TTATCACTAT ATTATTATAG
 51  ATAAACCGGC CAAACAACCA AATCGGGGCG CAAAGGAGAG CCGGGGCGTG
101  GATTTAAACC ATTTTTGGAA AAACAAAAGG AAAACCTGCT TGTAAAAAGA
151  TGTTTTCGCG AAACGAAAGC GGGAATAGTA CCTTTGTTCT CTTCGCCTTT
201  TGTCATGCTT AAAATCATAA TTGATTGAAA ATTTTTTCAT GTTCACTTAT
251  ACTAAACGCA TCAACTATTA CTTCTTTTGG AAGGGGCAGT TTCCATGGGG
301  ATCC
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130 **TABLE S1.** Oligonucleotide primers used in this study.

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Oligonucleotide	gene	RES[a] introduced	Sequence[b]
P1	*lox66*	SalI, NheI	CCCGTCGACGCTAGCTACCGTTCGTATAATGTATGC TATACGAAGTTATGTGGATAAGACAACAGGATTCG
P2	*lox71*	BglII	CGCAGATCTTACCGTTCGTATAGCATACATTATACG AAGTTATCCTTCTTCAACTAACGGGGCAGGTTAG
P3	Δ*sigF*	SalI	CCCGTCGACGTTGCCGACAAAACAGTGAAAC
P4	Δ*sigF*	NheI	CCCGCTAGCCGGCACGACTCCTTAATTGC
P5	Δ*sigF*	EcoRI	CGCGAATTCAAATACTTGAAGTGATGAAAGAGCGC
P6	Δ*sigF*	XhoI	CCGCTCGAGTGAATCGTTCCGTCCTGGAC
P7	*tetK*	SalI	CCGGTCGACACAAAATATAAGAATTTGATAAAAGA AATTTCG
P8	*tetK*	BglII	GCCAGATCTGAGCTCTGCGAGGCTTAAACC
P9	*ldhA*	RcaI	GACAATTCATGACTAAAATTTTTGC
P10	*ldhA*	XbaI, PstI	GGATTTCTCTAGACTGCAGTTAGCCAACCTTAA
P11	*lacZ*	-	TGCAACCGTGTCCAGAGTTCTGAAT
P12	*lacZ*	-	CGTCCTTGTCAACCGGAAGCGAATC
P13	*lacZ*	-	GGGTCCGTCCGGTAATGCCTATCAA
P14	*lacZ*	-	TGCGGCTTGGCGTGGATAATTCCTG
P15	*lacZ*	NcoI	GAGCCATGGACTTGGAGGAATGCGTGAT
P16	*lacZ*	-	AACACGCAAACAGACCGTAG
P17	*spoIIAA*	XbaI	GAGTCTAGAAGTGAAACGCGCAGGCTGGT
P18	*spoIIAA*	NcoI	ACGCCATGGGCCTCCTTCGCTGTTAATA
P19	*dacF*	XbaI	GAGTCTAGATATGCGAAATTCCATCCAAG
P20	*dacF*	NcoI	ACGCCATGGAACTTGTAAAATCGTAAAAC
P21	*cotE*	XbaI	GAGTCTAGATTCTTCCGGCACCCTCCAC
P22	*cotE*	NcoI	ACGCCATGGTCCTCATCTATTTTCAATTC
P23	*spoIID*	XbaI	GAGTCTAGAGAAAGGGTCGACGACGAAG
P24	*spoIID*	NcoI	ACGCCATGGTGATCGAAGTGGTGGAAAG
P25	*pta*	XbaI	GAGTCTAGATTGCCGGAATTCTTTCACAG
P26	*pta*	NcoI	ACGCCATGGACTCCTCGTATAACGGTATC
P27	*ldhL*	XbaI	GAGTCTAGAAGCCTCATCGCCGGTTTCCC
P28	*ldhL*	NcoI	ACGCCATGGATCTTCCTCCCCATCAAAAG
P29	*pgi*	XbaI	GAGTCTAGAGCTCCCGACCGCGTTAAATG
P30	*pgi*	NcoI	ACGCCATGGAGCCGGTAAATCATTGCC

[a]RES denotes restriction endonuclease site.
[b]Introduced RES underlined in sequence.

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TABLE S2. β-galactosidase activity in Δ*lacZ and* Δ*lacZ*Δ*sigF* strains. Cells were collected from overnight cultures for the measurements. Data are averages of 6 biological replicates.

Plasmid	β-galactosidase activity	
	B. coagulans Δ*lacZ*	*B. coagulans* Δ*lacZ*Δ*sigF*
Sporulation genes		
pSPOIIA-LAC	580 ±100	520 ±60
pSPOIID-LAC	240 ±40	11 ±3
pDACF-LAC	46 ±23	3.5 ±0.3
pCOTE-LAC	560 ±60	10.4 ±4.1
Metabolic genes		
pPTA-LAC	570 ±44	540 ±61
pLDH-LAC	450 ±48	410 ±38
pPGI-LAC	18.1 ±4.9	14.7 ±5.5

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