Integration of a lipase gene into the *Bacillus subtilis* chromosome: Recombinant strains without antibiotic resistance marker

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Abstract
A new system is presented for the generation of recombinant *Bacillus subtilis* strains without antibiotic markers. This system is based on two plasmids constructed in *Escherichia coli*. The first plasmid pHM30 contains an incomplete *hisI* gene, the last gene in the histidine biosynthesis operon of *B. subtilis* and part of the genes *yvcA* and *yvcB* of unkown function flanking *hisI* at the 3´-end. The spectinomycin resistance gene is inserted between *hisI* and the downstream *yvcAB* region. Transformation of *B. subtilis* with this plasmid pHM30 led to spectinomycin resistant, histidine auxotrophic strains. The integrated parts of pHM30 act like a docking station for the second plasmid pHM31. The plasmid pHM31 contains the same *yvcAB* region but a complete copy of the *hisI* gene and no antibiotic resistance marker. Heterologous genes to be expressed in *B. subtilis* were inserted into a multiple cloning site between *hisI* and the downstream region. Transformants of *B.subtilis*/pHM30 with pHM31 derivatives were selected on minimal medium without histidine. By double crossovers during homologous recombination the heterologous genes were integrated, replacing the defect copy of *hisI* and the spectinomycin resistance gene. The plasmids were also successfully applied in the chromosomal integration of the lipase gene of *Bacillus thermocatenulatus* under a *B. subtilis* glucose regulated promotor/antiterminator system.
Keywords: Chromosomal integration; Antibiotic marker free strains; Food-grade organisms.

INTRODUCTION

The introduction of heterologous genes in organisms by plasmids, viruses or integration into the chromosomes is usually selected by antibiotic resistance markers. The occurrence of multiple antibiotic resistances in pathogenic organisms is a growing problem and there are concerns that antibiotic resistance genes in transgenic plants and microorganisms used for food production might cause a further spread of this problem. Today the main method to remove an antibiotic resistance gene from a chromosome is to use antibiotic resistance gene cassettes flanked by recognition sites for site-specific recombinases, for instance the Cre or FLP recombinases. When the recombinase genes are transiently expressed in the recombinant organism, the antibiotic resistance genes are excised. This has been done successfully in plants as well as in bacteria (Marx and Lindstrom, 2004; Kopertekh *et al*., 2004). There are only a few other methods reported that generate recombinant strains free of antibiotic resistance genes. One method was described by Brans *et al*. (2004). They brought the lysine biosynthesis gene *lysA* of *Bacillus subtilis* under the control of a β-lactamase promotor. Then they introduced the β-lactamase repressor gene *blaI* together with an antibiotic resistance marker and the gene of interest into the chromosome of this strain which made the cells conditionally auxotrophic for lysine. The *blaI* gene and the resistance marker were flanked by long direct repeats allowing the loss of the cassette by a single crossover. The eviction of the *blaI* and antibiotic resistance genes were identified simply by selecting lysine prototrophic strains. In another method previously described by Fabret *et al*. (2002), a gene cassette with an antibiotic resistance marker and a gene for counterselection were integrated into the chromosome. As before, the cassette was flanked by direct repeats which led to spontaneous eviction of the cassette via homologous recombination. The antibiotic sensitive strains were selected by the counterselection marker *upp* (uracil

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phosphoribosyl-transferase) which make cells sensitive to 5-fluorouracil. Fabret *et al*. (2002) used this method for generating chromosomal deletions and chromosomal point mutations, but in principle it can also be used to introduce foreign genes into a chromosome too. This was shown by Zhang *et al*. (2006) who used the same strategy, but with a different counterselection gene (an IPTG inducible *E. coli mazF* gene) to integrate a gene of interest into the chromosome or to generate point mutations in chromosomal genes. All three methods were developed for *B. subtilis.*

Bacillus subtilis is classified as a food-grade organism, i. e. it produces no endotoxins and other toxic substances and can be safely used for production of enzymes for food processing or for pharmaceuticals. Besides *E. coli,* it is the most deeply investigated bacterium. The genome is completely sequenced (Kunst *et al*., 1997) and all except 271 essential genes were knocked out recently (Kobayashi *et al*., 2003). A major advantage of *B. subtilis* is its genetic ability to take up any double-stranded DNA from the environment. This type of DNA is degraded to a single strand during uptake and efficiently integrated into the chromosome via homologous recombination (Dubnau, 1991). Such natural competence for transformation was exploited in the methods described above for generating recombinant strains without antibiotic resistance markers. The disadvantage of all three methods is the need for tight regulation systems or well functioning counterselection markers. In the following study, we describe a new way for generating recombinant *B. subtilis* strains without antibiotic resistance marker which, in contrast to the known methods does not need any sophisticated regulatory system or counterselection.

MATERIALS AND METHODS

Strains and culture conditions: *Escherichia coli* JM109 was used as host for transformation (Yanisch-Perron *et al*., 1984). *Bacillus subtilis* 3NA was transformed by taking advantage of its natural competence. Competent cells were prepared and transformed according to protocol No 3.8 (Bron, 1990) with plasmid DNA from *E. coli* linearized with *Sac*I. *E. coli* and *B. subtilis* cells were grown at 37ºC on LB agar plates and in LB liquid medium or minimal liquid medium and agar plates [2 g/l $(NH_4)SO_2$, 14.8 g/l K_2HPO_4, 5.4 g/l KH_2PO_4, 1.9 g/l trisodium citrate, 0.2 g/l $Mg_2SO_4.2H_2O$, 0.02 % (w/v) casamino acids, 15 g/l agar; protocol No 3.8] with 0.5 % w/v glucose and 0.5 % glycerol, respectively. The media were supplemented with spectinomycin (100 µg/ml) and histidine (20 µg/ml) when necessary.

Molecular techniques: All standard molecular techniques such as restriction enzyme analysis, ligation, PCR, transformation of *E. coli* were carried out as described in Ausubel *et al*. (1994). For Southern blots the DNA was blotted onto nitrocellulose and hybridized with pHM31 DNA and λ DNA labeled with digoxigenin, according to the manufacturer's instructions (Roche, Germany).

Plasmid constructions: The plasmids pHM30 and pHM31 were constructed from pIC20HE (Altenbuchner *et al*., 1992). A DNA fragment from *B. subtilis* containing the C-terminal part of *hisF*, the complete downstream *hisI* gene, which are the last two genes in the *his* operon (http://genolist.pasteur.fr/SubtiList/), were amplified with the primers S3597 (5′-CGC GGA TCT CGA AGC TC-3′) and S3598 (5′-AAA AAA GCT AGC ACC CAA TAT AAA TCT AAA TAC-3′), cleaved with endoR *Mlu*I and *Nhe*I and inserted between the *Mlu*I/*Nhe*I sites of pIC20HE to give pJOE4476.1. From same PCR fragment a *Mlu*I/*Bsa*AI fragment was inserted into pIC20HE cut with *Mlu*I and *Bsa*AI to give pJOE4475.2. Hereby the C-terminal end of *hisI* was deleted. A 1.3 kb fragment containing the C-terminal end of *yvcA* and N-terminal end of *yvcB* downstream of *hisI* was amplified by PCR using the primers S3599 (5′-GGA TGC AGT ATG AAT GAC AA-3′) and S3600 (5′-AAA AAA GCA TGC GCG GGT CAT CTT TTG AGA T-3′), cleaved with endoR *Bam*HI and *Sph*I and inserted into pIC20HE to give pJOE4482.1. From pJOE4482.1 the cloned PCR fragment was isolated again together with vector DNA as a *Bam*HI/*Sca*I fragment to replace the corresponding restriction fragment in pJOE4476.1 to give pHM31 and in pJOE4475.2 leading to pJOE4519.1. Finally, an *Eco*RI/*Eco*RV fragment from plasmid pDG1730 (Guérout-Fleury *et al*., 1996) encoding a spectinomycin resistance gene was inserted between the two *Sac*II sites of pJOE4519.1 leading to formation of the plasmid pMH30. Hereby a 108 bp *Sac*II fragment was deleted from pJOE4519.1. The lipase gene (*lip*) without signal sequence for export was isolated from plasmid pT-BTL-2 (Rua *et al*., 1998) as a *Nde*I/*Bam*HI fragment and first inserted into the *E. coli* expression vector pJOE4042.1 giving plasmid pJOE4615.1. For regulated expression of the lipase gene in *Bacillus*, the *B. subtilis ptsGHI* promotor (Stülke *et al*., 1997)

together with the corresponding antiterminator gene *glcT*, the terminator/antiterminator sequence and ribosomal binding site of *ptsG* were amplified with the primers S4163 (5′-AAA AAA CAA TGG CCC GGG AAG GAC AGC CGA TTG AAA-3′) and S4164 (5′-AAA AAA CAT ATG AAT TGA CCT CCT CTT TTT-3′). The resulting PCR fragment was inserted between the *Mfe*I/*Nde*I sites of pJOE4615.1. Finally, the *glcT-lip* fusion fragment was isolated again as a *Xma*I-fragment and inserted into pHM31 giving the pHM67 plasmid.

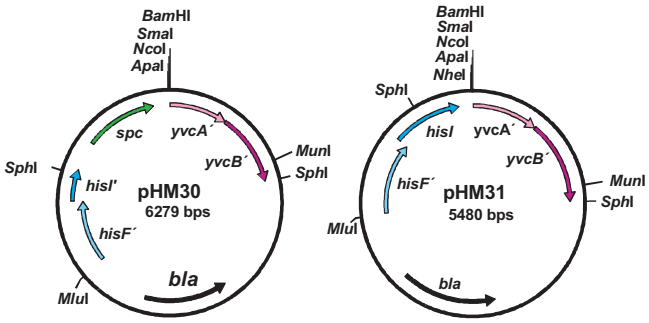

Figure 1. Restriction map of plasmids pHM30 and pHM31.

Induction kinetics of the *Bacillus thermocatenulatus* lipase: Lipase activity in the strains 3NA and 3NA/pHM67 was determined as follows. Cells were grown at 37ºC in minimal medium with 0.5% w/v glycerol to an optical density (OD_{550}) of 0.4. Glucose was added to a final concentration of 0.5% w/v and the cells were further incubated. Samples were taken immediately and after 2, 4 and 6 h, the cells were washed in 0.05 M sodium phosphate puffer, pH 7.0 and lysed by ultrasonication. The crude extract was cleared by centrifugation and the lipase activity was determined by adding 10 µl of crude extract to 990 µl of reaction buffer (0.05 mM $NaPO_4$, 5 mM Na-desoxycholate, 0.8 mM *p*-nitrophenyl palmitate). The resulting change in absorption was measured in a spectrophotometer at 410 nm for 1 min at 65ºC. One unit of lipase corresponds to the release of 1 µM *p*-nitrophenol (molar extinction coefficient: 15.200 mol^{-1} cm^{-1}) per min (Kaufmann and Schmitt-Dannert, 2001). Protein concentrations were determined according to Bradford (1970), using bovine serum albumin as standard.

RESULTS

Basically, to obtain marker-free recombinant strains DNA must be integrated into the *B. subtilis* chromosome by homologous recombination with an incomplete *B. subtilis* gene from the histidine biosynthesis operon which leads to auxotrophy for histidine. This first event is selected by an antibiotic resistance marker. These auxotrophic strains can then be used for integration of the target genes by a second plasmid. This plasmid has now the complete *his* gene but lacks the antibiotic resistance marker. This allows a selection of the *his* prototrophic strains, and by homologous recombination the target genes are integrated and the antibiotic resistance marker replaced. For this purpose we constructed two plasmids which are shown in Figure 1. Both plasmids pHM30 and pHM31 were constructed from the *E. coli* plasmid pIC20HE (Altenbuchner *et al*., 1992) which can not replicate in *B. subtilis*. The plasmid pHM30 contains the C-terminal part of *hisF* and the N-terminal part of *hisI* gene, the last two genes in the *his* operon. Downstream of the *his* operon in *B. subtilis*, the *yvcA* and *yvcB* genes of unknown function are located. A 1.3 kb fragment containing the C-terminal end of *yvcA* and N-terminal end of *yvcB* was amplified by PCR and fused with the fragment containing the incomplete *hisF/I* genes. Finally, a spectinomycin resistance gene was inserted between the *his-yvc* region to give pHM30. The plasmid pHM31 contains the same *yvc*-region and the same C-terminal end of *hisF* but has the complete *hisI* gene and lacks the antibiotic marker. Therefore, integration of pHM30 into the *B. subtilis* chromosome by two crossovers should lead to spectinomycin resistant, *his* auxotrophic mutants. The insertion of pHM31 into this mutant must lead to a spectinomycin sensitive, *his* prototrophic strain.

To demonstrate that this system is useful for integration and expression of heterologous genes, a lipase gene (*lip*) from *B. thermocatenulatus* was inserted into the *B. subtilis* chromosome via pHM30/pHM31. The lipase gene without a signal sequence for export was obtained from plasmid pT-BTL-2 (Rua *et al*., 1998). For regulated expression of the lipase gene, the *B. subtilis ptsGHI* promotor (Stülke *et al*., 1997) was inserted upstream of the lipase gene. The *ptsGHI* operon is controlled by a antiterminator gene *glcT* located upstream of the *ptsGHI* operon. Transcription of *ptsGHI* starts from a constitutive *ptsGHI* promotor within the C-terminal end of *glcT* and ends at a terminator sequence between the *glcT* and *ptsG* gene. In the presence of glucose the GlcT antiterminator protein binds between the promotor and the *ptsG* gene at a transcriptional antiterminator (RAT) sequence leading to transcription of *ptsGHI*. Therefore, the correspon-

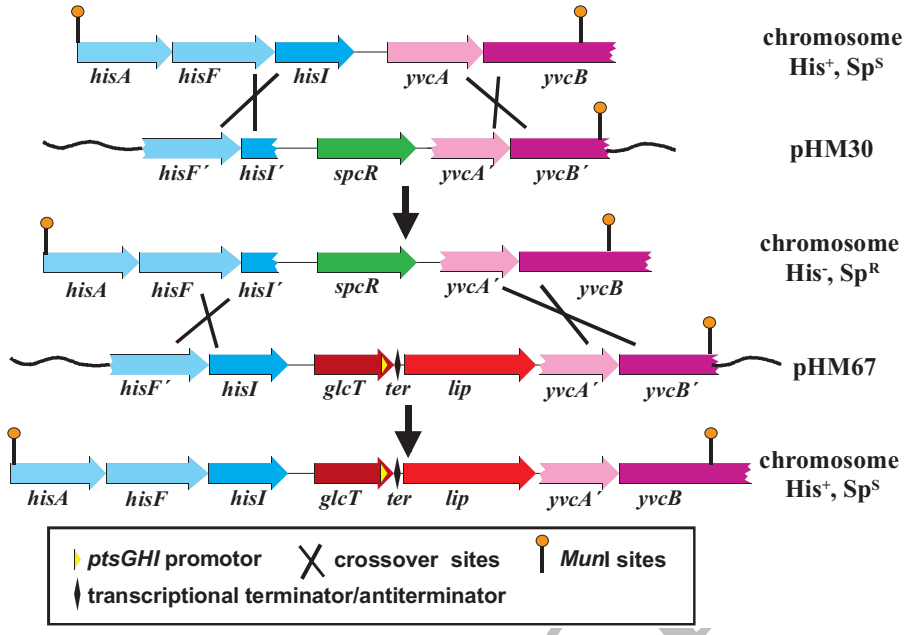

Figure 2. Schematic illustration of the two successive integration events of plasmids pHM30 and pHM67 into the *B. subtilis* 3NA chromosome.

ding *glcT* gene was amplified together with the promotor, the terminator/antiterminator sequence and ribosomal binding site of *ptsG*, then fused with the lipase gene and inserted into pHM31 to give pHM67. A sporulation negative *B. subtilis* 3NA (genotype *spoA3*, Michel and Millet, 1970) was transformed by pHM30 and the resulting transformants were selected by spectinomycin resistance. The colonies turned out to be histidine negative as expected. One of the clones was transformed again with pHM67 and the transformants were selected on minimal glucose medium lacking histidine. Testing of these colonies showed that most of them were spectinomycin sensitive but positive for lipase. The homologous recombination events are illustrated in Figure 2. To see that there were no further DNA rearrangements the chromosomal DNA of *B. subtilis* 3NA, 3NA/pMH30 and 3NA/pHM67 were isolated and digested with endoR *Mun*I. The resulting fragments were then separated on an agarose gel, blotted on nitrocellulose filter and hybridized with digoxigenin labeled pHM31 DNA (Figure 3). A 3.58 kb *Mun*I band was observed in the wild type 3NA, a 4.13 kb band in 3NA/pHM30 and a 5.78 kb in 3NA/pHM67 as expected, which indicates that no other further rearrangements happened except for the homologous recombination events illustrated in Figure 2.

To see that the lipase gene was actively expressed in the recombinant strain, the wild type 3NA and the recombinant 3NA/pHM67 were grown in minimal medium with glycerol and the *ptsGHI* promoter was induced by addition of glucose. Every two hours samples of the induced cultures were harvested and the cells were lysed by ultrasonic treatment. Lipase activity was determined by incubation of the crude extracts with *p*-nitrophenyl palmitate and measuring the change in the absorption at 410 nm by a spectrophotometer. Only very low lipase activities were found in the *B. subtilis* 3NA wild type strain (0.013 U/mg protein) whereas the recombinant strain with pHM67

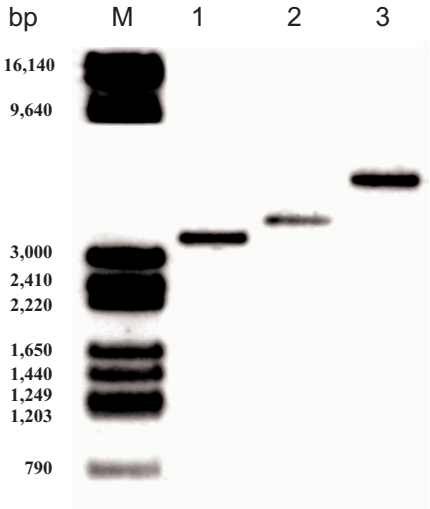

Figure 3. Southern blot of *B. subtilis* 3NA wild type (lane 1), 3NA/pHM30 (lane 2) and 3NA/pHM67 (lane 3) chromosomal DNA cleaved with *Mun*I. Molecular size marker (lane M) is λ DNA digested with *Bgl*I.

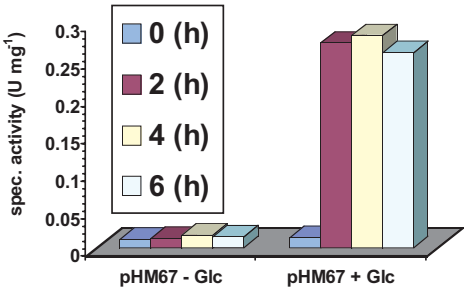

Figure 4. Induction kinetics of the *B. thermocatenulatus* lipase under the control of the *ptsGHI* promotor in strain 3NA/pHM67.

showed 0.25 U/mg of lipase activity. The induction kinetics of the lipase gene with glucose in *B. subtilis* 3NA/pHM67 is shown in Figure 4.

DISCUSSION

The successful integration and expression of the lipase gene into the *B. subtilis* chromosome and removal of the antibiotic resistance gene proves that this new system for genetic engineering of *B. subtilis* is efficient and easy to handle. With plasmid pHM30, any *B. subtilis* strain can be made histidine auxotrophic and ready for integration of any other gene which is inserted into pHM31. By just one further transformation, one gets recombinant strains free of antibiotic resistance genes. The same principle might be used for insertions of recombinant genes into other biosynthetic or catabolic operons where inactivation leads to auxotrophic or any other growth negative mutants, allowing the engineering of *B. subtilis* with multiple insertions. In addition this method could be extended to any other bacterial strain able to take up linear DNA.

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References

Altenbuchner J, Viell P, Pelletier I (1992). Positive selection vectors based on palindromic DNA sequences. *Methods Enzymol*. 216: 457-466.

Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (1994). *Curr Prot Mol Biol*. John Wiley and Sons, Inc.

Bradford M.M. 1976. A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem*. 72: 248-251.

Brans A, Filée P, Chevigné A, Claessens A, Joris B (2004). New integrative method to generate *Bacillus subtilis* recombinant strains free of selection markers. *Appl Env Microbiol*. 70: 7241-7250.

Bron S (1990). Plasmids. In: Harwood C.R. and S.M. Cutting (eds) *Molecular Biological Methods for Bacillus*. John Wiley and Sons Ltd, Chichester, England.

Dubnau D (1991). Genetic competence in *Bacillus subtilis*. *Microbiol Rev.* 55: 395-424.

Guérout-Fleury AM, Frandsen N, Stragier P (1996). Plasmids for ectopic integration in *Bacillus subtilis. Gene* 180: 57-61.

Fabret C, Ehrlich SD, Noirot P (2002). A new mutation delivery system for genome-scale approaches in *Bacillus subtilis*. *Mol Microbiol*. 46: 25-36.

Kaufmann I, Schmitt-Dannert C (2001). Conversion of *Bacillus thermocatenulatus* lipase into an efficient phospholipase with increased activity towards long-chain fatty acyl substrates by directed evolution and rational design. *Protein Engin.* 14: 919-928.

Kobayashi K, Ehrlich SD, Albertini A *et al*. (2003) Essential *Bacillus subtilis* genes. *Proc Natl Acad Sci USA.* 100: 4678-4683.

Kopertekh L, Juttner G, Schiemann J (2004). PVX-Cre-mediated marker gene elimination from transgenic plants. *Plant Mol Biol*. *55*: 491-500.

Kunst F, Ogasawara N, Moszer I *et al*. (1997). The complete genome sequence of the gram-positive bacterium *Bacillus subtilis*. *Nature* 390: 249-256.

Marx JM, Lidstrom ME (2004). Development of an insertional expression vector system for *Methylobacterium extorquense* AM1 and generation of null mutants lacking *mtdA* and /or *fch*. *Microbiology* 150: 9-19.

Michel JF, Millet J (1970). Physiological studies on early-blocked sporulation mutants of *Bacillus subtilis. J Appl Bacteriol*. 33: 220-227.

Rua ML, Atomi H, Schmitt-Dannert C, Schmid RD (1998). High-level expression of the thermoalkalophilic lipase from *Bacillus thermocatenulatus* in *Escherichia coli*. *Appl Microbiol Biotechnol.* 49: 405-10.

Stülke J, Martin -Verstraete I, Zagorec M, Rose M, Klier A, Rapoport G (1997). Induction of the *Bacillus subtilis ptsGHI* operon by glucose is controlled by a novel antiterminator, GlcT. *Mol Microbiol*. 25: 65-78.

Yanisch-Perron C, Viera J, Messing J (1984). Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. *Gene* 33: 103-109.

Zhang X-Z, Yan X, Cui Z-L, Hong Q, Li S-P (2006). *mazF*, a novel counter-selectable marker for unmarked chromosomal manipulation in *Bacillus subtilis*. *Nucleic Acids Res*. *34* (9): *e71* doi:10.1093/nar/gkl358.