Construction of a single-copy integration vector and its use to study gene expression in *Bacillus licheniformis*

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A versatile system consisting of an integrational vector and a bacitracin (Bt)-producing β -galactosidase (β -Gal)-negative (Lac^-) *Bacillus licheniformis* TLH strain was constructed to quantify promoter activity and to study gene regulation in a single-copy set-up. The vector pTLH utilizes the promoterless *Escherichia coli lacZ* gene derived from pQF52 and contains the pBR322 origin of replication and a kanamycin-resistance gene for selection in both *B. licheniformis* and *E. coli*. The vector also contains an inner part of the first gene of the Bt synthetase (*bts*) operon which enables its integration into the *bts* of *B. licheniformis* by Campbell-type recombination. This recombination event can be easily tested on a *Micrococcus flavus* lawn where loss of Bt production, i.e. no clearing zone on the lawn, is indicative of the proper integration. The Lac^- *B. licheniformis* TLH strain was developed by elimination of the natural β -Gal activity of *B. licheniformis* strain ATCC 10716 UM12 using NTG mutagenesis.

Keywords: NTG mutagenesis, Campbell-type recombination, bacitracin synthetase, *lacZ* fusions, gene expression

INTRODUCTION

Bacillus licheniformis is used for the industrial production of the peptide antibiotic bacitracin (Bt), α -amylase, β -lactamase, alkaline protease and alkaline phosphatase (Kleinkauf & von Döhren, 1987; Harwood, 1992). In the framework of a strain improvement and genetic manipulation programme, the construction of a system to study gene expression and to analyse regulatory elements in *B. licheniformis* is indispensable.

Prokaryotic gene regulation is frequently studied using operon fusions carried on plasmids (for a review see Slauch & Silhavy, 1991). Some of these, with the *Escherichia coli lacZ* gene as an indicator, have found widespread application in selecting or screening for transcriptional control signals and in studying regulation of various promoters. There are more advantages to using the *lacZ* gene than other reporter genes: its product, β -galactosidase (β -Gal), can be easily and sensitively measured by an assay for β -Gal activity

reported by Miller (1972), and there are various indicators that can be incorporated into plates to generate a colour change (e.g. X-Gal). In addition, the fluorogenic substrate MUG (4-methylumbelliferyl- β -D-galactopyranoside) is inexpensive and gives a product that can be visualized by UV light and is an exceedingly sensitive indicator (Errington, 1990). The use of antibiotic-resistance reporter genes is less versatile because of the expense and limited sensitivity of the available assay systems (Farinha & Kropinski, 1990).

In *Bacillus subtilis*, a number of integrational vectors have been created which allow the construction of fusions with the *lacZ* gene at either the transcriptional or translational level. The advantage of using integrational vectors is that obtaining the fusions does not necessarily result in gene disruption, and thus the pattern of regulation of essential genes can be monitored (Perego, 1993). Additionally, using integrational vectors, transcriptional and translational fusions can be analysed in single copy integrated via a Campbell-type mechanism into the wild-type locus of the cloned insert, or in single copy integrated into a heterologous, defined locus (Lewandoski & Smith, 1988; Perego, 1993). The latter is thought to be more advantageous because: (i) the study

Abbreviations: Bt, bacitracin; β -Gal, β -galactosidase; MCS, multi-cloning site.

of the transcriptional potential of a specific DNA fragment is accomplished in a transcriptionally neutral region of the chromosome, therefore the sequence at the normal chromosomal location of the gene used to create the fusion remains intact; (ii) deletion analysis to study the role of segments surrounding the promoter in gene regulation can be easily carried out; and (iii) *lacZ* fusions constructed of genes from organisms completely dissimilar to *B. subtilis* can be integrated. As a consequence, this approach, employing the *amyE* locus as the integration site, is frequently used in *B. subtilis* (Shimotsu & Henner, 1986; Lewandoski & Smith, 1988; Perego, 1993; Axel *et al.*, 1996).

With full knowledge of the above advantages, the need to analyse the expression of regulated genes in *B. licheniformis* has motivated the development of a system which allows us to study gene expression and gene regulation in a heterologous, defined locus. To achieve this approach, we took advantage of the *B. licheniformis* ATCC 10716 UM12 strain, whose Bt production can be used for the easy testing of integrational events at the Bt synthetase (*bts*) locus. If the vector carrying an internal homologous fragment integrates into the *bts* operon by a Campbell-type mechanism, due to gene disruption, there will be no zone of clearing on the indicator lawn of *Micrococcus flavus*, which is sensitive to Bt. Furthermore, the rationale for the selection of the *B. licheniformis* ATCC 10716 UM12 strain is that it can be transformed with high efficiency by various methods (McCuen & Thorne, 1971; Prágai *et al.*, 1994). Thus the method that was based on the application of two +ori-pUB110 sequences in the same orientation on the parental plasmid, and used for integration of DNA sequences in the chromosome of poorly transformable bacilli (Tangney *et al.*, 1995), is avoidable.

The use of the *lacZ* gene as a reporter gene in *B. licheniformis*, however, incurs a major problem: all *B. licheniformis* strains, including *B. licheniformis* ATCC 10716 UM12, have natural β -Gal activity. Hence, to be able to study gene expression in a single-copy integrational state in *B. licheniformis* ATCC 10716 UM12 based on *lacZ* complementation, the elimination of its natural β -Gal activity is necessary.

In this article, we report the construction of a versatile system for studying gene expression in single copy integrated via a Campbell-type mechanism into the heterologous *bts* locus consisting of a promoter-probe pTLH vector and a Lac⁻ mutant derivative of *B. licheniformis* ATCC 10716 UM12 isolated by NTG mutagenesis.

METHODS

Bacterial strains, bacteriophage, plasmids and media. *B. licheniformis* ATCC 10716 UM12 is a Lac⁺, Bt-producing strain which is cured of phage LP52 (Thorne & Kowalski, 1976). Its derivative, *B. licheniformis* TLH, is Lac⁻ and was acquired by NTG mutagenesis. *M. flavus* ATCC 10240 (OKI) is a sensitive indicator strain for Bt and was used to detect Bt production by *B. licheniformis* strains. *E. coli* DH5 α

[F⁻/endA1 *hsdR17*(r_k⁻ m_k⁺) *supE44* *thi-1* *recA1* *gyrA* (Nal^r) *relA1* Δ (*lacIZYA-argF*)U169 *deoR* (ϕ 80*dlac* Δ [*lacZ*]M15)] was used for cloning experiments. *B. licheniformis* ATCC 9800 (OKI) and virulent bacteriophage BL11, which infects most *B. licheniformis* strains (Holczinger, 1986), were sources of various promoters. Plasmids used and constructed in this work are shown in Table 1. Strains were grown in YTA medium (Sik *et al.*, 1980). If necessary, YTA medium was supplemented with ampicillin (100 μ g ml⁻¹), kanamycin (10 μ g ml⁻¹) and X-Gal (40 μ g ml⁻¹ for *E. coli* and 80 μ g ml⁻¹ for *B. licheniformis*). For the transformation of *B. licheniformis* protoplasts, media were used as described previously by Prágai *et al.* (1994b).

DNA techniques. Total DNA was extracted from *B. licheniformis* by the method of Bron (1990). Preparation of plasmid and phage DNA, restriction endonuclease digestion, filling-in of cohesive ends, ligation, agarose gel electrophoresis and transformation of *E. coli* competent cells were applied as described by Sambrook *et al.* (1989). NTG mutagenesis was carried out according to Cutting & Vander Horn (1990). The PEG-induced transformation procedure for *B. licheniformis* protoplasts was reported previously by Prágai *et al.* (1994). Southern hybridization was performed using the DIG DNA Labeling and Detection kit as described in the protocol (Boehringer Mannheim). Enzymes were purchased from MBI Fermentas and Pharmacia Biotech and used according to the manufacturers' instructions.

Assay for Bt production. The colonies were picked onto plates containing *M. flavus*, which is sensitive to Bt. The Bt⁺ and Bt⁻ phenotypes can be distinguished easily. Around colonies of Bt⁺ strains, a zone of clearing indicates growth inhibition of *M. flavus*, which becomes visible after incubation for 24 h at 37 °C. In the case of the Bt⁻ phenotype, this effect cannot be seen.

Assay of β -Gal activity. Overnight cultures were diluted 100-fold in fresh YTA medium and cultivated to the early exponential growth phase (OD₆₀₀ = 0.24; UV1101 Biotech Photometer). The cultures were again diluted 100-fold and grown at 37 °C with intensive agitation (in an incubated shaker at 250 r.p.m.). Samples were collected at hourly intervals from the third hour for OD₆₀₀ readings and β -Gal activity determinations. The β -Gal assay and the calculation of activities (expressed as units per OD₆₀₀) were carried out as described by Miller (1972).

RESULTS AND DISCUSSION

Isolation of the Lac⁻ *B. licheniformis* TLH strain

After mutagenesis of strain *B. licheniformis* ATCC 10716 UM12 with NTG, the mutagenized stock was screened for mutants unable to express β -Gal. From approximately 5000 surviving colonies, one white colony, called *B. licheniformis* TLH (Lac⁻ mutant), was found on X-Gal plates. The β -Gal activity of this mutant was measured in extracts from cells grown overnight, and found to be approximately five times lower than that of the wild-type strain. To determine the reversion frequency, the *B. licheniformis* TLH mutant was grown for 20 generations in YTA broth. The cells were then washed with sterile distilled water and the dilutions were plated on YTA agar containing X-Gal. No Lac⁺ revertants were observed among 9.2×10^8 c.f.u. ml⁻¹, therefore the reversion rate was less than 10^{-9} per

Table 1. Plasmids

Plasmid	Relevant characteristics	Reference
pQF52	6.8 kb, carrying promoterless <i>lacZ</i> gene, Ap ^r	McLean <i>et al.</i> (1997)
pBR322	4.4 kb, Ap ^r Tc ^r	Bolivar <i>et al.</i> (1977)
pBE1	12.1 kb, carrying the left arm of Tn917PF1 and the flanking part of the <i>bts</i> region (<i>btsA'</i>), Ap ^r Km ^r Cm ^r	Prágai (1994)
pGP100	9.1 kb, carrying bacteriophage SPO2 promoter, Km ^r Tc ^r	Meima <i>et al.</i> (1996)
pKSA11	6.2 kb, carrying the promoter of the <i>B. subtilis</i> 168 pyrimidine biosynthetic (<i>pyr</i>) gene cluster, Ap ^r	Prágai <i>et al.</i> (1997)
pBRQF	5.6 kb; pBR322 replicon with the <i>lacZ</i> reporter gene carried on the 3.8 kb <i>NdeI</i> – <i>ScaI</i> fragment from pQF52, Km ^r	This study
pTLH	8.6 kb; pBRQF with 3 kb <i>EcoRI</i> – <i>BamHI</i> fragment carrying an internal part (' <i>btsA'</i> ') of the <i>bts</i> region and the left end of Tn917PF1 from pBE1, Km ^r	This study
pTLHspo2	8.9 kb; 0.3 kb <i>EcoRI</i> fragment from pGP100 carrying P _{SPO2} promoter in pTLH, transcriptional P _{SPO2} – <i>lacZ</i> fusion, Km ^r	This study
pTLHpyr	9.4 kb; 0.8 kb <i>XhoI</i> – <i>HincII</i> fragment from pKSA11 carrying P _{pyr} promoter in pTLH, transcriptional P _{pyr} – <i>lacZ</i> fusion, Km ^r	This study
pTLHbli	9.6 kb, carrying a 1 kb <i>Sau3A</i> fragment with promoter activity from ATCC 9800, Km ^r	This study
pTLH11	9.8 kb, carrying a 1.2 kb <i>Sau3A</i> fragment with promoter activity from bacteriophage BL11, Km ^r	This study

generation. The NTG mutagenesis did not affect Bt production of the *B. licheniformis* TLH strain. Hence, *bts* can be used as the integration site and then the production of Bt is used for testing integrational events.

Construction of the pTLH vector

To construct versatile integrational vector pTLH for studying gene expression in *B. licheniformis* TLH, we took advantage of the promoterless *lacZ* gene derived from pQF52, which is a promoter-probe vector and capable of replicating in a wide range of Gram-negative bacteria (McLean *et al.*, 1997), and of the multi-cloning site (MCS) placed upstream of the *lacZ* gene. In the latter construct, the first 15 bases of the *lacZ* gene were replaced with the Shine–Dalgarno sequence and the first 12 bases of the *E. coli* lipoprotein (*lpp*) gene (Kalnins *et al.*, 1983; Nakamura & Inouye, 1982; Ruther *et al.*, 1981). This region also contains translational stop codons in all three reading frames located between the MCS and the translation initiation codon of the indicator gene. The above topology prevents initiation of translation from other start sites into the indicator gene which may have unwanted effects on its expression. The hybrid β -Gal is enzymically indistinguishable from the native protein, which is one amino acid longer.

The *lacZ* gene from pQF52 was excised as a 3.8 kb *NdeI*–*ScaI* fragment and ligated to pBR322 cut with the same enzymes, creating a 5.6 kb pBRQF, which is smaller than pQF52 (6.8 kb), because the *ori*₁₆₀₀ (Olsen *et al.*, 1982) was deleted. As a next step, the 3 kb

fragment containing the Km^r gene and the flanking internal part ('*btsA'*') of the *bts* operon in pBE1 (Prágai, 1994) was purified upon digestion with *EcoRI* and *BamHI*. The ends of this fragment were filled in, and the resulting fragment was ligated to pBRQF digested with *ScaI*, creating the final product, pTLH, which is 8.6 kb in size (Fig. 1). The two *trpA* terminators placed upstream of the MCS were sufficient to prevent read-through transcription from promoters located in the pQF52 region of the plasmid (Farinha & Kropinski, 1990).

Testing of the pTLH–*B. licheniformis* TLH system

To demonstrate that the pTLH–*B. licheniformis* TLH system can be used to investigate gene expression in a single copy, the indicator *lacZ* gene was fused with the P_{SPO2} promoter (a constitutive promoter derived from the *B. subtilis* bacteriophage SPO2) and with the P_{pyr} promoter of the *B. subtilis* 168 pyrimidine biosynthetic (*pyr*) gene cluster. To construct the P_{SPO2}–*lacZ* transcriptional fusion, the 0.3 kb *EcoRI* fragment carrying P_{SPO2} was deleted from plasmid pGP100 (Meima *et al.*, 1996), the ends of the fragment were made blunt and inserted into pTLH digested with *StuI*, creating the pTLHspo2 plasmid. To construct the P_{pyr}–*lacZ* transcriptional fusion, the P_{pyr} promoter from pKSA11 (Prágai *et al.*, 1997) was transferred as a 0.8 kb *HincII*–*XhoI* fragment to pTLH cut with *StuI* and *XhoI*, creating the pTLHpyr plasmid. *E. coli* cells were transformed, and *lacZ* fusions were detected on X-Gal

Fig. 1. Schematic presentation of the construction of pTLH. The open arrow represents part (*btsA'*) of the *bts* operon and the open box represents the internal part (*'btsA'*). The *lacZ* gene is marked by a black arrow. The *Ap^r*, *Km^r*, *Tc^r* and *Cm^r* markers are shown as hatched arrows and the replication origins are shown as hatched boxes. The small black box represents the two *trpA* terminators (*T*). Non-unique restriction sites in the MCS are indicated by asterisks.

indicator plates, where promoter-positive clones manifested themselves as blue colonies. Subsequently, the two transcriptional fusions were integrated via a Campbell-type integration into the *bts* operon of *B. licheniformis* TLH using kanamycin for selection in protoplast transformation of this strain. The promoterless insertions served as controls. Insertion by a Campbell-type recombination at the *bts* was verified by the *Bt⁻* phenotype of the cells, which could be identified on an indicator lawn of *M. flavus*.

The efficiency of transformations was $1-2 \times 10^2$ *Km^r* and β -Gal-producing transformants per μ g plasmid DNA, which is approximately 10^4 times lower than that (1.1×10^6) with plasmids carrying Gram-positive replication origins (Prágai *et al.*, 1994). Accordingly, the frequency of integration was approximately 10^{-4} per μ g plasmid DNA. Transformation of *E. coli* with total DNA extracted from *Km^r* *Bt⁻* integrants resulted in no transformants, indicating the stable integration of the pTLH, pTLHspo2 and pTLHpyr plasmids in the *bts* operon of the *B. licheniformis* integrants and also the lack of autonomously replicating plasmids. The presence of a single copy of pTLH, pTLHspo2 and pTLHpyr

in the first *bts* gene of *B. licheniformis* integrants was confirmed by Southern hybridization analysis (data not shown).

One integrant was chosen from each plasmid type and subsequently assayed quantitatively for the activity of the *lacZ* reporter gene product. The results, presented in Fig. 2, showed that in the absence of a promoter, enzymic activities were barely detectable, contrasted with the presence of the promoters, especially in the case of the constitutive P_{SPQ2} promoter, where enzymic activities increased significantly (Fig. 2).

The test concluded above showed that this system is suitable for studying gene expression in *B. licheniformis* strain TLH. The advantage of the *bts* operon as the integration site is that it provides an easily detectable phenotype for integration events. To the best of our knowledge, the system described above is the first versatile system for analysing regulatory elements in single-copy set-up in *B. licheniformis*.

In addition, pTLH is a useful tool for selection of promoters from various sources. To demonstrate this practical use, fragments with promoter activity from *B.*

Fig. 2. Testing of the pTLH-*B. licheniformis* TLH system. *B. licheniformis* strain TLH carrying a promoterless pTLH as a control ($\blacktriangle$), a P_{SPO2} -*lacZ* transcriptional fusion ($\bullet$), a P_{pyr} -*lacZ* transcriptional fusion ($\blacksquare$), a P_{bli} -*lacZ* transcriptional fusion ($\blacklozenge$) and a P_{11} -*lacZ* transcriptional fusion ($\triangle$) was grown in YTA medium. Samples were collected at hourly intervals for OD_{600} readings ($\square$, growth curve, log scale) and for assaying β -Gal activities. Data are expressed as means of three independent experiments.

licheniformis ATCC 9800 and bacteriophage BL11 were cloned by linearizing pTLH with *Bam*HI and ligating *Sau*3A fragments from *B. licheniformis* ATCC 9800 and phage BL11 to the linearized vector. Insertion of the *Sau*3A fragments carrying promoters can be selected by plating transformants of *E. coli* on media supplemented with X-Gal. Promoter-positive clones manifested themselves as blue colonies. One randomly selected clone from each type, called pTLHbli and pTLH11, carrying P_{bli} -*lacZ* and P_{11} -*lacZ* transcriptional fusions, respectively, was integrated via a Campbell-type mechanism into the *bts* operon. The integrants were checked for Km^r and Bt^- phenotype. Southern hybridization was used to confirm the presence of a single copy of pTLHbli and pTLH11 in the first *bts* gene of *B. licheniformis* integrants. The β -Gal activity of these integrants was also investigated and shown in Fig. 2. The Km^r Bt^- integrants carrying various transcriptional fusions at the *bts* locus, including P_{bli} -*lacZ*, are very stable and can be maintained without kanamycin.

The promoter-probe pTLH plasmid can also be used to screen for regulatory mutants. If the transcriptional fusion is negatively regulated, expression in the wild-type background will be low, resulting in a low β -Gal activity. In contrast, inactivation of the repressor gene will lead to increased β -Gal activity. If the transcriptional fusion is positively regulated, expression will be high and inactivation of the gene encoding the activator will result in low or even no β -Gal activity.

We have shown in this paper that the Lac^- *B. licheniformis* TLH mutant, a derivative of *B. licheniformis* ATCC 10716 UM12 isolated by NTG mutagenesis, is extremely stable. The NTG mutagenesis did not affect the ability of *B. licheniformis* strain TLH

to produce Bt. Thus, *bts* can be used as the integration site and the production of Bt is then applied for testing integrational events. We constructed the promoter-probe pTLH vector carrying a promoterless *lacZ* reporter gene, the pBR322 replicon and a Km^r gene for selection in both *B. licheniformis* and *E. coli*. This vector also contains an internal part of the first gene of the *bts* operon, allowing insertion of transcriptional fusions into the *B. licheniformis* TLH *bts* by a Campbell-type recombination. Successful integration can be visualized on a *M. flavus* lawn and directly selected for with kanamycin. Using different promoters fused to the *lacZ* reporter gene to test the system consisting of the pTLH vector and the *B. licheniformis* TLH strain, we showed that the system is suitable for studying gene expression. pTLH can also be used as a promoter-probe vector.

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REFERENCES

- Axel, M., Hayward, R. & Schumann, W. (1996). Integrative vectors for constructing single-copy transcriptional fusions between *Bacillus subtilis* promoters and various reporter genes encoding heat-stable enzymes. *Gene* 182, 33–36.
- Bolivar, F., Rodriguez, R. L., Greene, P. J., Betlach, M. C., Heynecker, H. L., Boyer, H. W., Crosa, J. H. & Falkow, S. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* 2, 95.
- Bron, S. (1990). Plasmids. In *Molecular Biological Methods for Bacillus*, pp. 75–174. Edited by C. R. Harwood & S. M. Cutting. Chichester: Wiley.
- Cutting, S. M. & Vander Horn, P. B. (1990). Genetic analysis. In *Molecular Biological Methods for Bacillus*, pp. 27–74. Edited by C. R. Harwood & S. M. Cutting. Chichester: Wiley.
- Errington, J. (1990). Gene cloning techniques. In *Molecular Biological Methods for Bacillus*, pp. 175–220. Edited by C. R. Harwood & S. M. Cutting. Chichester: Wiley.
- Farinha, M. A. & Kropinski, A. M. (1990). Construction of broad-host-range plasmid vectors for easy visible selection and analysis of promoters. *J. Bacteriol* 172, 3496–3499.
- Harwood, C. R. (1992). *Bacillus subtilis* and its relatives: molecular biological and industrial workhorses. *Trends Biotechnol* 10, 247–256.
- Holzinger, A. (1986). *Improvement of bacitracin production and bacteriophages of Bacillus licheniformis*. PhD thesis, Budapest Technical University.
- Kalnins, A., Otto, K., Ruther, U. & Muller-Hill, B. (1983). Sequence of the *lacZ* gene of *Escherichia coli*. *EMBO J* 2, 593–597.
- Kleinkauf, H. & von Döhren, H. (1987). Biosynthesis of peptide antibiotics. *Annu Rev Microbiol* 41, 259–289.

- Lewandoski, M. & Smith, I. (1988). Use of a versatile *lacZ* vector to analyze the upstream region of the *Bacillus subtilis* *spoOF* gene. *Plasmid* 20, 148–154.
- McCuen, R. W. & Thorne, C. B. (1971). Genetic mapping of genes concerned with glutamyl polypeptide production by *Bacillus licheniformis* and a study of their relationship to the development of competence for transformation. *J Bacteriol* 107, 636–645.
- McLean, B. W., Wiseman, S. L. & Kropinski, A. M. (1997). Functional analysis of σ^{70} consensus promoters in *Pseudomonas aeruginosa* and *Escherichia coli*. *Can J Microbiol* 43, 981–985.
- Meima, R., Venema, G. & Bron, S. (1996). A positive selection vector for the analysis of structural plasmid instability in *Bacillus subtilis*. *Plasmid* 35, 14–30.
- Miller, J. H. (1972). *Experiments in Molecular Genetics*, pp. 352–355. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Nakamura, K. & Inouye, M. (1982). Construction of versatile expression cloning vehicles using the lipoprotein gene of *Escherichia coli*. *EMBO J* 1, 771–775.
- Olsen, R. H., DeBusscher, G. & McCrombie, W. R. (1982). Development of broad-host-range vectors and gene banks: self-cloning of the *Pseudomonas aeruginosa* PAO chromosome. *J Bacteriol* 150, 60–69.
- Perego, M. (1993). Integrational vectors for genetic manipulation in *Bacillus subtilis*. In *Bacillus subtilis and Other Gram-Positive Bacteria*, pp. 615–624. Edited by A. L. Sonenshein, J. A. Hoch & R. Losick. Washington, DC: American Society for Microbiology.
- Prágai, Z. (1994). *Transposon mutagenesis and protoplast transformation of Bacillus licheniformis*. PhD thesis, Hungarian Academy of Sciences.
- Prágai, Z., Holczinger, A. & Sík, T. (1994). Transformation of *Bacillus licheniformis* protoplasts by plasmid DNA. *Microbiology* 140, 305–310.
- Prágai, Z., Tjalsma, H., Bolhuis, A., van Dijk, J. M., Venema, G. & Bron, S. (1997). The signal peptidase II (*lsp*) gene of *Bacillus subtilis*. *Microbiology* 143, 1327–1333.
- Ruther, U., Koenen, M., Otto, K. & Muller-Hill, B. (1981). pUR222, a vector for cloning and rapid chemical sequencing of DNA. *Nucleic Acids Res* 9, 4087–4098.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Shimotsu, H. & Henner, D. J. (1986). Construction of a single-copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. *Gene* 43, 85–94.
- Sík, T., Horváth, J. & Chatterjee, S. (1980). Generalized transduction in *Rhizobium meliloti*. *Mol Gen Genet* 178, 511–516.
- Slauch, J. M. & Silhavy, T. J. (1991). Genetic fusions as experimental tools. *Methods Enzymol* 204, 213–248.
- Tangney, M., Jørgensen, P. L., Diderichsen, B. & Jørgensen, S. T. (1995). A new method for integration and stable DNA amplification in poorly transformable bacilli. *FEMS Microbiol Lett* 125, 107–144.
- Thorne, C. B. & Kowalski, J. B. (1976). Temperate bacteriophages for *Bacillus licheniformis*. In *Microbiology*, pp. 303–314. Edited by D. Schlessinger. Washington, DC: American Society for Microbiology.

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