Novel methods for genetic transformation of natural Bacillus subtilis isolates used to study the regulation of the mycosubtilin and surfactin synthetases

Duitman, Erwin H.; Wyczawski, Dobek; Boven, Ludolf G.; Venema, Gerard; Kuipers, Oscar; Hamoen, Leendert W.

Published in:
Applied and Environmental Microbiology

DOI:
10.1128/AEM.02751-06

SUPPLEMENTAL MATERIAL

Construction of reporter gene fusions

To measure the expression and regulation of the *srfA* and *myc* operons, *lacZ*-reporter gene fusions were made. For this the plasmid pLGW300 was used, which contains the ribosomal binding site of the *B. subtilis spoVG* gene fused to a promoter-less *lacZ* (3). For the construction of the transcriptional *myc-lacZ* fusion in *B. subtilis* ATCC6633 (BV12E12) an internal part of *mycA*, obtained by PCR using primers MA1 and MA2, was digested with *Bam*HI and *Eco*RI, and cloned into pLGW300, linearized with the same restriction enzymes. Using *E. coli* as an intermediate host, the obtained plasmid was transformed to *B. subtilis* ATCC6633, and transformants were selected on minimal agar plates containing 5 μg/ml kanamycin and 4 μg/ml 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal). Transformants were tested for the absence of mycosubtilin production, and proper integration was verified by PCR using primers LZ1 and MA1.

In order to determine the possible effects of mycosubtilin production on expression of *myc*, a transcriptional *lacZ* fusion was made downstream of *myc,* without disrupting the operon (BV12E14). To this end the 3'-part of *myc*, obtained by PCR using primers MC1 and ML1, and digested with *Bam*HI, was ligated into *Bam*HI and *Sma*I linearized pLGW300, and transformed to *E. coli*. Plasmid DNA was isolated from this cloning host to transform *B. subtilis* ATCC6633, and transformants were selected on minimal agar plates containing 5 μg/ml kanamycin and 4 μg/ml X-gal. Transformants were tested for the production of mycosubtilin, and correct integration was verified by PCR using primers LZ1 and MC2.

To study expression and transcriptional regulation of *myc* in *B. subtilis* 168 we inserted a transcriptional fusion of the *myc* promoter region with *lacZ* into the genome of *B. subtilis* 168 derivative strain 8G5 (here denoted as 168-8G5), obtaining strain BV12E27. The *myc* promoter-*lacZ* fusion was inserted between *dacC* and *ppsA*, identical to the position occupied by *myc* in *B. subtilis* ATCC6633 (1). As the sequence homology between

ATCC6633 and 168-8G5 is about 98 % in this region, the following approach was used to obtain efficient recombination. A PCR product containing the *dacC* region of *B. subtilis* 168-8G5 using primers DC1 and YA1, and plasmid pLGW300 were both restricted with *Pst*I and *Xho*I, and after ligation the ligation products were transformed to *E. coli*. The correct orientation of the insert was verified with PCR using primers LZ1 and YA1. The correct plasmids were linearized with *Eco*RI and *Pst*I, and ligated to an *Eco*RI and *Pst*I restricted *myc* promoter fragment obtained by PCR using primers DC2 and FF1, and *B. subtilis* ATCC6633 chromosomal DNA as template. Because plasmids containing the mycosubtilin promoter are not stable in *E. coli*, this ligation was performed in the presence PEG-8000, as described above, and the multimeric ligation products were directly transformed to competent 168-8G5 cells. Transformants were selected on minimal agar plates containing 5 μg/ml kanamycin and 4 μg/ml X-gal. Integration of the *lacZ*-reporter gene fusion was verified with PCR using primers LZ1 and YA2.

To examine whether the expression and transcriptional regulation of *srfA* in *B. subtilis* ATCC6633 is comparable to that in *B. subtilis* 168-8G5, transcriptional fusions of *srfA* with *lacZ* were made in both strains (BV12E13 and BV12E15). For this the 3'-end of the *srfAD* gene, obtained by PCR using primers SD1 and SD2, was restricted with *Bcl*I and the correct 340 basepairs fragment was ligated into *Bam*HI-linearized pLGW300. The ligation products were transformed to *E. coli,* and the expected orientation of the inserts was determined with PCR using primers SD1 and LZ1. The resulting plasmid was used to transform competent *B. subtilis* ATCC6633, 168-8G5, and 168-7G5. The latter strain contains a wild type *sfp* gene that is essential for surfactin synthesis (2). Transformants were selected on minimal agar plates containing 5 μg/ml kanamycin and 4 μg/ml X-gal, and correct integration of the *lacZ*-reporter gene fusion was verified with PCR using primers SD2 and LZ1. Surfactin production was tested on blood-agar plates as described below.

Construction of regulatory mutant strains

To determine the possible involvement of the proteins AbrB, CodY, ComA, CssS, DegU, SinR, Spo0H and Spo0K in the transcriptional regulation of *srfA* and *myc* in *B. subtilis* ATCC6633, the genes encoding these proteins were mutated (BV12E16, BV12E18, BV12E20, BV12E22, BV12E24, BV12E29, BV12E31, BV12E32, BV12E33, BV12I11 and BV12I37). This was mostly done by transformation with chromosomal DNA of *B. subtilis* strains already harbouring the desired mutation, marked with an antibiotic resistance gene, except for *codY* and *degU*. Deletions of *abrB, comA, cssS, sinR, spo0H* and *spo0K* were verified by PCR using primers AB1, AB2, CA1, CA2, CM1, CM2, CS1, CS2, DC1, SH1, SH2, SK1, SK2, SK3, SK4, SR1 and SR2, respectively.

A *codY* knockout was obtained as follows. Plasmid pUC18 was digested with *Nde*I and *Eco*RI, made blunt with Klenow enzyme, and the linearized plasmid was ligated to a DNA fragment containing *codY*, flanked by 1000 bp to effect replacement recombination. This fragment was obtained with PCR using primers CY1 and CY2, and *B. subtilis* ATCC6633 chromosomal DNA as template. The resulting plasmid was used as template for PCR, using primers CY3 and CY5 located at the 5'- and 3'-ends of *codY*, which produced a linearized plasmid that harbours the flanking sequences of *codY* but lacks the *codY* open reading frame. A chloramphenicol resistance marker, obtained from pUC19C by restriction with *Hind*II, was inserted between the flanking regions. Plasmids in which the chloramphenicol marker had the same orientation as the deleted gene were isolated and used to transform *B. subtilis* strains harboring the *myc-lacZ or srfA-lacZ* reporter gene fusions. Integration and deletion of the *codY* gene was verified by PCR using primers CY1, CY2, CM1 and CM2.

A *degU* deletion was obtained as follows. Plasmid pUC18 was digested with *Nde*I and *Eco*RI, made blunt with Klenow enzyme, and the linearized plasmid was ligated to a DNA fragment containing *degU*, flanked by 1000 bp to effect replacement recombination. This fragment was obtained by PCR using primers DU1 and DU2, and *B. subtilis* ATCC6633 chromosomal DNA as template. Within the *degU* gene a chloramphenicol resistance marker from pUC19C was inserted. Plasmids that contain the chloramphenicol marker in the same

orientation as *degU* were isolated and used to transform *B. subtilis* strains harbouring *myc-lacZ or srfA-lacZ* reporter gene fusions. Integration and deletion of the *degU* gene was verified with PCR using primers DU1, DU2, CM1 and CM2.

REFERENCES

1. **Duitman, E. H., L. W. Hamoen, M. Rembold, G. Venema, H. Seitz, W. Saenger, F. Bernhard, R. Reinhardt, M. Schmidt, C. Ullrich, T. Stein, F. Leenders, and J. Vater.** 1999. The mycosubtilin synthetase of *Bacillus subtilis* ATCC6633: a multifunctional hybrid between a peptide synthetase, an amino transferase, and a fatty acid synthase. Proc Natl Acad Sci U S A **96:**13294-9.

2. **Nakano, M. M., N. Corbell, J. Besson, and P. Zuber.** 1992. Isolation and characterization of *sfp*: a gene that functions in the production of the lipopeptide biosurfactant, surfactin, in *Bacillus subtilis*. Mol Gen Genet **232:**313-21.

3. **van Sinderen, D., S. Withoff, H. Boels, and G. Venema.** 1990. Isolation and characterization of *comL*, a transcription unit involved in competence development of *Bacillus subtilis*. Mol Gen Genet **224:**396-404.

Table S1. Oligonucleotides

Primer:	gene or region:	sequence:
AB1	*abrB*	5'- GGA AAC CCT CAC TGC GAA AGA AC -3'
AB2	*abrB*	5'- GCT GTT ATT TCG GTA GTT TCC AAG AC -3'
CM1	*cm*^{R}	5'- GAC AAT TGG AAG AGA AAA GAG -3'
CM2	*cm*^{R}	5'- GAA TGT TTT AGA TAC ACC ATC AAA AAT TG -3'
CS1	*cm*^{R} *	5'- CTC TTT TCT CTT CCA ATT GTC -3'
CS2	*cm*^{R} *	5'- TAC ATC ATT CTG TTT GTG ATG -3'
CA1	*comA*	5'- ACC TGG CCT CGC CGC GGT TTC -3'
CA2	*comA*	5'- CAT TCC ATG AGC ATG GGG CTT TCT G -3'
CY1	*codY*	5'- AGC TGG CTT TAG GAG AGC TCG AAG -3'
CY2	*codY*	5'- AGA ACG ATT CAC CTT TTG ACT GAA G -3'
CY3	*codY*	5'- ATC GAG TCT AGA TCA TTA GGA ATG -3'
CY4	*codY*	5'- TGC CGC AGC TTG CAG CAT GGA G -3'
CS1	*cssS*	5'- GCT CTA GAA TTG CCG TCT CCT CGT ATCG -3'
CS2	*cssS*	5'- CGC GGA TCC AGC AGA CCT TGT CAG AGA A -3'
DC1	*dacC*	5'- ATA TCC TCG AGA AGG ATA AAC ACA ACC ATC TTC AC -3'
DC2	*dacC*	5'- ACA CCG AAT TCC GGA AGC GTA CTG TTT AAC -3'
DF1	*dacC-FenF*	5'- CCT CCA CCT AGA GCT ATT CAA C -3'
DF2	*dacC-FenF*	5'- GAT GCG TTT GTA TGT CTC TGA GGG TG -3'
DU1	*degU*	5'- CTA AAA ACA ACC TGG AGA GGG ATC -3'
DU2	*degU*	5'- CAT TCG GCT TGC TGG GCA TGA AAG -3'
FF1	*fenF*	5'- TCC GTC TGC AGG TCT TGT AGC GGT TGG ATA TAA AC -3'
FF2	*fenF*	5'- CAT GCT GTT GAT CCG AGT TC -3'
LZ1	*lacZ*	5'- CCA GCT GGC GAA AGG GGG -3'
MA1	*mycA*	5'- AAT TTG ATG ATA TCT ATT CTC -3'
MA2	*mycA*	5'- CCG GAA TTC GAC CAC TTT CTG TCT CTG G -3'
MC1	*mycC*	5'- TTC TGG ATC CAG CAC CTT ATG TTC GAT CAG -3'
MC2	*mycC*	5'- CTA TGG CAC ACG CTA TTC AAG -3'
ML1	*mycC*	5'- TGG GAC GAA TCG ATC ATC AGG -3'
PA1	*ppsA*	5'- ATA TCC TCG AGA AGG ATA AAC ACA ACC ATC TTC AC -3'
SR1	*sinR*	5'- GAA GCT ACA GAG TGG AAC GGC TTG -3'
SR2	*sinR*	5'- GGT TGA ATT AAT GGT GGA AGC CAA AG -3'
SH1	*Spo0H*	5'- GAA ATC GGC CCG GGA GCT TC -3'
SH2	*Spo0H*	5'- GAG CTG TAT GTG AAT TGC AAG TAC -3'
SK1	*Spo0K*	5'- GAA TGT TCT GCA TGG CCT AGG CTC -3'
SK2	*Spo0K*	5'- CTG AGG ATT TAG CCG TAA GGA GCTG -3'
SK3	*Spo0K*	5'- TTG TGA CGA GGA CTC CTG CTA AAG -3'
SK4	*Spo0K*	5'- GAG CCT AGG CCA TGC AGA ACA TTC -3'
SD1	*srfAD*	5'- GGC GGA ATG ATC ACC TTC AG -3'
SD2	*srfAD*	5'- CAG CCG CCA TGA CGA TTC CC -3'
YA1	*yoxA*	5'- TAT TTG GGA ACG CCG GCC ATC AAA G -3'

Underlined bases indicate restriction enzyme recognition sites and asterisks (*) indicates a Cy5-labeled primer.