Development of a markerless gene deletion system for *Bacillus subtilis* based on the mannose phosphoenolpyruvate-dependent phosphotransferase system

Marian Wenzel and Josef Altenbuchner

Institut für Industrielle Genetik, Universität Stuttgart, Allmandring 31, 70569 Stuttgart, Germany

To optimize *Bacillus subtilis* as a production strain for proteins and low molecular substances by genome engineering, we developed a markerless gene deletion system. We took advantage of a general property of the phosphoenolpyruvate-dependent phosphotransferase system (PTS), in particular the mannose PTS. Mannose is phosphorylated during uptake by its specific transporter (ManP) to mannose 6-phosphate, which is further converted to fructose 6-phosphate by the mannose-6-phosphate isomerase (ManA). When ManA is missing, accumulation of the phosphorylated mannose inhibits cell growth. This system was constructed by deletion of *manP* and *manA* in *B. subtilis* D6, a 168 derivative strain with six large deletions of prophages and antibiotic biosynthesis genes. The *manP* gene was inserted into an *Escherichia coli* plasmid together with a spectinomycin resistance gene for selection in *B. subtilis*. To delete a specific region, its up- and downstream flanking sites (each of approximately 700 bp) were inserted into the vector. After transformation, integration of the plasmid into the chromosome of *B. subtilis* by single cross-over was selected by spectinomycin. In the second step, excision of the plasmid was selected by growth on mannose. Finally, excision and concomitant deletion of the target region were verified by colony PCR. In this way, all nine prophages, seven antibiotic biosynthesis gene clusters and two sigma factors for sporulation were deleted and the *B. subtilis* genome was reduced from 4215 to 3640 kb. Despite these extensive deletions, growth rate and cell morphology remained similar to the *B. subtilis* 168 parental strain.

INTRODUCTION

*Bacillus subtilis* is an important producer of industrial enzymes, such as amylases and proteases, with favourable features including high protein secretion capability, GRAS (generally recognized as safe) status, natural competence, a long tradition in fermentation and an extensive existing knowledge base of its genetics, physiology and biochemistry. Up-to-date information is available on SubtiWiki (Michna et al., 2014). Strain optimization to remove adverse properties such a sporulation, lysogenic prophages, production of antibiotics and extracellular proteases (Krishnappa et al., 2013; Stein, 2005; Westers et al., 2003, 2004) or to introduce new metabolic routes, requires powerful tools for genetic engineering.

An easy method for chromosomal deletions is to construct deletion cassettes comprising an antibiotic resistance marker flanked by sequences that normally flank the region to be deleted. The successful integration of the cassette via homologous recombination can then be selected by the antibiotic resistance. For multiple modifications of the chromosome, recognition sites for site-specific recombinases, such as Xer/dif, Flp/FRT or Cre/loxP (Bloor & Cranenburgh, 2006; Hoang et al., 1998; Marx & Lidstrom, 2002), allow the precise excision of the marker gene, and the cassette can be used again. The risk of unintended large chromosomal deletions or inversions can be avoided by using chimeric recognition sites like *lox71/lox66* or *mroxP* (Warth & Altenbuchner, 2013a, b). However, a second transformation and curing of the plasmid providing the recombinase are needed. Last but not least, all such methods leave ‘scars’ in the chromosome.

The development of markerless systems deals with this problem. Here, the antibiotic resistance gene is located outside the cloned flanking sequences on a suicide vector. The vector is integrated via single cross-over recombination. Spontaneous excision of the vector either leads to removal of just the vector, or to removal of the vector and the target region. However, this is a rare event. By integration of a β-galactosidase gene into the vector, it is possible to

Abbreviation: PTS, phosphoenolpyruvate-dependent phosphotransferase system.

Two supplementary tables are available with the online Supplementary Material.
detect such rare events by blue-white screening, but this needs strains lacking β-galactosidase activity and the screening of thousands of colonies (Leenhouts et al., 1996). Therefore, counter-selection marker systems have been developed, like the popular fusaric acid (tetAR), streptomycin (rpsL) and sucrose (sacB) sensitivity systems (reviewed by Reyrat et al., 1998). Another takes advantage of the endonuclease I-SceI of Saccharomyces cerevisiae (Martinez-García & de Lorenzo, 2011; Montelhet et al., 1990). However, the application of these systems has not been proven effective or even applicable in B. subtilis due to natural resistances, lack of suitable vectors, etc.

A rather powerful system based on the sensitivity against 5-fluorouracil (5-FU; upp) has been developed for B. subtilis and successfully adapted for several other micro-organisms (Fabret et al., 2002; Goh et al., 2009; Graf & Altenbuchner, 2011; Keller et al., 2009; Kristich et al., 2005; Tanaka et al., 2013). Besides the toxicity of 5-FU, a major disadvantage is the occurrence of diverse suppressor mutations as already stated by Fabret et al. (2002). Other systems based on the action of toxins and antitoxins such as CcdB and MazF from Escherichia coli (Bernard et al., 1994; Zhang et al., 2006) require a perfect and sensitive toxin/antitoxin equilibrium. As these systems deal with toxic molecules, the occurrence of suppressor mutations is also very likely.

Alternatively, temperature-sensitive plasmids allow selection of their integration at their non-permissive temperature. Returning to its permissive temperature, the plasmid starts replicating again, and excision is selected (Arnaud et al., 2004; Zakataeva et al., 2010). However, the cells might also survive by slow growth and one has to make sure that the plasmid was indeed integrated.

Thus, there is a clear need for a less toxic, but powerful and reliable, counter-selection system for B. subtilis in order to deal with upcoming questions from large scale omics-data and analyses (Buescher et al., 2012; Goelzer et al., 2008; Kobayashi et al., 2001; Petersohn et al., 2001; Price et al., 2001; Yoshida et al., 2001). In this study, we describe the development and application of a novel, markerless method for multiple subsequent gene deletions in B. subtilis. We took advantage of a general property of the phosphoenolpyruvate-dependent phosphotransferase system (PTS), and particularly the mannose PTS. With this method we were able to construct a strain without several adverse properties and a reduced genome. The resulting strain was examined with regard to its growth rate.

**METHODS**

**Plasmids, bacterial strains and growth conditions.** Relevant bacterial strains and plasmids used in this study are summarized in Table 1 and Table S1, available in the online Supplementary Material. Standard recombinant DNA techniques were used (Sambrook et al., 1989). Cloning steps were performed with E. coli JM109 (Yanisch-Perron et al., 1985). E. coli JM109 was transformed with plasmid DNA using the TSS heat shock method as described by Chung et al. (1989). B. subtilis was transformed according to the modified ‘Paris method’ (Harwood & Cutting, 1990). All strains were grown at 37 °C, using LB medium (Bertani, 1951) or M6. The M6 minimal medium used for the first of the two-step growth phase for competence development and for comparing the growth rates of B. subtilis contained per l: 2 g (NH4)2SO4, 6 g KH2PO4, 14 g K2HPO4, 1 g sodium citrate, 0.2 g MgSO4, 5 g glucose, 200 mg Casamino acids, 50 mg tryptophan (if necessary) and 1 ml of a 1000-fold trace element solution (Wenzel et al., 2011). Antibiotics were used in the following concentrations: ampicillin (amp), 100 μg ml⁻¹; spectinomycin (spc), 100 μg ml⁻¹; erythromycin (erm), 5 μg ml⁻¹.

**Materials.** Mannose was purchased from AMRESCO. DNA modifying enzymes including restriction endonucleases were purchased from New England Biolabs. PCRs were run with High Fidelity PCR Enzyme Mix and DreamTaq DNA polymerase (Thermo Fisher Scientific) on a TPersson Thermocycler (Biometra). Synthetic DNA oligonucleotides (Table S2) were purchased from Eurofins MWG Operon. DNA sequencing was performed by GATC Biotech.

**Construction of plasmids pJOE6743.1 and pJOE6577.1.** The plasmid pJOE6743.1 is derived from pJOE4786.1 (Jeske & Altenbuchner, 2010). The spectinomycin resistance gene was isolated from pMW168.1 (Wenzel et al., 2011) by endoR AgeI and EcoRI, the protruding ends of the fragment filled in by Klenow polymerase treatment and inserted into the SfoI site of pJOE4786.1 (pJOE6562.4). The manP gene together with its promoter was amplified from B. subtilis 168 by PCR using primers s6477 and s6401, cleaved with NdeI and inserted into the NdeI site of pJOE6562.4 to give pJOE6743.1. The plasmid pJOE6577.1 is derived from pSUN346.1 (Sun & Altenbuchner, 2010). The plasmid pSUN346.1 again is derived from pJOE4786.1 and contains the C-terminal end of manR, the manA gene, followed by an erythromycin resistance gene (erm), ydpF and a spectinomycin resistance gene (aad9). The manA gene was removed by cutting pSUN346.1 with BamHI and PmeI and after Klenow treatment ligated to plasmid pJOE6577.1.

**Protocol for the chromosomal deletion formation.** Derivatives of pJOE6743.1 with deletion cassettes were used to transform a manPA negative B. subtilis strain. Colonies with the integrated plasmid (via single cross-over) were selected on LB agar plates containing spectinomycin. Five of the transformants were picked and streaked on a new LB agar plate containing spectinomycin. One single colony obtained from each of the five transformants was used to inoculate LB liquid medium. After incubation at 37 °C for about 8 h, the cultures were diluted 10⁻⁴ in LB liquid medium containing 6.5 % (w/v) mannose. After incubation at 37 °C overnight, 10⁻³ dilutions were plated on LB agar plates containing 6.5 % (w/v) mannose. The manose used in the last two steps helps to select the cells which have lost the integrated vector (counter-selection). Four colonies from each plate (20 clones in total) were picked and streaked on LB agar plates with and without spectinomycin. Spectinomycin-sensitive colonies were finally checked by colony PCR.

**Colony PCR.** After selection for chromosomal plasmid excision, colonies were streaked out on LB agar plates. A small amount of cells was taken from these plates and resuspended in 100 μl H₂O (deionised). After heating the suspension for 10 min at 99 °C, a cold shock for 20 min at −70 °C followed by a further heating step (10 min at 99 °C) was performed. After centrifugation of the cell suspension, 2 μl of the supernatant containing chromosomal DNA was used in a 30 μl PCR preparation using DreamTaq DNA polymerase. As a control, the same procedure was performed with two colonies still containing the integrated plasmid.
Table 1. Bacterial strains used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype/properties</th>
<th>Deletion from bp–bp*</th>
<th>Reference/source</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. subtilis 168</td>
<td></td>
<td>NA</td>
<td>Bacillus Genetic Stock Center</td>
</tr>
<tr>
<td>B. subtilis 3NA</td>
<td>trpC2</td>
<td>NA</td>
<td>Bacillus Genetic Stock Center</td>
</tr>
<tr>
<td>B. subtilis A6</td>
<td>sp0A3</td>
<td>NA</td>
<td>Westers et al. (2003)</td>
</tr>
<tr>
<td>IIG-Bs1</td>
<td>trp+</td>
<td>1272608–1275639</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs2</td>
<td>ΔmanPA : : erm</td>
<td>529495–604658</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs3</td>
<td>Δproφ2</td>
<td>1879831–1886598</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs4</td>
<td>Δproφ5</td>
<td>2055447–2079118</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs5</td>
<td>Δproφ6</td>
<td>1781877–1859956</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs8</td>
<td>ΔCmrR</td>
<td>3835926–3842999</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs9</td>
<td>Δsubtilosin</td>
<td>1960195–1997960</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs10</td>
<td>Δplipastin</td>
<td>3867368–3874329</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs11</td>
<td>Δbacillosin</td>
<td>2708926–2742191</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs12</td>
<td>Δproφ7</td>
<td>3121673–3124005</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs13</td>
<td>Δbacilloscin</td>
<td>3464131–3467474</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs14</td>
<td>ΔBacillus toxin</td>
<td>1599076–1606455</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs20</td>
<td>Protease, sporulation (sigE, sigG)</td>
<td>1272643–1279510</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs20-1</td>
<td>ΔmanPA : : erm</td>
<td>1263599–1270416</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs20-2</td>
<td>Δproφ4</td>
<td>1125000–1130795</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs20-3</td>
<td>Δ3,3'-neotrehalosodiamine</td>
<td>377215–390803</td>
<td>This study</td>
</tr>
<tr>
<td>IIG-Bs20-4</td>
<td>AsrfAA-srfX (except comS)</td>
<td>391074–408169</td>
<td>This study</td>
</tr>
</tbody>
</table>

NA, Not applicable.

RESULTS

Plasmid and strain construction for manP-based counter-selection

In order to develop a new efficient counter-selection system for B. subtilis, we took advantage of the fact that mannose-6-phosphate dehydrogenase mutants (manA) were sensitive to the presence of mannose (Sun & Altenbuchner, 2010; Wenzel et al., 2011). Cells developed a bubble-like morphology and stopped growing about 1 h after addition of mannose to the culture medium (Wenzel et al., 2011). The sensitivity to mannose is dependent on the presence of manP and absence of manA. A strain without both genes is resistant to mannose. The introduction of manP to such a strain would reestablish the sensitivity to mannose. Taking advantage of these observations, we developed the idea of using manP as a counter-selectable marker.

For this concept to work, one has to delete the manPA genes in the B. subtilis chromosome and to develop a vector which: (i) carries the manP gene; (ii) does not replicate in B. subtilis; (iii) encodes an antibiotic resistance gene for selection in B. subtilis; and (iv) has restriction sites for cloning of the deletion cassettes. The deletion cassettes, two sequences of about 700 bp flanking the target region, enable integration of the vector into the B. subtilis chromosome via single cross-over. This is selected for by the antibiotic resistance. After integration, cells are cultured in the presence of mannose. Only cells which have spontaneously lost the vector, alone or together with the target region, form colonies on mannose-containing agar plates. The colonies are screened for loss of the antibiotic resistance, and the antibiotic-sensitive ones finally tested by colony PCR for loss of the target region.

To use manP for multiple deletions in the chromosome of B. subtilis, the vector pJOE6743.1 was constructed (Fig. 1). This is a cloning vector derived from pJOE4786.1 (Jeske & Altenbuchner, 2010). Two long inverted repeats, separated by a lacPOZs fragment, allow blue/white screening in strains like E. coli JM109. When the two repeats come together, the plasmid cannot be replicated (Altenbuchner et al., 1992). Therefore, a positive selection occurs on the replacement of the lacPOZs fragment. The vector is a pUC derivative and carries the ampicillin resistance gene for selection in E. coli, a spectinomycin resistance gene for selection in both B. subtilis and E. coli, and the B. subtilis manP gene encoding the mannose PTS transporter under control of its own promoter regulated by ManR (Wenzel & Altenbuchner, 2013).

Before this vector can be used in B. subtilis, the manPA genes had to be removed. B. subtilis A6, a derivative of B. subtilis 168 with a series of already deleted prophages and antibiotic biosynthesis genes, was chosen as the
Markerless gene deletion system for Bacillus subtilis

Fig. 1. Plasmid map of the positive cloning and integration vector pJOE6743.1. Selected double cutters for insertion of the amplified homologous regions were added to the map. Binding sites for SP6 and T7 standard primers used for sequencing are shown as white boxes. manP, B. subtilis mannoside PTS transporter; ter, transcription terminator from phage λ; amp, ampicillin resistance; SpcR, spectinomycin resistance; lacPOZ, lac promoter, lac operator and lacZ α fragment.

starting strain (Westers et al., 2003). For easier handling, the tryptophan auxotrophic strain was transformed with chromosomal DNA from B. subtilis 3NA (Michel & Millet, 1970) to replace the trpC2 allele present in the B. subtilis 168 derivatives (Table 1; for more details, see Table S1). To make sure that the new strain designated IIG-Bs1 still contained the deletions conducted by Westers et al. (2003), PCR was performed with primers shown in Table S2.

Next, the manPA genes had to be deleted in the IIG-Bs1 chromosome. This was done by cloning the upstream manose regulatory gene and the downstream yjdF gene to both sides of an erythromycin resistance gene in an E. coli pJOE4786.1 derivative. In addition, this plasmid had a spectinomycin resistance gene inserted downstream of yjdF (plasmid pJOE6777.1). This plasmid DNA was used to transform B. subtilis IIG-Bs1. The resulting strain, designated IIG-Bs2, had the manPA genes replaced by an erythromycin resistance gene and served as the initial strain for further deletions using the new counter-selection system. Later, the erythromycin resistance gene was deleted, together with yjdF and several further genes downstream of yjdF (IIG-Bs20-1; Tables 1 and S1).

Development and application of the new counter-selection method

First, the flanking regions of the target DNA to be deleted were amplified by PCR. The fragments were combined and cloned into the vector pJOE6743.1 in three different ways: (i) restriction sites were added by the primers to both sides of the amplified fragments and the fragments cloned into the vector by a three-fragment ligation reaction; (ii) amplified fragments were fused by overlap extension PCR using the outer primers (Ho et al., 1989) and inserted blunt-ended into the SmaI or EcoRV cut vector; and (iii) restriction sites were added to the outer ends of the amplified fragments, the fragments fused by overlap PCR, cut at the corresponding restriction sites and inserted into the vector cleaved with the same restriction enzyme. Tables S1 and S2 summarize the constructed plasmids and oligonucleotides used for PCR. Using a pJOE6743.1 derivative with a deletion cassette, a manPA-negative strain was transformed. In principle, the integration of the plasmid in the chromosome was selected by spectinomycin and in a second step, the excision was selected, first by growing the cells in LB liquid, in LB liquid containing mannose and on mannose-containing LB plates. The excision and deletion formation were checked by colony PCR. In theory, half of the clones should have lost the region of interest, while the other half should have restored the original state. In fact, we observed that on average, 30–70 % of the clones (mean value) had lost the region of interest.

Application of the new manP counter-selection system for B. subtilis genome engineering

Ten large AT-rich regions were identified in the B. subtilis 168 chromosome, which represent the temperate phage SPβ (Zahler et al., 1977), the defective prophage PBSX (Wood et al., 1990), the integrative conjugative element ICEBs1 (Auchtung et al., 2003), skin (sigma K intervening) integrated in sigK and six prophage-like elements (Westers et al., 2003). Lysogenic phages and cryptic prophages might cause severe problems by spontaneous or induced phage activation during fermentation. Therefore, it is advantageous to remove all phage elements. In B. subtilis Δ6, the phages SBβ and PBSX, as well as the cryptic prophages proφ1, proφ3 and skin have already been deleted. With the new counter-selection method described in the section above, we were able to successively delete ICEBs1 together with a large flanking region (IIG-Bs3), proφ5 (IIG-Bs4), proφ6 (IIG-Bs5), proφ4 (IIG-Bs20-2) and proφ7 (IIG-Bs12) (see Tables 1 and S1).

About 4–5 % of the B. subtilis genome is devoted to antibiotic production (Stein, 2005). There are two lantibiotic biosynthesis gene clusters producing sublancin 168 (Paik et al., 1998) and subtilosin A (Zheng et al., 2000). Two
further gene clusters are responsible for the production of the non-ribosomal lipopeptides surfactin and plipastatin (synonymous to fengycin). The pks operon encodes the synthesis of an unknown polyketide (Kunst et al., 1997). Due to a mutation in sfp, the phosphopantetheine transferase, the non-ribosomal peptides and the polyketide are not produced in *B. subtilis* 168 (Mootz et al., 2001). Nevertheless, the pathways can be reactivated by simple transformation with DNA of *B. subtilis* WT strains (Nakano et al., 1992; Tsuge et al., 1999). There are three other antibiotics produced by *B. subtilis* 168: the dipeptidyl bacilysin (Inaoka et al., 2003), the phospholipid bacilyscin (Tamehiro et al., 2002) and the amino sugar 3,3′-neotrehalsadiamine (Inaoka et al., 2004). The sublancin 168 gene cluster is located on plasmid SPβ and was already deleted in Δ6. The pks operon was deleted as well in Δ6 and replaced by a chloramphenicol resistance gene. To get rid of this antibiotic resistance gene, the original deletion was slightly extended, leading to IIG-Bs8. The subtilosin A production genes were removed by deletion of genes *sboA*, *sboX* and *albA–albG* (IIG-Bs9) with the new method.

In case of plipastatin, the genes *ppsA–ppsE* (Steller et al., 1999) were deleted (IIG-Bs10). In case of surfactin, the situation was more complicated (Marahiel et al., 1993). Within *srfA*, encoding the nonribosomal peptide synthase, there is a second small gene *comS* needed for competence. (Turgay et al., 1998). Hence, *srfAA*, *srfAB* (except *comS*), *srfAC*, *srfAD* and additional genes up to *sfp* were deleted. The *srfA*-promoter and *comS*, however, were retained (IIG-Bs20-4).

The bacilysin production was abolished by deletion of the genes *bacABCDEF* (IIG-Bs11), the bacilysin synthesis by deletion of *ytpA* (IIG-Bs13) and finally the 3,3′-neotrehalsadiamine biosynthesis by deletion of *ndtR* (*yjhm*), *ndtA*, *ndtB*, *ndtC* and *gcp* (IIG-Bs20-3). In the end, all known antibiotic biosynthesis clusters on the *B. subtilis* chromosome were deleted in strain IIG-Bs20-4.

Endospore formation in *B. subtilis* is triggered by nutrient limitations. The master regulator for differentiation into spores is Spo0A, which becomes activated by phosphorylation (Fujita & Losick, 2005). Activated Spo0A triggers two parallel and interconnected programs of gene expression, one in the forespore by activating sigma factor σ^F^, and one in the mother cell by activating sigma factor σ^E^ (Eichenberger et al., 2003; Molle et al., 2003; Wang et al., 2006). Deletion of spo0A would be the simplest way to inhibit sporulation. On the other hand, this would inactivate the natural transformation capability. Therefore, we deleted in one step sigma factors *sigE* and *sigG* together with *spoG* responsible for pro-σ^E^ activation and a gene for the extracellular protease Bpr (IIG-Bs20).

During transition from exponential growth to sporulation, some differentiating cells show fratricidal behaviour (González-Pastor, 2011). They produce toxins which kill sibling cells. There are two independent gene clusters for toxin production: *skf* for sporulation killing factor, and *sdp* for sporation delaying protein (Allenby et al., 2006). The *skf* gene cluster was already removed by deleting prophage 1. Similarly to the *skf* operon, the *sdp* gene cluster produces a toxic peptide which is modified and exported by enzymes encoded by genes in two convergent operons, *sdpABC* and *sdpRI* (Ellermeier et al., 2006; Engelberg-Kulka et al., 2006). The two *sdp* operons were deleted together in strain IIG-Bs14 in order to get rid of potential killer cells.

**Comparison of the growth characteristics of IIG-Bs20-4 with *B. subtilis* 168**

The chromosomal deletions in IIG-Bs20-4 might affect the growth rate of this strain. Therefore, we compared the growth curves of IIG-Bs20-4 with the ones from *B. subtilis* 168 in LB and minimal media. For LB, overnight cultures in LB at 37 °C were used to inoculate 10 ml fresh LB medium in shake flasks to an OD_{600} of 0.05. The cultures were incubated at 37 °C on a rotary shaker at 150 r.p.m. The OD_{600} was determined hourly. For minimal media, MG1 medium supplemented with trace elements was used. Fresh MG1 medium was inoculated by an overnight culture in MG1 to an OD_{600} of 0.1 and the growth determined spectroscopically like in LB.

Independent of the medium used, both strains behaved similarly (Fig. 2). Using LB or MG1, the two strains showed nearly identical growth rates (Table 2). Thus, even a deletion of as much as 13.6 % of the *B. subtilis* genome did not compromise the viability of the bacteria, suggesting that the constructed strain IIG-Bs20-4 is a suitable chassis for industrial applications.

**DISCUSSION**

A counter-selection system is essential for markerless genome engineering. The method described in this study allows the introduction of point mutations, deletions of various lengths and gene insertions into the *B. subtilis* chromosome. There is a shortage of reliable counter-selection systems, especially ones which do not use toxic compounds. The use of the manP PTS transporter in a manPA mutant turned out to be a highly reproducible and efficient way to produce a series of deletions in the *B. subtilis* genome. No spontaneous resistance to mannose, for example by mutation of the mannose activator gene or by induction of a phosphosugar phosphatase, was observed. The deletions from *B. subtilis* Δ6 to IIG-Bs20-4 were checked by colony PCR. Another strain derived from IIG-Bs20 with many more deletions, made for a different purpose with this new counter-selection method, clearly showed after sequencing that all deletions occurred exactly as planned and there were no DNA rearrangements or single base mutations found (D. Reuss, personal communication). Since many bacteria use PTS systems for sugar uptake, it should be easy to adapt such a system for genome engineering in other bacterial species.

In a series of deletions we were able to considerably reduce the *B. subtilis* genome from 4215 to 3640 kb. Another
B. subtilis 168 strain (MGB874) with reduced genome was constructed previously (Morimoto et al., 2008). In MGB874, depleted of 874 kb (20 %) of its genome sequence, the purpose was to delete a large number of dispensable genes without altering the growth rate and other strain-specific properties like sporulation or antibiotic production. Since many of the dispensable genes are located in regions identified as prophages or genomic islands, the strains MGB874 and IIG-Bs20-4 have many deleted genes in common. Nevertheless, IIG-Bs20-4 has the advantage that it does not produce spores, antibiotics or bacteriocins, properties which are desired for a production strain for enzymes or low molecular substances.

The new strain provides a valuable basis for further optimization concerning protein production and secretion, high cell-density fermentation or metabolic engineering.

ACKNOWLEDGEMENTS

We thank Annette Schneck and Sina Blessing for their assistance in creating the deletion strains and Kambiz H. Morabbi for helpful discussions. The project was partly financed by Lonza AG and thus we would like to thank especially Christoph Kiziak for support and helpful discussions. We are grateful to Jan Maarten van Dijl for providing the strain B. subtilis A6.

REFERENCES


Edited by: J. Stülke