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Published in:
Applied and Environmental Microbiology

DOI:
10.1128/AEM.03060-09

Publication date:
2010

Applied and Environmental Microbiology, June 2010, p. 4085–4088 Vol. 76, No. 12
0099-2240/10/$12.00 doi:10.1128/AEM.03060-09

Genetic Tool Development for a New Host for Biotechnology, the Thermotolerant Bacterium *Bacillus coagulans*▽†

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Received 18 December 2009/Accepted 10 April 2010

Bacillus coagulans **has good potential as an industrial production organism for platform chemicals from renewable resources but has limited genetic tools available. Here, we present a targeted gene disruption system using the Cre-*lox* system, development of a LacZ reporter assay for monitoring gene transcription, and heterologous D-lactate dehydrogenase expression.**

White biotechnology holds the promise to replace part of today's petrochemicals through the production of biofuels and green chemicals from renewable resources (19). Facultative anaerobic thermophilic *Bacillus* species, including the lactic acid-producing *Bacillus coagulans* (4, 10), appear to be attractive alternatives to mesophilic production hosts as potential next-generation microbial production organisms. Compared to the mesophilic and often aerobic strains, their fermentation requires less cooling and mixing and they can be used in a biorefinery concept (15, 18).

The development of genetic tools for *B. coagulans* has just emerged. Electroporation protocols that yield very small numbers of transformants have been described (22, 23), and an integration system, based on the thermosensitive lactococcal pSH71/pWV01 replicons, that works in combination with low transformation efficiencies is available (23). Still, metabolic engineering of *B. coagulans* is hampered by the availability of only a limited number of selective markers (23) and would greatly benefit from having a convenient markerless integration system. The Cre-*lox* system was previously applied for this purpose in *B. subtilis* (26). Here, we report the use of this system in the thermophilic *B. coagulans* for multiple genomic modifications and the reuse and removal of selectable markers.

Spore formation of genetically modified organisms is undesirable from both the biological safety and the industrial processing viewpoint. Therefore, we applied the Cre-*lox* system to construct a sporulation-deficient *B. coagulans* derivative by targeting the *sigF* (*spoIIAC*) gene, which has been successfully targeted for this purpose in *Bacillus licheniformis* and *B. subtilis* (7, 25, 27).

First an integration vector, pMH77 (Table 1), was constructed, carrying a *lox66-cat-lox71* cassette encoding chloramphenicol resistance and flanked by restriction sites that can be used to clone the regions for homologous recombination (see the supplemental material). This vector is based on pNZ124 (21) that carries a thermosensitive replicon for *B. coagulans* (23) and has the *cat* gene under the control of a *B. coagulans* promoter. Mutant *loxP* sites, *lox66* and *lox71*, that flank the *cat* gene can be recognized by the Cre recombinase, resulting in a double mutant and an inactive *lox72* site (1). Subsequently, a plasmid for introduction of the Cre recombinase, pMH66, was constructed (see the supplemental material). The *cre* gene, encoding Cre recombinase, under the control of a *Lactobacillus plantarum* promoter (14) was cloned in a pNZ124 derivative with *cat* replaced by *tetK*, encoding tetracycline resistance.

The *sigF*-flanking regions were cloned in integration vector pMH77, resulting in pMH79, which was introduced into *B. coagulans* DSM 1 by electroporation (see the supplemental material for details) (Fig. 1A). One transformant having the presence of pMH79 confirmed by plasmid isolation was selected for integration (see the Fig. 1 legend for details). Single colonies were tested for integration, and both single and double crossovers were detected by PCR. The Cre resolvase-encoding plasmid pMH66 was transformed to a selected double crossover strain, DSM 1 Δ*sigF*::*cat*, while selecting for tetracycline resistance (Fig. 1B). By PCR, we detected the absence of *cat* in all tested transformants, indicating that Cre activity upon introduction was sufficient to cure *cat* from the transformant colonies and no extended incubation is required. Plasmid pMH66 was cured from the *cat*-free Δ*sigF* strain. Plasmid-free colonies were obtained, and one was selected as DSM 1 Δ*sigF*.

The *B. coagulans* DSM 1 cells were grown in a specially developed SM medium to quantify sporulation, a phenomenon which is rarely observed for this strain in Luria broth or BC broth (unpublished observations). Wild-type cells showed 3.1×10^{-4} sporulation efficiency, while the Δ*sigF* strain contained no visible spores when examined using confocal microscopy and gave no colonies in spore tests (described in the supplemental material). After staining of the cell membrane using FM 9-95 dye [*N*-(3-trimethylammonium propyl)-4-6-4-diethylamino phenol], the wild-type strain showed round spores, while the Δ*sigF* strain showed the presence of one or two asymmetric septa (Fig. 2), typical of *sigF* mutants of *B. subtilis* and *B. licheniformis* (3, 7).

We wanted to be able to monitor promoter activities of

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† Supplemental material for this article may be found at http://aem.asm.org/.

▽ Published ahead of print on 16 April 2010.

TABLE 1. Strains and plasmids

Strain or plasmid	Relevant feature(s)[a]	Reference or source
B. coagulans strains		
DSM 1	Type strain	DSMZ collection
DSM 1 Δ*sigF*	DSM 1 Δ*sigF*	This study
DSM 1 Δ*lacZ*	DSM 1 Δ*lacZ*	This study
DSM 1 Δ*lacZ* Δ*sigF*	DSM 1 Δ*lacZ* Δ*sigF*	This study
Plasmids		
pNZ124	2.8 kb, Cm^r Ts, Gram-positive-*E. coli* shuttle vector	21
pNZ8048	3.3 kb, Cm^r, pNZ124-based cloning vector for the use of the NICE system	13
pNW33n	4.2 kb, Cm^r, *Geobacillus-E. coli* shuttle vector	Bacillus Genetic Stock Centre
pMH66	5.1 kb, Tet^r Ts, pNZ124-based Cre-encoding plasmid	This study
pMH77	3.0 kb, Cm^r Ts, pNZ124-based cloning vector with *lox66-cat-lox71* cassette	This study
pMH79	5.0 kb, Cm^r Ts, pMH77-based integration vector for *sigF* deletion	This study
pJS27	5.5 kb, Cm^r, pNW33n derivative containing P_{amy}-*ldhA*	This study
pLAC	5.1 kb, Cm^r Ts, pMH77-based integration vector for *lacZ* deletion	This study
pNZlac	5.5 kb, Cm^r Ts, pNZ124-based cloning vector with *B. coagulans* DSM 1 *lacZ* gene	This study
pSPOIIAA-LAC	5.7 kb, Cm^r Ts, pNZ8048 containing P*spoIIAA-lacZ* fusion	This study
pSPOIID-LAC	5.9 kb, Cm^r Ts, pNZ8048 containing P*spoIID-lacZ* fusion	This study
pDACF-LAC	5.8 kb, Cm^r Ts, pNZ8048 containing P*dacF-lacZ* fusion	This study
pCOTE-LAC	5.7 kb, Cm^r Ts, pNZ8048 containing P*cotE-lacZ* fusion	This study
pPTA-LAC	5.8 kb, Cm^r Ts, pNZ8048 containing P*pta-lacZ* fusion	This study
pLDHL-LAC	6.7 kb, Cm^r Ts, pNZ8048 containing P*ldhL1-lacZ* fusion	This study
pPGI-LAC	6.2 kb, Cm^r Ts, pNZ8048 containing P*pgi-lacZ* fusion	This study

[a] Cm^r, chloramphenicol resistant; Tet^r, tetracycline resistant; Ts, thermosensitive replicon in *B. coagulans*.

selected genes to assess the effect of the *sigF* deletion. After unsuccessfully testing several reporter systems (GFPmut1 and GFPuv [9], cyan fluorescent protein and yellow fluorescent protein [24], and anaerobic BsFbFP [*B. subtilis* FMN-based fluorescent protein] [5]), we decided to use the *B. coagulans lacZ* gene as a reporter gene. The promoterless *B. coagulans lacZ* gene identified in the 3.2-Mb draft genome sequence (unpublished results) was cloned, together with its ribosome-binding site (GGAGGAATGCGTG**ATG** [start codon of *lacZ* is in bold]), in a pNZ124 derivative, resulting in the reporter vector pNZlac (for construction, see the supplemental material). To enable the use of the *lacZ* reporter system, we eliminated the *lacZ* gene from the genome by cloning the *lacZ* upstream and downstream fragments in the integration vector pMH77 (see the supplemental material). After electroporation of the resulting construct, single recombination integration was selected for by chloramphenicol resistance, followed by screening for white colonies on 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal)-containing plates, resulting in double-recombi-

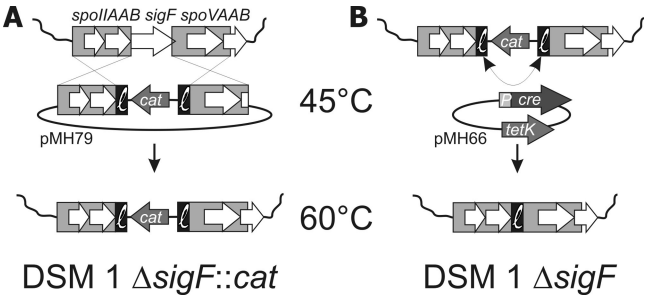

FIG. 1. Schematic overview of Cre-*lox*-based chromosomal deletion system for construction of *B. coagulans* DSM 1 Δ*sigF*. (A) Exchange of *sigF* for *cat* by double crossover. The *lox66-cat-lox71* cassette flanked by the homologous regions for crossover is introduced into *B. coagulans* on a thermosensitive plasmid, pMH79, at the permissive temperature of 45°C and with selection for chloramphenicol resistance (7 μg · ml^{-1}). Double crossover recombination events are selected for by plating at 60°C, a nonpermissive temperature for the integration plasmid, while maintaining the antibiotic resistance pressure. (B) Removal of *cat*. The Cre-encoding plasmid is introduced on a thermosensitive plasmid (pMH66) carrying a tetracycline resistance gene for selection (1 μg · ml^{-1}). Cre recombines the *lox66* and *lox71* sites to a *lox72* site that is no longer recognized by Cre. The Cre-encoding plasmid is cured by cultivation at 60°C and subsequent plating of a dilution series.

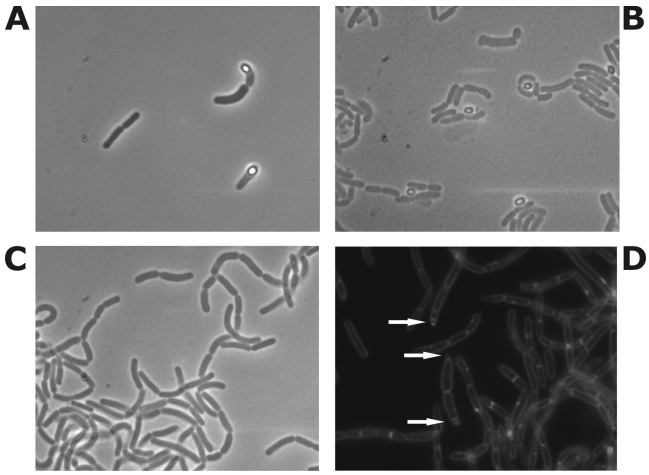

FIG. 2. Microscopic observation of wild-type *B. coagulans* (A and B) and the Δ*sigF* strain (C and D). Bright spores are observed in wild-type cells using confocal microscopy during early (A) and late (B) stationary phase. No spores are observed in the Δ*sigF* strain (C), while asymmetric septa can be visualized using FM 9-95 membrane stain during fluorescence microscopy (D).

TABLE 2. Fermentation products of *B. coagulans*

Strain	Amt of indicated organic acid produced[a]		
	Lactic acid	Acetic acid	Formic acid
B. coagulans DSM 1	3.5 ± 0.3	0.06 ± 0.02	0.12 ± 0.05
B. coagulans DSM 1 Δ*sigF*	3.7 ± 0.2	0.03 ± 0.00	0.04 ± 0.00

[a] Values are given as the percentage by weight and represent means ± standard deviations for 3 consecutive fermentations using an initial glucose concentration of 50 g · liter^{-1} and a 10% inoculum. Values for other organic acids were below the detection limit. The density of the fermentation broth was approximately 1.1 g · ml^{-1}.

TABLE 3. Chiral purity of lactic acid produced by *B. coagulans*[a]

Strain	% of lactic acid that was:		Total amt of lactic acid produced[b]
	D Form	L Form	
DSM 1	0.2, 0.3	99.8, 99.7	2.5, 2.8
DSM 1(pNW33n)	0.4, 0.3	99.6, 99.7	2.3, 2.8
DSM 1(pJS27)	15.7, 16.9	84.3, 83.1	2.6, 2.7

[a] Data are from two independent fermentations using initial glucose concentrations of 30 g · liter^{-1}.

[b] Values are given as the percentage by weight. The density of the fermentation broth was approximately 1.1 g · ml^{-1}.

nant strains lacking the *lacZ* gene. The Δ*lacZ* mutation was also introduced into the DSM 1 Δ*sigF* strain, enabling a direct comparison between wild-type and Δ*sigF* mutant strains with the *lacZ* reporter system.

For evaluation of the effect of the *sigF* deletion on sporulation gene expression, we cloned promoters of the *spoIIA* operon, containing *sigF* (P_{spoIIA}), *spoIID* (P_{spoIID}), *dacF* (P_{dacF}), and *cotE* (P_{cotE}), whose homologues in *B. subtilis* code for proteins involved in sporulation (6, 20), in pNZlac. We determined the β-galactosidase activities as described before (12), using cultures of Δ*lacZ* and Δ*lacZ* Δ*sigF* strains grown overnight (see Table S2 in the supplemental material). While the promoter activity of $P_{spoIIAA}$ was not significantly changed in the Δ*sigF* strain, the β-galactosidase activities transcribed from P_{spoIID}, P_{cotE}, and P_{dacF} in the Δ*sigF* strain were reduced 25, 10, and 50-fold, respectively, compared to those of the wild type. This is in line with the regulation reported for *B. subtilis* (6).

The effect of the *sigF* deletion on central metabolism was first determined by cloning the promoter regions of *pgi*, *pta*, and *ldhL*, encoding glucose-6-phosphate isomerase, phosphotransacetylase, and lactate dehydrogenase, respectively, in pNZlac. The reporter activities of the resulting reporter plasmids in the two strain backgrounds showed no significant differences (see Table S2 in the supplemental material), indicating that the *sigF* deletion has no major impact on central metabolism. This was further evaluated by analyzing the fermentation performance of wild-type and Δ*sigF B. coagulans* cultivated in parallel in serial-batch lactic acid fermentations. In 3 consecutive fermentations, there were no significant differences in the fermentation times and the organic acid profiles of the two strains (Table 2), demonstrating that the *sigF* deletion has no major effect on the fermentation performance.

Lactic acid can serve as a building block for the production of poly(lactic acid) (PLA). Industrially produced lactic acid is mainly the L enantiomer, but stereocomplex PLA also requires the availability of low-cost D-lactic acid (8). Although several production strains for D-lactic acid are described in the literature (e.g., see references 2, 11, 16, 17, and 28), all are mesophilic. To demonstrate that *B. coagulans* may be used for thermophilic production of D-lactate, the *Lactobacillus delbrueckii* LMG 6901 *ldhA* gene was fused to the *B. coagulans* amylase gene promoter in the thermophilic cloning vector pNW33n, resulting in plasmid pJS27, which was transformed into strain DSM 1. Fermentation studies (Table 3) showed that the lactate produced by *B. coagulans* DSM 1 and *B. coagulans* DSM 1 harboring pNW33n was almost enantiopure in the L form, while a significant part of the lactate produced by *B. coagulans* DSM 1 harboring pJS27 was in the D form. No differences in lactic acid concentration or by-product formation were detected. These results demonstrate that D-lactate production by *B. coagulans* can be achieved by the introduction of a heterologous D-lactate dehydrogenase gene.

B. coagulans shows great potential as a next-generation production platform for building block chemicals or biofuels from renewable resources. We have extended the metabolic engineering toolkit, and we have demonstrated that *B. coagulans* can produce D-lactate. The construction of an industrial *B. coagulans* D-lactate production strain requires replacing the *ldhL* gene with the heterologous *ldhA* gene for D-lactate production and disruption of *sigF* for a sporulation-deficient phenotype.

Nucleotide sequence accession numbers. Sequences used in this study have been deposited in GenBank under accession numbers GU323910 (P_{pMH71}), GU323911 (P_{spoIIA}), GU323912 (P_{spoIID}), GU323913 (P_{cotE}), GU323914 (P_{dacF}), GU323915 (P_{pta}), GU323916 (P_{ldhL}), GU323917 (P_{pgi}), and GU323918 (P_{lacZ}-*lacZ*).

We thank Jurgen Snelders and Esther van Mullekom for their contributions to cloning and fermentation activities.

Part of this work was financially supported by a SenterNovem subsidy, no. IS044081. A.T.K. was financially supported by grant 818.02.004 from the ALW-NWO Open program. This project was carried out within the research program of the Kluyver Centre for Genomics of Industrial Fermentation, which is part of the Netherlands Genomics Initiative/Netherlands Organization for Scientific Research.

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