Nonribosomal peptide synthesis in Bacillus subtilis

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
2003

Link to publication in University of Groningen/UMCG research database

Citation for published version (APA):

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Chapter three

Molecular tools to facilitate transformation of *Bacillus subtilis*

Abstract

Here we describe two methods that facilitate transformation of *Bacillus subtilis*. The first method strongly increases competence development and hence transformation efficiencies of *B. subtilis* ATCC6633, *B. subtilis* ATCC21332, and probably other *B. subtilis* strains that are difficult to transform, because of low competence development. For this purpose we made use of the low-copy plasmid pGSP12, which contains the *comK* gene under control of its own promoter. Introduction of pGSP12 in *B. subtilis* ATCC6633 and *B. subtilis* ATCC21332 increased the transformation efficiency of these strains 60 to 200 fold and also enables transformation of these strains when cultured in rich media. The second method facilitates Campbell-type genomic integrations in *B. subtilis* of DNA ligation mixtures without the prior need of subcloning in *E. coli*. In this method Polyethyleneglycol 8000 (PEG8000) is used for the production of large linear multimeric ligation products that appeared to be efficient substrates for Campbell-type integrations in competent *B. subtilis* cells.

Introduction

*Bacillus subtilis* exhibits a number of interesting features such as synthesis and secretion of degradative enzymes, production of peptide antibiotics, sporulation and development of competence (Sonenshein *et al.*, 1993). The latter makes this bacterium easily accessible to genetic analysis, and is one of the main reasons why *B. subtilis* became a popular prokaryotic model organism. Competent *B. subtilis* cells are able to take up exogenous DNA, and subsequently, to integrate this DNA into their genomes via homologous recombination (Dubnau, 1991). Competence in *B. subtilis* develops optimally in minimal medium at the beginning of the stationary growth phase, when the culture has reached a critical cell density. A complex signal transduction cascade integrates the various external- and internal signals, and carefully regulates expression of ComK, the pivotal competence transcription factor necessary for the expression of the genes encoding the proteins involved in DNA uptake and recombination (Grossman, 1995; van Sinderen and Venema, 1994; van Sinderen *et al.*,1995). In cultures of the commonly used laboratory strain, *B. subtilis* 168 (Kunst *et al.* 1997), about 10 % of the total cell population develops competence under optimal conditions. This relatively high percentage of competence development is one of the main reasons why this strain, or derivatives of this strain, became such a popular laboratory strain. Most other *B. subtilis* strains, many of which are producers of important industrial products, have been selected on other criteria and develop very low or no competence at all. The failure of such strains to develop sufficient levels of competence may be caused at any level of
### Table 3.1: Strains and plasmids.

<table>
<thead>
<tr>
<th>Strains:</th>
<th>Relevant genotype/characteristics</th>
<th>Source or reference</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. subtilis</em> ATCC21332</td>
<td>low competence development, surfactin⁺</td>
<td>Mulligan <em>et al.</em>, 1984.</td>
</tr>
<tr>
<td><em>B. subtilis</em> ATCC6633</td>
<td>low competence development, surfactin⁺, mycosubtilin⁻</td>
<td>Garrido <em>et al.</em>, 1982.</td>
</tr>
<tr>
<td><em>B. subtilis</em> BV02J07</td>
<td><em>Km⁺</em>, <em>sfp⁺</em></td>
<td>This work</td>
</tr>
</tbody>
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<table>
<thead>
<tr>
<th>Plasmids:</th>
<th></th>
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<tbody>
<tr>
<td>pGSP12</td>
<td>derivative of pH12, Em⁺, contains <em>comK</em> under the control of its own</td>
<td>Van Sinderen <em>et al.</em>, 1995.</td>
</tr>
<tr>
<td>pKM1</td>
<td><em>Km⁺</em>.</td>
<td>Kiel <em>et al.</em>, 1987</td>
</tr>
<tr>
<td>pLGW300</td>
<td>integration vector, Km⁺, contains a promoterless <em>spo0V-lacZ</em> fusion.</td>
<td>Van Sinderen <em>et al.</em>, 1990.</td>
</tr>
</tbody>
</table>

The complex regulation cascade underlying competence development. Alternative transformation methods for *B. subtilis*, such as electroporation and protoplast transformation, have been developed (Chang and Cohen, 1979; Chassy *et al.*, 1988). However, these methods are relatively inefficient and can not be used for mutating specific genes via homologous recombination, since the proteins required for this process are only produced during competence development (Haijema *et al.*, 1995).

In addition to transcriptional activation of several competence-related genes, ComK is also required for the expression of its own gene, and it has been shown that introduction of *comK* on the low copy plasmid pGSP12 resulted in overproduction of ComK, presumably due to an effective autostimulatory response (van Sinderen *et al.*, 1995). This overproduction resulted in competence development, which was partially insensitive to medium- and growth phase control. We assumed that the increased expression of *comK* bypasses several essential regulatory steps in the competence signal transduction cascade and consequently, that introduction of pGSP12 might stimulate development of competence in *B. subtilis* strains which normally exhibit low levels of competence. In this study we show that the transformation efficiencies of the poorly transformable *B. subtilis* strains ATCC6633 and ATCC21332, both producers of several lipopeptides with antiviral and antifungal activities (Garrido *et al.*, 1982; Mulligan *et al.*, 1984), was strongly stimulated by the introduction of pGSP12.

Campbell-type integrations, often used for interruption of genes or construction of reporter gene fusions, require circular DNA. Binding of DNA to the cell envelope of competent *B. subtilis* cells is accompanied by DNA cleavage. Therefore, Campbell-type single cross-overs with linearized monomeric molecules will result in chromosome breakage, as no circular molecules can be formed after uptake of the DNA, and integration will only result from rare double cross-over events (Dubnau and Cirigliano, 1972; Venema *et al.*, 1965). This complication can be overcome by using multimeric DNA, which after uptake, circularizes on the basis of
Transformation tools

redundant sequence homologies. Usually, certain *E. coli* strains are used as intermediate host for the production of high amounts of multimeric DNA. However, a disadvantage of the use of *E. coli* is that plasmids containing *B. subtilis* DNA fragments are often unstable in this bacterium. Multimeric DNA can also be obtained by means of enzymatic ligation *in vitro*, yet the efficiency of Campbell-type integrations of ligation products is extremely poor due to the low percentage of multimeric DNA that is formed during the ligation reaction. Addition of high concentrations of polyethylene glycol 8000 (PEG8000) to the reaction mixture causes macromolecular crowding, which strongly enhances blunt- and cohesive-end ligations and, most importantly, prevents intramolecular circularization, thus facilitating the formation of large linear multimeric products are formed during the ligation reaction (Pfeiffer and Zimmerman, 1983; Zimmerman and Pfeiffer, 1983). Here we show that PEG8000-stimulated formation of multimeric DNA enables the direct use of ligation products as efficient substrates for Campbell-type integrations in *B. subtilis*.

Materials and methods

**General materials and methods.** Strains and plasmids used in this study are listed in Table 3.1. Molecular cloning and PCR procedures were carried out using standard molecular biology techniques described by Sambrook *et al.* (Sambrook *et al.*, 1989). TY-medium for growth of *E. coli* and *B. subtilis* was prepared as described by Biswal *et al.* (Biswal *et al.*, 1967). Minimal medium for growth of *B. subtilis* was prepared as described by Spizizen (Spizizen, 1958). Plasmid DNA and *B. subtilis* chromosomal DNA were purified according to protocols of Birnboim *et al.* and Venema *et al.* (Birnboim *et al.*, 1979; Venema *et al.*, 1965), respectively. Enzymes and chemicals were obtained from Roche diagnostics GmbH (Mannheim, Germany), Sigma (St. Louis, USA) and Merck KGaA (Darmstadt, Germany). Oligonucleotides used for PCR were obtained from Gibco BRL (Paisley, UK).

**Transformation in the presence of pGSP12.** Transformation of competent *B. subtilis* was essentially performed as described by Spizizen (Spizizen, 1958). Poorly competent *B. subtilis* strains were first transformed with pGSP12, a low-copy plasmid containing *comK*, using protoplast transformation, and transformants were selected on regeneration-agar plates containing 5 µg/ml erythromycin (Chang and Cohen, 1979). *B. subtilis* cells containing pGSP12 were grown to competence as follows. An overnight culture was grown in minimal medium with 2.5 µg/ml erythromycin at 37ºC under continuous shaking at 300 rpm. After 100-fold dilution of the overnight culture in the same medium, growth was continued and followed in time. Two hours after the transition from exponential to stationary growth, 1 µg of *B. subtilis* BD1807 chromosomal DNA, containing a chloramphenicol resistance marker at the *abrB* locus, was added to 0.5 ml of cells. After 20 minutes, 0.3 ml of TY medium was added and growth was continued for another 30 minutes, after which the cells were plated on TY-agar plates containing 5 µg/ml chloramphenicol. From a number of chloramphenicol resistant colonies chromosomal DNA was isolated to verify the insertion of the chloramphenicol resistance marker. The presence and position of this marker was determined by means of PCR using the following primers; Cm1: 5’- GAC AAT TGG AAG AGA AAA GAG -3’, Cm2: 5’- CAT CAC AAA CAG AAT GTA C -3’, AbrB1: 5’- GGA AAC CCT CAC TGC GAA AGA AC -3’, and AbrB2: 5’-
GCT GTT ATT TCG GTA GTT TCC AAG AC -3'.

**Campbell-type integration using DNA ligation products.** The plasmid used to measure the efficiency of Campbell-type integration of ligation products, pLGW300, is a general integration vector often used to introduce *lacZ*-reporter fusions via Campbell-type integrations in *B. subtilis* (van Sinderen et al., 1990). All ligations were performed overnight at room temperature using T4 ligase and buffer obtained from Roche diagnostics GmbH (Mannheim, Germany), in a total volume of 30 µl. For PEG-ligations, PEG<sub>8000</sub> was added to a final concentration of 15% (Pfeiffer and Zimmerman, 1983; Zimmerman and Pfeiffer, 1983). To monitor Campbell-type integration, a DNA fragment containing the *dacC* region of *B. subtilis* strain168 was ligated to pLGW300, which was linearized by digestion with *EcoRI* and *XhoI*. The *dacC* region was obtained by PCR using the following primers; DacC-up1: 5'- ATA TCC TCG AGA AGG ATA AAC ACA ACC ATC TTC AC -3' (*XhoI* recognition site is underlined), and DacC2: 5'- ACA CCG AAT TTC CCT CAC ATG TCA TCT ATT TG -3', and Km1: 5'- CGC GGA TCC GTC GAC CAT ATT TA -3', Km2: 5'- CCG GAA TTC GGG ACC CCT ATC TAG CGA AC -3', for the 3'-part of *sfp* and the kanamycin resistance marker, respectively (*BamHI* and *EcoRI* recognition sites are underlined).

Prior to ligation both PCR products were digested with *BamHI* and *EcoRI* to ensure the desired orientation of both genes in the multimeric ligation products. Transformants were selected on TY-agar plates containing 5 µg/ml kanamycin, and surfactin production was tested using a halo-assay on blood-agar plates as described by Mulligan et al. (1984). The correct insertion of the kanamycin resistance marker was determined by means of PCR using the following primers; Km1, Km2, SFP3: 5'- TTG TTC TGC GCT GGA CAT TT -3', and sfp4: 5'- GTC CAG CTT TG GGT AAG GGG -3'.

**Results and discussion**

**Stimulating transformation by introduction of plasmid-located *comK*.** Both *B. subtilis* ATCC6633 and ATCC21332 produce lipopeptides with antiviral and antifungal activities, such as surfactin and mycosubtilin (Garrido et al., 1982; Mulligan et al., 1984). In order to study the expression and regulation of the operons
Figure 3.1. pGSP12-stimulated transformation of *B. subtilis* ATCC6633 and ATCC21332. A) Growth curves of *B. subtilis* ATCC6633 (▲/△ with pGSP12 and ■/□ without pGSP12) and *B. subtilis* ATCC21332 (●/○ with pGSP12 and ◆/◇ without pGSP12). Closed symbols refer to growth in minimal medium (MM) and open symbols refer to growth in rich medium (TY). The time scale indicates hours before and after transition from the exponential to the stationary growth phase and arrows indicate the time of transformation. B) Number of transformants obtained after transformation with 1 µg chromosomal DNA.

encoding the surfactin- and mycosubtilin synthetases, we had to introduce transcriptional *lacZ* fusions and specific mutations in these strains. However, the low levels of competence development in these *B. subtilis* strains precluded this approach. The low copy plasmid pGSP12, containing the *comK* gene under the control of its own promoter, stimulates competence development in *B. subtilis* 168 derivatives, and bypasses the requirements for medium composition essential for optimal competence development (van Sinderen and Venema, 1994). To examine whether pGSP12 also would increase competence development, and hence the transformation efficiencies, of *B. subtilis* ATCC6633 and ATCC21332, this plasmid was introduced into these strains by means of conventional protoplast transformation. Indeed, the number of transformants obtained in minimal medium after introduction of pGSP12 increased dramatically for both *B. subtilis* ATCC6633 and ATCC21332 (Fig. 3.1). This figure also shows that similar results were obtained when both strains were cultured in rich medium, although the total number of transformants was lower. These results clearly demonstrate that pGSP12 is a very useful tool to enhance transformation in *B. subtilis* strains which do not normally develop sufficient levels of competence for genetic studies.

Two other methods have been described, which could be used to stimulate competence development in *B. subtilis*: knockout mutations in *mecA* or *clpC*, and overexpression of ComS by means of a high copy plasmid harbouring *comS* (Hahn, *et al*., 1995; Kong *et al*., 1993; Liu *et al*., 1996). MecA, in consort with the protein chaperon ClpC, binds ComK and presents the latter for degradation by the protease ClpP. During the development of competence this
process is inhibited due to the accumulation of ComS in the cell. ComS binds to MecA and prevents binding of MecA to ComK and, as such, the degradation of ComK. Both mutations in mecA or clpC, as well as overproduction of ComS result in an overproduction of ComK and increased levels of competence. However, the introduction of mecA or clpC mutations to raise competence will be very difficult if not impossible, in such poorly transformable strains. In addition, mutations in mecA or clpC are accompanied by a number of undesirable effects resulting in decreased viability, and knockout mutations of these genes do not bypass a possible mutation in the comK gene. The advantage of pGSP12 over a comS bearing plasmid is that pGSP12 is able to bypass possible adverse mutations in the original comK resulting in poor competence development.

**Producing Campbell-type integration by ligation products.** Campbell-type integrations of suitable cloning vectors require transformation of competent *B. subtilis* cells with multimeric DNA. Usually, plasmid multimers are first isolated from *E. coli*. However, subcloning in *E. coli* may be impossible if the DNA fragments to be cloned are deleterious to the host. The production of multimers by means of *in vitro* DNA ligation could overcome this problem, were it not that standard ligation reactions are rather inefficient and result in a substantial amount of closed circular monomeric DNA products. Ligation in the presence of high concentrations of PEG8000 have been shown to strongly stimulate the ligation reaction, and in addition, PEG8000 inhibits the formation of closed circular monomeric DNA, thus resulting in a high percentage of large linear multimeric DNA molecules (Pfeiffer and Zimmerman, 1983; Zimmerman and Pfeiffer, 1983). To examine whether ligation products produced in the presence of PEG8000 might serve as more efficient substrates for Campbell-type integrations in competent *B. subtilis*, we tested the integration efficiency of the integration vector pLGW300 in the *dacC* region.
region of *B. subtilis*. Linearized pLGW300 and a PCR product of the *dacC* region were ligated in the presence and absence of 15% PEG\textsubscript{8000}, and the DNA ligation products were transformed to competent *B. subtilis* cells. Clearly, addition of PEG\textsubscript{8000} strongly stimulated the ligation efficiency, and resulted in the formation of large multimeric DNA complexes which did not enter the gel matrix (Fig. 3.2A). Most importantly, PEG\textsubscript{8000} increased the transformation efficiency of DNA ligation products 30 to 60 fold (Fig. 3.2B). In conclusion, PEG-ligation increases the suitability of DNA ligation products as substrate for Campbell-type integrations in competent *B. subtilis*. It should be mentioned however, that multimeric DNA obtained from *E. coli* is still a more efficient substrate producing a 10-fold higher number of transformants compared to PEG-ligation products (data not shown).

The use of ligation products for Campbell-type integrations in *B. subtilis* renders the use of shuttle vectors redundant. This was demonstrated by converting *B. subtilis* 168 into a surfactin producing strain, just by using a single kanamycin resistance gene to the 3'-end of the wild-type *sfp* gene (Fig. 3.3). *B. subtilis* 168 does not produce surfactin due to a point mutation in the 3'-end of the *sfp* gene. This gene encodes a phosphopantetene transferase, which couples the essential cofactor phosphopantetene to the surfactin synthetase. The 3'-end of a wild-type *sfp* gene from the surfactin producing strain *B. subtilis* ATCC21332, obtained by PCR, was ligated in the presence of PEG\textsubscript{8000} to a kanamycin resistance marker obtained by PCR as well. To ensure the desired orientation of both genes in the multimeric ligation products both PCR products were digested with *Bam*HI and *Eco*RI prior to ligation. The obtained ligation products were directly transformed to competent *B. subtilis* 168 cells. Most of the obtained kanamycin resistant colonies produced surfactin and the majority of transformants contained a single integrated kanamycin resistance

**Figure 3.3.** Schematic representation of the strategy to convert *B. subtilis* 168 into a surfactin producing strain. The 3'-end of wild-type *sfp* gene (*sfp*) and the kanamycin resistance marker (*KmR*) were obtained by PCR, and digested with *Bam*HI and *Eco*RI to ensure proper orientation. After ligation in the presence of PEG\textsubscript{8000}, the ligation products were transformed into *B. subtilis* 168. A Campbell-type integration will result in 2 possible phenotypes; kanamycin resistant colonies which do not produce surfactin (*sfp-mut*), and kanamycin resistant colonies which do produce surfactin (*sfp-wt*); a and b in figure 3, respectively.
marker adjacent to sfp (data not shown). Another effect of macro-molecular crowding is that the activity of T4-polynucleotide kinase is strongly stimulated as well (Harrison and Zimmerman, 1986). We have repeated the integration of the kanamycin resistance marker at the sfp locus, yet without digestion of the PCR fragments prior to ligation. In addition to PEG8000, we added T4-polynucleotide kinase to the ligation mixture. Transformation of the ligation products to competent B. subtilis 168 gave almost the same results to that described in figure 2. A substantial number of transformants produced surfactin and contained a single kanamycin resistance marker at the sfp locus (data not shown). These experiments demonstrate that a single antibiotic resistance marker can be specifically integrated into the genome of B. subtilis when using PEG-ligation.

Both methods are now routinely used in our laboratory and together these methods enabled us to study the expression and transcriptional regulation of the mycosubtilin- and surfactin synthetase operons in B. subtilis ATCC6633 and B. subtilis 168 (Cosmina et al., 1993; Duitman et al., 1999). In addition, both methods could increase the genetic accessibility of other poorly transformable B. subtilis strains, many of which are producers of important industrial products.

Acknowledgements

This work was supported by the European Union (EU Grant PL950176).

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