

Magnesium aminoclay-based transformation of *Paenibacillus riograndensis* and *Paenibacillus polymyxa* and development of tools for gene expression

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Abstract Members of the genus *Paenibacillus* are widespread facultative anaerobic, endospore-forming bacteria. Some species such as *Paenibacillus riograndensis* or *Paenibacillus polymyxa* fix nitrogen and may play an important role in agriculture to reduce mineral nitrogen fertilization in particular for non-legume plants. The genetic manipulation of *Paenibacillus* is an imperative for the functional characterization, e.g., of its plant growth-promoting activities and metabolism. This study showed that *P. riograndensis* and *P. polymyxa* can be readily transformed using physical permeation by magnesium aminoclays. By means of the fluorescent reporter genes *gfpUV*, *mcherry*, and *crimson*, a two-plasmid system consisting of a theta-replicating plasmid and a rolling circle-replicating plasmid was shown to operate in both species. Xylose-inducible and mannitol-inducible fluorescent reporter gene expression was demonstrated in the compatible two-plasmid system by fluorescence-activated cell scanning. As a metabolic engineering application, the biotin requiring *P. riograndensis* was converted to a biotin-prototrophic strain based on mannitol-inducible expression of the biotin biosynthesis operon *bioWAFDBI* from *Bacillus subtilis*.

Keywords *Paenibacillus* · Theta-replicating plasmid · Magnesium aminoclays · Transformation · Biotin prototrophy

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Introduction

The genus *Paenibacillus* includes several diazotrophic species broadly distributed in the environment, for example, in different types of soil, the rhizosphere and plant tissues (Beneduzi et al. 2013). They may play an important role in the agriculture since the use of diazotrophic bacteria inoculants can reduce the mineral nitrogen fertilization that represents a significant cost in non-legume cultures. The diazotroph *Paenibacillus riograndensis* SBR5 is a Gram-positive, rod-shaped, endospore-forming rhizobacterium that was isolated from the rhizosphere of *Triticum aestivum* in southern Brazil (Rio Grande do Sul; Beneduzi et al. 2010). The genome of *P. riograndensis* SBR5 was sequenced and fully annotated and consists of a single chromosome of 7,893,056 base pairs containing 6705 protein coding, 87 tRNA and 27 rRNA genes (Brito et al. 2015). Simple mineral salts media support the growth of SBR5. Since SBR5 is auxotrophic for biotin, it is necessary to add this vitamin to the minimal medium (Brito et al. 2015). Previous studies have shown that this bacterium exhibits nitrogen fixation activity (Fernandes et al. 2014) and other plant growth-promoting characteristics such as indol-3-acetic acid and siderophore production have been described (Beneduzi et al. 2010; Sperb et al. 2016).

Despite its potential as a plant growth promoting rhizobacterium (PGPR), some of the PGPR and metabolic activities of *P. riograndensis* SBR5 still remain to be studied (e.g., its function in the soil phosphorus cycle). Since the genetic tools are not well developed for this species, functional genomics analyses are very difficult to perform. For this reason, an efficient method for transformation of *P. riograndensis* SBR5 would be beneficial to further study its PGPR activities and metabolism.

Some transformation methods for related *Paenibacillus* species have been reported previously, most of them based

on electroporation. For example, a transformation efficiency of 1.9×10^5 transformants/ μg of plasmid DNA has been achieved for *Paenibacillus larvae* (Murray and Aronstein 2008). An electroporation method was shown to function efficiently for the plant growth-promoting *Paenibacillus polymyxa*, but much less efficiently for the related *Paenibacillus azotofixans* (Rosado et al. 1994). For *P. larvae*, a polyethylene glycol-based protoplast transformation method was also reported (Bakhiet and Stahly 1985).

A simple, inexpensive and efficient bacterial transformation method based on physical permeation using magnesium aminoclays was recently shown to be functional for both the Gram-positive *Streptococcus mutans* and the Gram-negative *Escherichia coli* (Choi et al. 2013) as well as for the microalga *Chlamydomonas reinhardtii* (Kim et al. 2014). Unlike chemical transformation, electro-transformation, biolistic transformation, or sonic transformation, this method is based on the Yoshida effect (Yoshida et al. 2001). Sliding friction applied to a colloidal solution with a nanosized acicular material and bacterial cells increases the frictional coefficient rapidly, and the resulting complex increases in size and penetrates the bacterial cells, which results in the uptake of exogenous DNA (Yoshida and Sato 2009). Transformation of *Paenibacillus* based on physical permeation using magnesium aminoclays has not yet been reported. In the present work, the magnesium aminoclay-based transformation method was adapted to *P. riograndensis*. The heterologous *gfpUV*, *mcherry*, and *crimson* reporter genes were functionally expressed in *P. riograndensis* SBR5 under the control of constitutive or inducible promoters from either theta-replicating or rolling circle-replicating plasmids. As an example of a biotechnological application, *P. riograndensis* was rendered biotin prototrophic by inducible expression of the *bioWAFDBI* operon from *B. subtilis*. Moreover, the transformation method and the plasmids developed for *P. riograndensis* were shown to be transferable to *P. polymyxa* DSM-365.

Materials and methods

Strains, plasmid DNA and primers

P. riograndensis SBR5, *P. polymyxa* DSM-365, and *Bacillus methanolicus* MGA3 were used as hosts for heterologous fluorescence genes expression. SBR5 was kindly provided by Prof. Luciane Passaglia, Genetics Department in Universidade Federal do Rio Grande do Sul (UFRGS, Brazil), DSM-365 purchased from DSMZ and *B. methanolicus* MGA3 obtained from Prof. Trygve Brautaset, SINTEF, Trondheim, Norway (Table 1). Information about the plasmids used as empty vectors in this work is available in Table 1; they were two rolling circle-replicating plasmids conferring chloramphenicol resistance and containing a methanol-inducible promoter from

B. methanolicus, named pNW33Nmp and pTH1mp (pRE) and third theta-replicating plasmid pHCMC04 here named pTE containing the xylose-inducible promoter PxylA and the gene encoding the xylose regulator XylR amplified from the genome of *Bacillus megaterium* (Biedendieck et al. 2011). All the empty vectors were obtained from SINTEF, Trondheim. Sequences for origin of replication of *E. coli* and *B. subtilis* are present in all the shuttle vectors. The primers used for strain construction are presented in Table S1.

Medium and growth conditions

For the cultivation of *Paenibacillus* transformants, the cells were routinely grown at 30 °C and 120 rpm, in medium Caso broth (medium 220 from DSMZ) containing: peptone from casein (15 g L⁻¹), peptone from soymeal (5 g L⁻¹), yeast extract (3 g L⁻¹), and NaCl (5 g L⁻¹) with pH adjusted to 7.15 with NaOH. Antibiotics were added accordingly to the antibiotic resistance of the plasmid in use, 5.5 $\mu\text{g mL}^{-1}$ of chloramphenicol and 10 $\mu\text{g mL}^{-1}$ of ampicillin. *E. coli* strains were routinely cultivated at 37 °C in lysogeny broth supplied with 10 $\mu\text{g mL}^{-1}$ of chloramphenicol and 100 $\mu\text{g mL}^{-1}$ of ampicillin when necessary. The strains of *B. methanolicus* were grown as described before (Irla et al. 2016).

To test the inducible systems, the transformant cells of SBR5 with reporter gene under control of mannitol-inducible system were grown in Caso broth supplemented with gradually increasing concentrations of mannitol (0, 20, 40, 80, and 160 mM). The transformant cells of SBR5 with reporter gene under control of the xylose-inducible system were grown in Caso broth supplemented with 0, 25, 50, 100, 200, or 400 mM of xylose, and DSM-365 transformants were grown in Caso broth supplemented with 0, 25, 50, or 100 mM xylose. In co-transformation with two inducible plasmids, the transformant cells of SBR5 and DSM-365 were grown in Caso broth with addition of both mannitol and xylose in concentration of 0, 25, or 50 mM. In the growth assay of the *P. riograndensis* SBR5(pRM2-*bioWAFDBI*), the recombinant cells were grown over night in Caso broth and centrifuged for 15 min at 4000 rpm. After washing the pellet for three times with NaCl 0.89 % solution, the cells were transferred to the PbMM *P. riograndensis* minimal medium (MVcMY without vitamin complex and yeast extract; Jakobsen et al. 2009) using 50 mM xylose as carbon source, supplemented or not with 0.1 mg L^{-1} biotin and 160 mM inducer (mannitol).

Plasmids construction and preparation of recombinant strains

Molecular cloning was performed as described by Sambrook (2001). Chemically competent cells of *E. coli* DH5 α were prepared for cloning (Hanahan and Harbor 1983). All the information about the polymerase chain reactions (PCRs) for plasmid

Table 1 Bacterial strains and plasmids used in this study

Bacteria	Characteristics	Reference or source
<i>Bacillus methanolicus</i>	MGA3	SINTEF
<i>P. polymyxa</i>	DSM-365	DSMZ
<i>Bacillus subtilis</i>	168	BGSC
<i>Escherichia coli</i>	DH5 α	Hanahan (1983)
<i>Paenibacillus riograndensis</i>	SBR5; biotin auxotrophic	UFRGS
Plasmid		
pRE	Cm ^R ; pTH1mp: rolling circle-replicating vector with methanol-inducible promoter of methanol dehydrogenase (Pmdh) derived from <i>Bacillus methanolicus</i> ; contains origin of replication (ORI) sequences from <i>Bacillus subtilis</i> and <i>E. coli</i>	Irla et al. (2016)
pR- <i>gfpUV</i>	Cm ^R ; pRE with <i>gfpUV</i> cloned downstream the Pmdh	Irla et al. (2016)
pTE	Cm ^R , Amp ^R ; pHCMC04: theta-replicating vector with xylose-inducible promoter from <i>B. megaterium</i> ; contains ORI sequences from <i>Bacillus subtilis</i> and <i>E. coli</i>	Nguyen et al. (2005)
pNW33Nmp	Cm ^R , Km ^R ; pNW33N derivative in which the Pmdh was inserted	Irla et al. (2016)
pR _{fc} - <i>crimson</i>	Cm ^R ; protein fusion of Crimson and the Cm ^R from pRE: amplification of pRE back bone excluding the TAA sequence of cm ^R including <i>crimson</i> sequence instead	This work
pR _{fc} - <i>mCherry</i>	Cm ^R ; protein fusion of <i>mCherry</i> and the Cm ^R from pRE: amplification of pRE back bone excluding the TAA sequence of cm ^R including <i>mCherry</i> sequence instead	This work
pR _{fc} - <i>gfpUV</i>	Cm ^R ; protein fusion of <i>GfpUV</i> and the Cm ^R from pRE: amplification of pRE back bone excluding the TAA sequence of cm ^R including <i>gfpUV</i> sequence instead	This work
pPpyk- <i>gfpUV</i>	Cm ^R ; pRE with Pmdh replaced by pyruvate kinase promoter (amplified from SBR5 genome) upstream <i>gfpUV</i>	This work
pPtuf- <i>gfpUV</i>	Cm ^R ; pRE with Pmdh replaced by the elongation factor Tuf promoter (amplified from SBR5 genome) upstream <i>gfpUV</i>	This work
pPgap- <i>gfpUV</i>	Cm ^R ; pRE with Pmdh replaced by glyceraldehyde-3-phosphate dehydrogenase promoter (amplified from SBR5 genome) upstream <i>gfpUV</i>	This work
pRM1- <i>gfpUV</i>	Cm ^R ; pRE- <i>gfpUV</i> with Pmdh replaced by <i>mtlA</i> mannitol-inducible promoter amplified from SBR5 genome	This work
pRM2- <i>gfpUV</i>	Cm ^R ; pRE- <i>gfpUV</i> with Pmdh replaced by <i>mtlR</i> mannitol-inducible promoter amplified from MGA3 genome	Irla et al. (2016)
pRM3- <i>gfpUV</i>	Cm ^R ; pRE- <i>gfpUV</i> with Pmdh replaced by <i>mtlA</i> mannitol-inducible promoter amplified from 168 genome	This work
pRX- <i>gfpUV</i>	Cm ^R ; pRE derivative for <i>gfpUV</i> expression under control of the xylose-inducible promoter from <i>B. megaterium</i>	Irla et al. (2016)
pTX- <i>crimson</i>	Cm ^R , Amp ^R ; pTE derivative with <i>crimson</i> sequence inserted in <i>EcoRV</i> GAT [^] ATC site	This work
pTX- <i>mCherry</i>	Cm ^R , Amp ^R ; pTE derivative with <i>mCherry</i> sequence inserted in <i>EcoRV</i> GAT [^] ATC site	This work
pEKEx3- <i>bioWAFDBI</i>	Spec ^R ; <i>C. glutamicum/E. coli</i> shuttle vector for IPTG-inducible expression of <i>bioWAFDBI</i> from <i>B. subtilis</i>	Peters-Wendisch et al. (2014)
pRM2- <i>bioWAFDBI</i>	Cm ^R ; pRM2- <i>gfpUV</i> in which <i>gfpUV</i> was replaced by <i>bioWAFDBI</i> amplified from pEKEx3- <i>bioWAFDBI</i>	This work

construction in this work is present in Table 1 and the oligonucleotide sequences described in Table S1. Genomic DNA of *P. riograndensis* was isolated as described by Eikmanns et al. (1994). *B. methanolicus* DNA isolation procedure, competent cells preparation, and transformation method are described in Irla et al. (2016). The NucleoSpin® Gel and PCR Clean-up kit (Machery-Nagel, Düren, Germany) was used for PCR clean-up

and plasmids were isolated using the GeneJET Plasmid Miniprep Kit (Thermo Fisher Scientific, Waltham, USA). Plasmid backbones and inserts were amplified using Phusion® DNA polymerase (New England Biolabs, Ipswich, England) and the overlapping regions joined by Gibson assembly (Gibson 2014). For colony PCR the Taq polymerase (New England Biolabs) was used.

Preparation of the magnesium aminoclays

The preparation of the magnesium aminoclays was done according to Han et al. (2011). An ethanolic solution of 200 mM $\text{MgCl}_2 \times 6\text{H}_2\text{O}$ was stirred for 20 min, and 13 mL of 3-aminopropyl triethoxysilane (Carl Roth, Karlsruhe, Germany) was added dropwise. The bulk solution was stirred at room temperature for 18 h. After stirring, the milky solution was centrifuged for 10 min at 4000 rpm and the white pellet washed with ethanol. The pellet was dried at 50 °C for 24 h, and the white product was grinded and autoclaved inside falcon tubes.

Magnesium aminoclay-based transformation method assay

The bacterial transformation method using magnesium aminoclays was developed and optimized by Choi et al. (2013). Here, we performed similar experiments by varying the parameters for adaptation of this method for *P. riograndensis* SBR5. The magnesium aminoclay solution was prepared by mixing 10 mg of magnesium aminoclays with 1 mL of deionized sterile water 1 day before the transformation for total dissolution. The plasmid DNA, in amounts of 0.05, 0.1, 0.3, 0.5, or 1 µg was mixed with 0.05 mL of the aminoclay solution, and the volume was completed to 0.5 mL with deionized sterile water. The bacterial cells were grown in Caso broth medium until reaching the exponential phase, when they were centrifuged at 4000 rpm for 10 min. The pellet was resuspended in pure sterile water ($\text{OD}_{600 \text{ nm}}$ adjusted to 1) and 0.5 mL of cell suspension was mixed to the aminoclay-plasmid solution. For mixing, we fixed the amount of plasmid DNA of 0.1 µg and two treatments were applied: vortexing the mixture for 10, 30, 60, 120, or 180 s or short-time ultrasonication, using amplitude of 40 % for 5, 10, 20, or 30 s. To test the friction force, Caso broth agar plates were prepared with 1.5 or 3 % of agar, and the remaining parameters were 0.1 µg of DNA and 60 s of vortexing. The spreading time of the 1.5 % agar varied being 30, 60, 120, or 180 s and on the plates with 3 % agar the spreading time (of 60 s) was not varied. After 48 h incubation at 30 °C, the colony-forming units were counted.

Recombinant *P. riograndensis* plasmid isolation and retransformation to *E. coli*

The plasmid isolation procedure used in this work was performed as following: an overnight culture (30 mL) of *P. riograndensis* SBR5 transformed with the plasmid pNW33mp was centrifuged for 15 min at 4000 rpm, and the pellet was washed and resuspended in 40 µL of the TE buffer (0.05 M Tris, pH 8.0, 0.01 M EDTA). The cell suspension was added to 600 µL freshly prepared lysis buffer (TE buffer with

4 % SDS, pH adjusted to 12.45) filled into an Eppendorf tube and the lysis was completed by the incubation of the mixture at 37 °C for 60 min. The lysate was neutralized by the addition of 30 µL of 2 M Tris, pH 7.0. For precipitation of the chromosomal DNA and proteins, 240 µL of 5 M NaCl was added to the lysate and the mixture was incubated in ice for 6 h. After the incubation, the lysate was centrifuged for 10 min at 11,000 rpm and the supernatant was transferred to a new tube. For DNA recovery, 10 % (v/v) of 3 M sodium acetate, pH 5.2 was added to the aqueous plasmid DNA solution and plasmid DNA was precipitated by addition of –20 °C cooled ethanol absolute. After 45 min centrifugation at 11,000 rpm, the DNA pellet was washed twice with ethanol 70 % solution and air dried for 10 min before resuspension in deionized water.

E. coli DH5 α was transformed with the isolated plasmid DNA via heat shock and the resulting transformants were used for a plasmid mini preparation kit (Macherey-Nagel) according to the manufacture specifications. The plasmid DNA isolated from SBR5 and the plasmid isolated from the *E. coli* transformed with plasmid DNA isolated from SBR5 were digested with the restriction enzyme *AclI* (Thermo Fisher Scientific) according to the manufacturer specifications, and the presence of digested plasmid DNA was confirmed by agarose gel electrophoresis.

Fluorescence measurement by fluorescence-activated cell scanning

To quantify the fluorescence intensities, transformants of *P. riograndensis* SBR5, *P. polymyxa* DSM-365, and *B. methanolicus* MGA3 were analyzed by flow cytometry. Routinely, the *P. riograndensis* SBR5 and *P. polymyxa* DSM-365 cells were grown until reaching the exponential phase and centrifuged for 10 min at 4000 rpm. The pellets were washed two times in NaCl 0.89 % solution and the $\text{OD}_{600 \text{ nm}}$ was adjusted to 0.5. The *B. methanolicus* cells were prepared as described before (Irla et al. 2016). The fluorescence of the cell suspension was measured using flow cytometer (Beckman Coulter, Brea, USA) and the data analyzed in the Beckman Coulter Kaluza® Flow Analysis Software. The settings for the emission signal and filters within the flow cytometer for detection of GfpUV, Crimson, and mCherry fluorescence were 550/525 bandpass FL9 filter, 710/660 bandpass FL6 filter, and 655/620 bandpass FL3 filter, respectively.

Results

Transformation of *Paenibacillus* using physical permeation by magnesium aminoclays

In order to be able to study the function of the genes with respect to their physiological roles in *P. riograndensis*, a

method for transformation of this bacterium using plasmid DNA has to be developed. In addition to transformation methods commonly used in bacteria such as heat shock or electroporation, a simple and efficient procedure for plasmid transformation by physical permeation using magnesium aminoclays has been recently developed (Yoshida and Sato 2009; Choi et al. 2013). In this method, a plasmid DNA and magnesium aminoclay supercomplex is formed and used to physically permeate the bacterial cell wall using sonication and/or spreading of the cell suspension on agar plates. Here, it was assessed whether this method, originally developed for *E. coli* and *Streptococcus mutans* (Choi et al. 2013), can be adapted for transformation of *Paenibacillus*. Two different plasmid backbones (pNW33Nmp and pTE) were tested. Transformation efficiency using physical permeation by magnesium aminoclays may depend on the concentration of plasmid DNA and physical parameters such as sonication, spreading time and the agar concentration. To this end, these parameters were varied for transformation of *P. riograndensis* SBR5. The cell-forming units (CFU) in the selective agar plates were counted. For both plasmids the highest transformation efficiencies (about $1.1 \cdot 10^3$ CFU/ μg pNW33Nmp DNA and about $1.8 \cdot 10^3$ CFU/ μg pTE DNA) were observed when 0.1 μg of plasmid DNA was added to the aminoclay solution, vortexed for 1 min with the cell suspension and spread for 1 min on a 1.5 % agar plate (Fig. S1). Extended duration of vortexing or spreading did not improve transformation efficiency. The use of short ultrasonication treatment to better mix the cell suspension with the plasmid-aminoclay solution did not result in higher transformation efficiency compared with the treatment using vortexing (Fig. S1).

To verify transformation, plasmid DNA from *P. riograndensis* transformed with pNW33Nmp was isolated using a classical plasmid DNA isolation method and subsequently used to transform *E. coli* via heat shock. The plasmid DNA isolated from the transformed *E. coli* cells was compared with that of transformed *P. riograndensis* cells by restriction enzyme digestion with the restriction enzyme *AcuI*. The agarose gel electrophoresis of cut plasmid DNA revealed the

expected two identical DNA patterns (1530 and 3776 base pairs), thus, indicating that intact DNA of plasmid pNW33Nmp could be isolated from *P. riograndensis* transformants and used for transformation into *E. coli* (Fig. S2).

Next, we tested if heterologous fluorescent reporter proteins can be produced in *P. riograndensis* transformants. For this reason, genes coding for fusion proteins were constructed by removing the stop codon of the chloramphenicol resistance cassette (Cm^R) from vector pRE and introduction of the genes coding for fluorescent reporter proteins (GfpUV, mCherry, or Crimson) downstream and in frame of this sequence. The resulting vectors were named pR_{fc}-gfpUV, pR_{fc}-mCherry, and pR_{fc}-crimson and used to transform *P. riograndensis* SBR5. The fluorescence of the transformants was quantified by flow cytometry analysis of populations with 20,000 transformed cells (Fig. 1). The chloramphenicol resistant *P. riograndensis* transformants expressed the reporter gene fusions since increased fluorescence was observed for Crimson (8.5 times higher than the empty vector carrying control), mCherry (5 times higher), and for GfpUV (5 times higher; Fig. 1). Thus, *P. riograndensis* could successfully be transformed by physical permeation using the magnesium aminoclay method and genes for fusion proteins of the chloramphenicol resistance marker protein with the fluorescent proteins Crimson, GfpUV and mCherry, respectively, could be functionally expressed.

A rolling circle-replicating plasmid for constitutive expression at different levels

In order to develop plasmids for constitutive gene expression of different promoter strengths, three different promoters were cloned upstream of the promoterless gene *gfpUV* on the rolling circle-replicating plasmid pRE: Ptuf, Pgap, and Ppyk. Since the orthologous promoters were characterized as strong in *Corynebacterium glutamicum* (Pátek and Nešvera 2011; Becker et al. 2013), the respective open reading frames from *C. glutamicum* were used as queries for nucleotide BLAST search (Altschup et al. 1990) against the genome sequence of

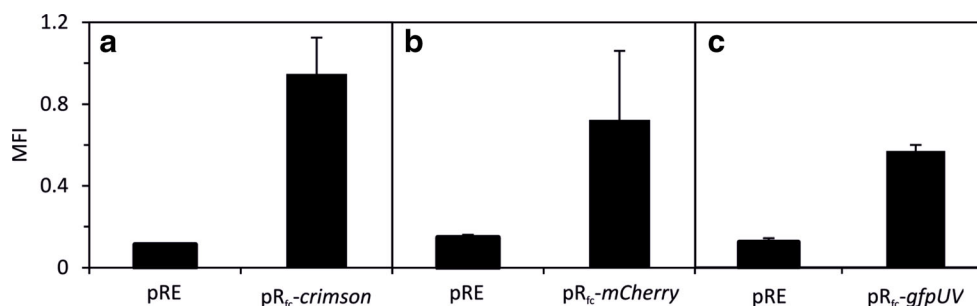


Fig. 1 Fluorescence analysis of *P. riograndensis* SBR5 cells carrying plasmids encoding protein fusions of the chloramphenicol resistance protein and either Crimson (a), mCherry (b), or GfpUV (c). Mean fluorescence intensities of populations of 20,000 cells analyzed by flow

cytometer are shown as means and standard deviation of biological triplicates. Transformants carrying the empty vector pRE were analyzed for comparison

Table 2 Sequences and positions of the –10 and –35 regions within the 300 base pairs sequences upstream of several *P. riograndensis* SBR5 genes

Promoter name	Gene ID	Spacing	–35 box	Spacing	–10 box	Spacing	Start codon
PgapA	PRIO_0184	N ₂₃₈	TTGACA	N ₁₃	GTCTTGAAT	N ₃₁	ATG
Ppyk	PRIO_2339	N ₁₉	CTCAAT	N ₁₂	CAGTATACT	N ₂₅₄	ATG
Ptuf	PRIO_6140	N ₁₆₆	TCTC CA	N ₃₀	TAACCT	N ₉₂	ATG

The promoters were predicted bioinformatically using the Bacterial Promoter Prediction (BPROM) tool on SoftBerry platform (Solovyev and Salamov 2011)

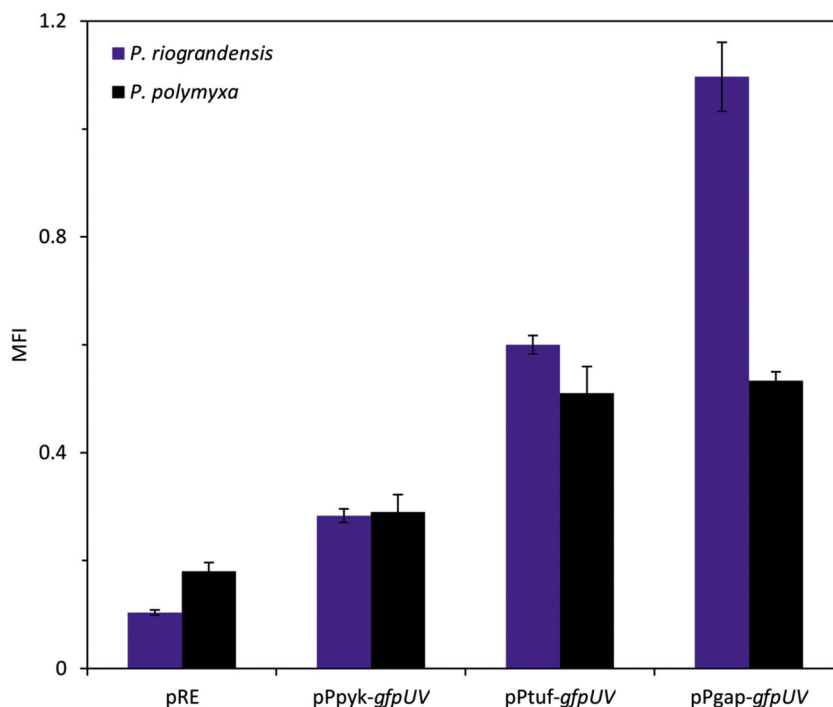
P. riograndensis SBR5. Thus, the identified *P. riograndensis* SBR5 genes PRIO_0184, PRIO_2339, and PRIO_6140 are annotated to encode glyceraldehyde-3-phosphate dehydrogenase, pyruvate kinase, and elongation factor G, respectively. The Bacterial Promoter Prediction (BPROM) tool on the SoftBerry platform (Solovyev and Salamov 2011) detected –10 and –35 hexamer regions in the 300 base pairs sequence upstream of the start codons of these genes (Table 2). The plasmids containing Ptuf, Pgap, and Ppyk upstream of the promoterless gene *gfpUV* were named pPgap-*gfpUV*, pPpyk-*gfpUV*, and pPtuf-*gfpUV* (Table 1) and used to transform *P. riograndensis*. GfpUV fluorescence was measured after growth in Caso broth for 6 h. *P. riograndensis* transformed with the empty vector showed a background median fluorescence intensity (MFI) of approximately 0.1, whereas GfpUV fluorescence of *P. riograndensis* transformed with pPgap-*gfpUV*, pPpyk-*gfpUV* or pPtuf-*gfpUV* was significantly higher (Fig. 2). Promoter strengths differed, and the fluorescence intensity of the analyzed strains increased in the following pattern: pPgapA-*gfpUV* (11-fold higher than the empty vector carrying control strain), pPtuf-*gfpUV* (6-fold), and pPpyk-

gfpUV (2.9-fold; Fig. 2). Thus, three different endogenous promoters are available to drive expression of heterologous genes with strengths at different levels.

Inducible and gradable expression system using the heterologous XylR system from *B. megaterium*

In order to develop a gene expression system that is inducible and gradable by an external trigger, the xylose-inducible XylR system from *B. megaterium* was tested in *P. riograndensis*. To this end, the pRE-based vector pRX-*gfpUV* was used (Irla et al. 2016). The transformants were cultivated in Caso broth supplemented with 0, 24, 50, 100, or 200 mM of xylose. GfpUV fluorescence of *P. riograndensis* SBR5(pRX-*gfpUV*) cells increased with increasing concentrations of the inducer xylose (Fig. 3a). Induction was about 6-fold higher when 24 mM xylose was added compared with the non-induced control and reached close to maximal values (about 12-fold higher in comparison with non-induced conditions) in the presence of 50 mM xylose.

Fig. 2 Reporter gene expression analysis of vectors with different constitutive promoters. GfpUV fluorescence of *P. riograndensis* SBR5 (blue) and *P. polymyxa* DM36 (black) cells carrying plasmids with *gfpUV* gene under control of three different constitutive promoters (Ppyk, Pgap, and Ptuf) or the empty vector plasmid pRE are given as means and standard deviations of biological triplicates measured by flow cytometer of 20,000 cells



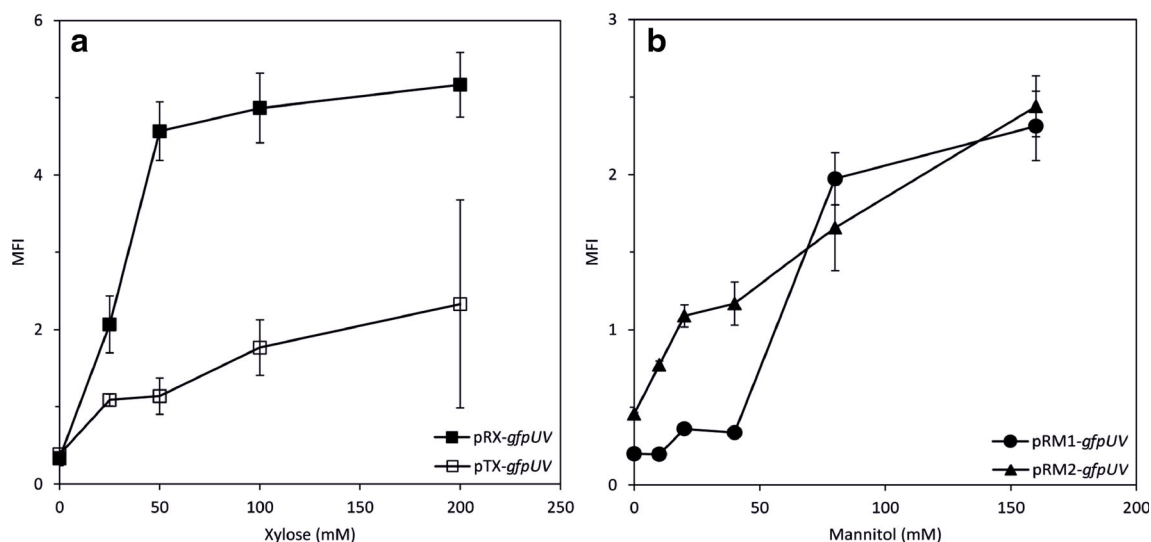


Fig. 3 Reporter gene expression analysis of plasmids with xylose-inducible (a) or mannitol-inducible (b) promoters. GfpUV fluorescence of *Paenibacillus riograndensis* SBR5 cells carrying (a) rolling circle-replicating, xylose-inducible plasmid pRX-*gfpUV* gene or theta-replicating, xylose-inducible plasmid pTX or (b) carrying plasmids with the *gfpUV* gene under control of mannitol-inducible promoter from

To test the xylose-inducible expression system in a theta-replicating vector, *gfpUV* was cloned into the multiple cloning site of the vector pTE, which also contains the xylose repressor gene *xylR* and xylose-inducible promoter. The resulting plasmid was named pTX-*gfpUV* and used to transform *P. riograndensis* SBR5. The transformants were cultivated in the presence of 0, 24, 50, 100, or 200 mM xylose. GfpUV fluorescence increased with increasing xylose concentrations (Fig. 3a). The GfpUV fluorescence levels were lower for the theta-replicating vector pTX-*gfpUV* than for rolling circle-replicating vector pRX-*gfpUV* (Fig. 3a). Taken together, theta-replicating and rolling circle replication plasmids for gradable, xylose-inducible gene expression were developed and shown to function in *P. riograndensis*.

Mannitol-inducible and gradable expression based on endogenous or heterologous promoter and activator genes

Genes of mannitol catabolism are typically regulated by the availability of the carbon source mannitol as for example shown for *B. methanolicus* (Jakobsen et al. 2009) and *B. subtilis* subsp. *subtilis* str. 168 (Heravi and Altenbuchner 2016). Based on microarray and RNAseq (Heggeset et al. 2012, Irla et al. 2015) analysis of mannitol-inducible genes in this bacterium, a mannitol-inducible gene expression system employing the promoter of the *mtlR* gene of *B. methanolicus* was developed (Irla et al. 2016). To identify potentially mannitol-inducible promoters, a BLAST analysis of the genome of *P. riograndensis* SBR5 using the upstream region of -35 sequence of *mtlR* gene of *B. methanolicus* as

P. riograndensis SBR5 (pRM1-*gfpUV*) or *B. methanolicus* MGA3 (pRM2-*gfpUV*) was analyzed by flow cytometry of populations of 20,000 cells. Gene expression was induced by 0, 25, 50, 100, and 200 mM xylose or addition of 0, 10, 20, 40, 80, and 160 mM mannitol added to the growth medium at inoculation. Means and standard deviations of biological triplicates are depicted

query was performed and revealed similarity to the upstream region of -35 sequences of *mtlA* from *P. riograndensis* SBR5. As a first test of this promoter, its expression was analyzed heterologously in *B. methanolicus* using plasmid pRM1-*gfpUV* and compared with the mannitol-inducible *mtlR* promoter from *B. methanolicus* (pRM2-*gfpUV*) and the mannitol-inducible *mtlA* promoter from *B. subtilis* subsp. *subtilis* str. 168 (pRM3-*gfpUV*). GfpUV fluorescence under non-inducing and inducing conditions was determined by flow cytometry. As shown in Table 3, the promoters from *B. methanolicus* and *P. riograndensis* were active and mannitol inducible in *B. methanolicus*, whereas the *mtlA* promoter from *B. subtilis* was not (Table 3). Thus, the *mtlA* promoter from *P. riograndensis* SBR5 allowed for mannitol-inducible gene expression in the heterologous *B. methanolicus*.

Table 3. GfpUV fluorescence of *B. methanolicus* MGA3 strains carrying plasmids with *gfpUV* gene under control of different mannitol-inducible promoters

Construct	Mean GfpUV fluorescence intensity		
	pRM1- <i>gfpUV</i>	pRM2- <i>gfpUV</i>	pRM3- <i>gfpUV</i>
0 mM mannitol	0.1 ± 0.0	0.2 ± 0.0	0.2 ± 0.0
50 mM mannitol	0.8 ± 0.1	1.1 ± 0.1	0.2 ± 0.0

The promoterless *gfpUV* gene was fused to putative mannitol-inducible promoters from *P. riograndensis* (pRM1-*gfpUV*), *B. methanolicus* (pRM2-*gfpUV*), or *B. subtilis* (pRM3-*gfpUV*). Gene expression was induced by addition of 50 mM mannitol to the growth medium. The table shows means and standard deviations of technical triplicates

To test if the *mtlA* promoter from *P. riograndensis* SBR5 is mannitol inducible in the native host, pRM1-*gfpUV* was used to transform *P. riograndensis* SBR5. As control, *P. riograndensis* SBR5(pRM2-*gfpUV*) with the mannitol-inducible promoter from *B. methanolicus* MGA3 was constructed. Both strains were analyzed by flow cytometry analysis after cultivation in Caso broth in the presence of 0, 20, 40, 80, or 160 mM of mannitol. This dose-response analysis revealed increasing GfpUV fluorescence with increasing mannitol concentrations and comparable maxima for both, SBR5(pRM1-*gfpUV*) and SBR5(pRM2-*gfpUV*) (Fig. 3b). Thus, the *mtlA* promoter from *P. riograndensis* SBR5 was shown to be mannitol inducible in the native host. An almost linear correlation between the inducer concentration and the mean GfpUV fluorescence intensity was only found for SBR5(pRM2-*gfpUV*) in the concentration range of 40 to 160 mM (Fig. 3b). Taken together, mannitol-inducible expression vectors carrying either an endogenous promoter or a heterologous promoter from *B. methanolicus* can be used for controlled gene expression in *P. riograndensis*. Mannitol induction of these promoters in *B. methanolicus* as well as in *P. riograndensis* relies on hitherto unknown trans-regulatory factors, likely activators, since these are not encoded on the gene expression vectors used.

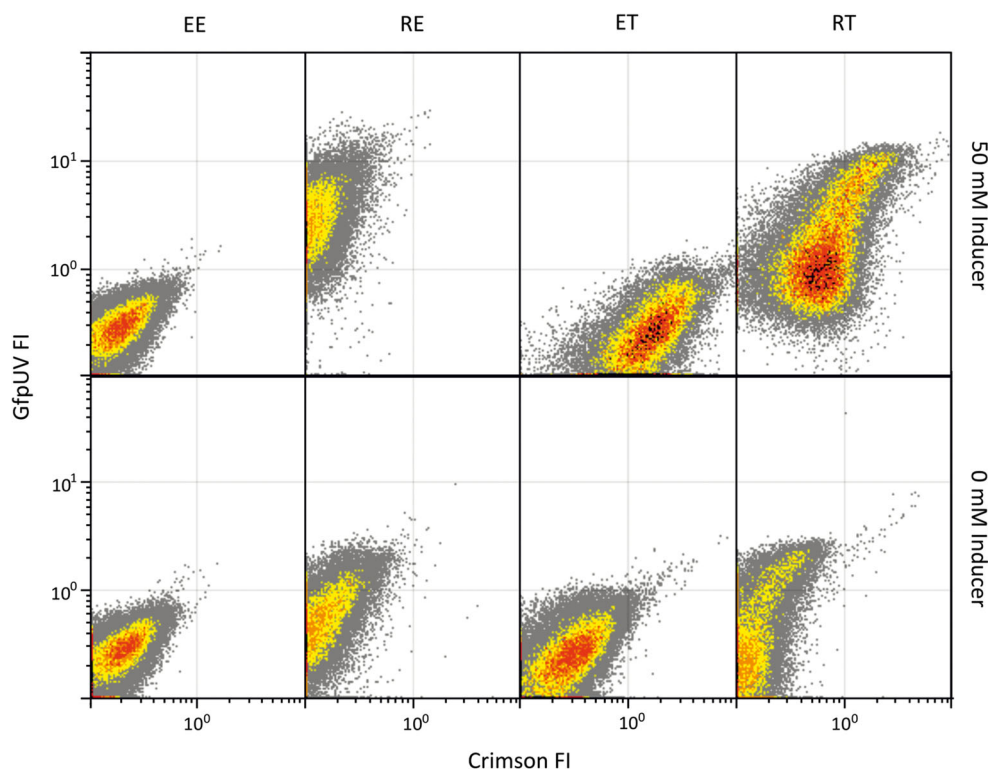
Inducible gene expression using a two vector system

In order to test if the compatible expression vectors pTX (based on theta-replicating plasmid pTE, carrying xylose-inducible gene expression system) and pRM2 (based on

rolling circle-replicating plasmid pRE, carrying mannitol-inducible promoter) allow for independently controllable gene expression in a single cell, *P. riograndensis* SBR5 was transformed with the following pairs of expression vectors: pRE and pTE, pRM2-*gfpUV* and pTE, pTX-*crimson* and pRE, or pRM2-*gfpUV* and pTX-*crimson*. The double transformants were cultivated under non-inducing conditions (in the absence of inducers) and under inducing conditions (in the presence of 50 mM xylose and 50 mM mannitol). The double transformants carrying the empty vectors showed background GfpUV and Crimson fluorescence of about 0.2 to 0.4 irrespective of the presence or absence of the inducers (Fig. 4; Table S2). Transformants carrying pTX-*crimson* showed increased mean Crimson fluorescence intensities of 1.2 to 2.1 under inducing conditions and transformants carrying pRX-*gfpUV* showed increased mean GfpUV fluorescence intensities of about 1.6 to 2.2 when induced (Fig. 4; Table S2). Double fluorescent cells (increased mean Crimson and GfpUV fluorescence intensities) were observed for transformants carrying pTX-*crimson* and pRX-*gfpUV* (Fig. 4; Table S2).

To test if mannitol- and xylose-inducible gene expression can be controlled independently, *P. riograndensis* SBR5(pRX-*gfpUV*)(pTX-*crimson*) was cultivated either without inducers, with 50 mM xylose and 50 mannitol, with 50 mM xylose alone, as well as with 50 mM mannitol alone. As expected, GfpUV fluorescence-positive, but Crimson fluorescence-negative cells were observed with mannitol alone, GfpUV-negative, but Crimson-positive cells with

Fig. 4 Reporter gene expression analysis of cells carrying two compatible expression vectors. GfpUV and Crimson fluorescence was analysed by flow cytometry of populations of 20,000 *P. riograndensis* cells carrying the two compatible plasmids pRE and pTE (EE), pRM2-*gfpUV* and pTE (RE), pTX-*crimson* and pRE (ET), or pRM2-*gfpUV* and pTX-*crimson* (RT), respectively. Cells were cultivated in the absence of inducers or in the presence of a mixture of 50 mM xylose and 50 mM mannitol



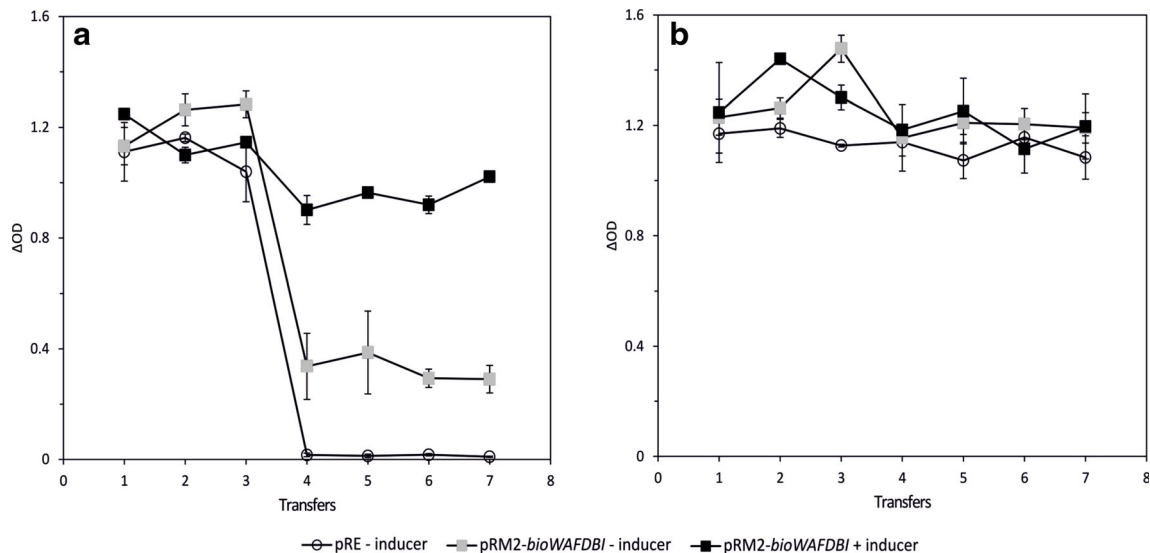


Fig. 5 Growth of *P. riograndensis* SBR5(pRE) and SBR5(pRM2-*bioWAFDBI*) in PbMM minimal medium lacking biotin (a) or containing 0.1 mg L⁻¹ biotin (b). SBR5(pRM2-*bioWAFDBI*) was cultivated without (gray squares) or with induction with 160 mM

mannitol (black squares). The biomass formed ($\Delta OD_{600 \text{ nm}}$) after growth for at least 24 h is shown for repeated transfers to fresh, biotin-free PbMM (a) or fresh biotin-containing PbMM (b). Means and standard deviations of biological triplicates are shown

xylose alone, and GfpUV and Crimson double-positive cells were only observed in the presence of both inducers (Fig. S3).

Controlled expression of heterologous *bioWAFDBI* genes rendered *P. riograndensis* SBR5 biotin-prototrophic

P. riograndensis lacks genes coding for biotin biosynthesis enzymes, thus, it requires biotin as supplement when grown in minimal media (Brito et al. 2015). The plasmid pEKEx3-*bioWAFDBI* (Peters-Wendisch et al. 2014) was used to subclone the *bioWAFDBI* operon from *B. subtilis* 168 into the expression vector pRM2. The resulting vector pRM2-*bioWAFDBI* was used to transform *P. riograndensis* SBR5. After pre-growth in the biotin-containing medium PbMM, *P. riograndensis* strains SBR5(pRE) and SBR5(pRM2-*bioWAFDBI*) were transferred repeatedly either to biotin-free or to biotin-containing medium in the absence of mannitol as inducer (Fig. 5). In addition, SBR5(pRM2-*bioWAFDBI*) was tested in the presence of 160 mM mannitol as inducer (Fig. 5). In biotin-containing minimal medium, both SBR5(pRE) and SBR5(pRM2-*bioWAFDBI*) grew to comparable biomass concentrations (given as $\Delta OD_{600 \text{ nm}}$) for seven serial transfers (Fig. 5b). As expected, *P. riograndensis* SBR5(pRE) failed to grow in biotin-free medium after the third transfer (Fig. 5a). By contrast, SBR5(pRM2-*bioWAFDBI*) grew for seven serial transfers to biotin-free medium when gene expression was induced by mannitol (Fig. 5a). Thus, induced pRM2-based expression of *bioWAFDBI* was sufficient to render *P. riograndensis* biotin prototrophic. It has to be noted that some growth of SBR5(pRM2-*bioWAFDBI*) was observed in the absence of the inducer mannitol upon repeated transfer to biotin-free medium ($\Delta OD_{600 \text{ nm}}$ of 0.3 without induction as

compared with $\Delta OD_{600 \text{ nm}}$ of 1.0 when induced), indicating possible promoter leakage.

Transfer of the transformation protocol and gene expression vector systems to *P. polymyxa* DSM-365

In order to test if the magnesium aminoclay-based transformation protocol and the constructed inducible expression vectors can be applied to another species of the genus *Paenibacillus*, *P. polymyxa* DSM-365 was transformed with the plasmids pNW33mp and pTE, respectively, using the conditions optimized for *P. riograndensis*. Transformation of both plasmids was successful; however, the transformation efficiency of approximately 1.0×10^2 transformants/ μg of DNA was about 10-fold lower than in *P. riograndensis*.

The constitutive expression plasmids pPpyk-*gfpUV*, pPtuf-*gfpUV*, and pPgap-*gfpUV* were used to transform *P. polymyxa* DSM-365 and GfpUV fluorescence was quantified by flow cytometry (Fig. 2). The *P. polymyxa* transformants showed higher than background GfpUV fluorescence intensities (Fig. 2). The strengths of promoters Ppyk and Ptuf were comparable in *P. polymyxa* and in *P. riograndensis* while, the promoter strength of Pgap was almost 2-fold lower in *P. polymyxa* than in *P. riograndensis* (Fig. 2).

The theta-replicating expression vector pTX carrying a xylose-inducible gene expression system was shown to allow for xylose-inducible gene expression in *P. polymyxa* DSM-365 using *mCherry* as reporter gene. After cultivation in Caso broth without added xylose, a background mCherry fluorescence of less than 0.2 was observed (Fig. 6a). With 50 mM and 100 mM xylose added as inducer, mCherry fluorescence increased about 2-fold and about 4-fold, respectively.

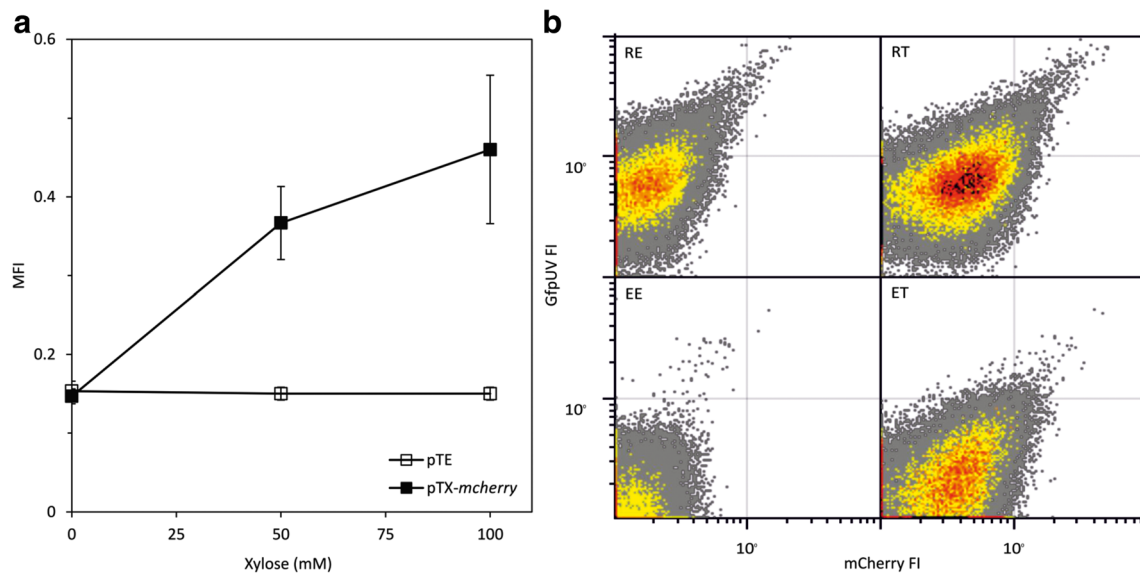


Fig. 6 GfpUV and mCherry fluorescence *Paenibacillus polymyxa* transformed with two compatible expression vectors. **a** mCherry fluorescence of DSM-365(pTE) and DSM-365(pTX.mCherry) induced with 0, 50 and 100 mM xylose added to the growth medium at inoculation. **b** Populations of 20,000 cells of *P. polymyxa* DSM-365

transformed with pRE and pTE (EE), pRM2-*gfpUV* and pTE (RE), pTX-*mcherry* and pRE (ET), or pRM2-*gfpUV* and pTX-*mcherry* (RT) cultivated in the presence of a mixture of 100 mM of xylose and mannitol were analyzed for GfpUV and mCherry fluorescence by flow cytometry. The figure shows means and standard deviation of biological triplicates

In the next step, *P. polymyxa* DSM-365 was transformed with the two compatible expression vectors pRM2-*gfpUV* and pTX-*mCherry* or the respective empty vectors pRE and pTE. *P. polymyxa* strains DSM-365(pRE)(pTE), DSM-365(pRM2-*gfpUV*)(pTE), DSM-365(pRE)(pTX-*mcherry*), and DSM-365(pRM2-*gfpUV*)(pTX-*mcherry*) were cultivated in Caso broth and fluorescent reporter gene expression was induced with a mixture of 100 mM xylose and 100 mM mannitol. GfpUV and mCherry fluorescence analysis revealed double fluorescence-negative cells for DSM-365(pRE)(pTE) and double fluorescence-positive cells for DSM-365(pRM2-*gfpUV*)(pTX-*mcherry*; Fig. 6b). DSM-365(pRM2-*gfpUV*)(pTE) only showed GfpUV fluorescence, whereas DSM-365(pRE)(pTX-*mcherry*) only showed mCherry fluorescence (Fig. 6b). Taken together, the transformation protocol and the gene expression tools developed for *P. riograndensis* were shown to be transferable to at least one other species of the genus *Paenibacillus*.

Discussion

Here, we optimized a simple and functional method using magnesium aminoclay for transformation of two *Paenibacillus* species. Moreover, efficient constitutive and inducible gene expression using compatible theta- and rolling circle-replicating vectors developed here enlarged the genetic toolbox for *Paenibacillus*. Besides characterization of these systems using fluorescent reporter genes, biotin-auxotrophic *P. riograndensis* was rendered prototrophic for biotin by inducible heterologous expression of the *bioWAFDBI* operon from *B. subtilis*.

Biotin is required as a co-factor for a diverse group of enzymes called biotin-dependent family enzymes (Jitrapakdee and Wallace 2003). Biotin is essential for *E. coli* although it possesses only a single biotin-containing enzyme, namely acetyl-CoA carboxylase catalyzing the formation of malonyl-CoA as essential precursor for fatty acid biosynthesis. The genome of the biotin-auxotrophic *P. riograndensis* SBR5 contains genes putatively encoding pyruvate carboxylase (PRIO_6030) and acetyl-CoA carboxylase (PRIO_2337). Biotin has to be added to the growth medium of auxotrophic *P. riograndensis* since it lacks genes for biotin biosynthesis. Of the proteins encoded in the biotin biosynthesis operon *bioWAFDBI* from *B. subtilis*, 6-carboxyhexanoate CoA ligase BioW is not required for de novo biotin synthesis but to activate pimelic acid to pimeloyl-CoA. *P. riograndensis* may possess a homolog of BioI since the PRIO_5347 encoded P450 enzyme shares similarity with BioI of *B. subtilis* but does not possess homologs of BioW, BioA, BioF, BioD, and BioB (Brito et al. 2015). As shown for other biotin auxotrophs, e.g., *C. glutamicum* ATCC 13032 (Peters-Wendisch et al. 2014; Ikeda et al. 2013), heterologous expression of the complete *bioWAFDBI* operon from *B. subtilis* led to biotin prototrophy of *P. riograndensis*. However, it remains to be shown if expression of all *bio* genes is required or if expression of a subset of these genes would be sufficient: *bioFI* in the case of *C. glutamicum* and possibly *bioAFDB* in the case of *P. riograndensis*. It is known that many root-associated bacteria are dependent on a supply of previously synthesized growth factors from plants. Indeed, biotin is commonly present in the root exudates of higher

plants (Jones et al. 1962). This may suggest that biotin synthesis by *P. riograndensis* has been lost during evolution.

The major advantage of the magnesium aminoclay method is its simplicity, whereas other methods may be superior with respect to the transformation efficiency. The transformation efficiencies obtained for *P. riograndensis* SBR5 ($10^3/\mu\text{g}$ of DNA) and *P. polymyxa* DSM-365 ($10^2/\mu\text{g}$ of DNA) are comparable with those obtained in *E. coli* and *S. mutans* by the magnesium aminoclay method (Choi et al. 2013). These transformation efficiencies are sufficient for strain construction but are too low to generate gene libraries in these species, e.g., when screening libraries of gene knockdowns using CRISPR interference (Cleto et al. 2016; Tong et al. 2015). Electroporation protocols developed for *P. polymyxa* (Rosado et al. 1994) and *P. larvae* (Murray and Aronstein 2008) are 100 to 1000 times more efficient; however, even these transformation efficiencies of $10^5/\mu\text{g}$ of DNA are too low to generate large gene libraries, which require transformation efficiencies in the order of 10^8 to $10^9/\mu\text{g}$ of DNA as obtained for *E. coli* (Hanahan and Jessee 1991). Taken together, the low transformation efficiency by the magnesium aminoclay method is compensated for by its simplicity, the fact that it does not involve the use of expensive material such as electroporation cuvettes or electricity, and the fact that laborious preparation of competent cells is not required since the use of an exponentially growing *Paenibacillus* culture is sufficient.

Biotechnological processes involving recombinant bacteria often face stability problems when using rolling circle-replicating plasmids as is seen also for Bacilli (Leonhardt 1990; Leonhardt and Alonso 1991; Irla et al. 2016). Besides their roles in plant growth promotion and bioremediation, *Paenibacilli* may find biotechnological application in the production of value-added compounds such as (R,R)-2,3-butanediol (Yu et al. 2011; Adlakha and Shams 2015) or of antimicrobials such as the lipodepsipeptide fusaricidin (Rim et al. 2014). Thus, the more stable theta-replicating plasmids may be valuable for applications using recombinant *Paenibacilli*. However, it has to be noted that due to the lower copy numbers of theta-replicating plasmids as compared with most rolling circle-replicating plasmids, overexpression of the endogenous or heterologous genes in theta-replicating plasmids requires stronger promoters and/or translation efficiency.

In this study, several constitutive promoters of various strengths as well as graded inducible gene expression systems were studied for gene expression in *P. riograndensis*. Based on a bioinformatics analysis of promoter sequences, three promoters expected to be strong and constitutive were chosen. As shown in Fig. 2, the expression of *gfpUV* in *P. riograndensis* was 3- (Ppyk), 6- (Ptuf), or about 10-fold (PgapA) higher than the autofluorescence background, and a mean fluorescence intensity of about 1.1 was obtained with expression vector pPgap-*gfpUV* (Fig. 2). However, in *P. polymyxa* the *gfpUV* expression from *P. riograndensis* promoter PgapA only led to mean fluorescence intensity of about 0.5 (Fig. 2). This is

commensurate with the sequence differences between the PgapA promoters from *P. riograndensis* and *P. polymyxa*, with six mismatches in the -10 box and one mismatch in the -35 box (BLAST analysis not shown), thus, high constitutive gene expression in *P. polymyxa* should be based on the endogenous PgapA promoter rather than the one from *P. riograndensis*.

When fully induced, gene expression from the mannitol-inducible promoter PmtlR and the xylose-inducible promoter PxylA reached higher levels (mean fluorescence intensities of about 2.5 for the mannitol-inducible gene expression vectors pRM1-*gfpUV* and pRM2-*gfpUV* and of about 5 for pRX-*gfpUV*; Fig. 3) than obtained with PgapA (mean fluorescence intensity of about 1.1; Fig. 2). The very high expression levels obtained with fully induced PmtlR and PxylA come at a cost, namely the requirement to add ≥ 100 mM xylose or ≥ 150 mM mannitol to the growth medium. Neither mannitol nor xylose are gratuitous inducers in *Paenibacillus* since they serve as carbon sources for growth (Beneduzi et al. 2010).

The induction patterns with respect to the inducer concentrations deviated from perfect linearity which may reflect that inducers were catabolized and their concentrations diminished during the growth of the recombinant strains, although they were added to complex Caso broth rather than minimal media and, thus, were not required as growth substrates. The observed induction patterns may also reflect all-or-none induction and the presence of induced and non-induced sub-populations. In *E. coli*, this phenomenon is known for arabinose-inducible gene expression from expression vectors and deletion of the arabinose utilization genes was required for the homogenous gene expression from the plasmid (Datsenko and Wanner 2000), a strategy which may be followed in *Paenibacillus* once gene deletion is possible in this bacterium. Homogenous gene expression may require that genes important for inducer uptake are expressed constitutively and independently from the inducer itself. In *E. coli*, for example, transcribing arabinose uptake gene *araE* from a constitutive promoter from *Lactococcus lactis* instead of its own arabinose-inducible promoter enabled homogenous graded arabinose induction (Khlebnikov et al. 2016).

In general, promoters from different *Bacillus* or *Paenibacillus* species are functional in other *Bacillus* or *Paenibacillus* species, e.g., the promoters PxylA from *B. megaterium* and PmtlA from *B. methanolicus* could be used in *Paenibacillus*. On the other hand, *B. subtilis* PmtlA hardly worked in *B. methanolicus* (Table 3). Likewise, PxylA was xylose inducible in *P. polymyxa* DSM-365; however, induced expression of the fluorescence reporter gene was about 10-fold lower than in *P. riograndensis* (Fig. 6a). Thus, transferability of the gene expression systems is achievable, however, to fully exploit the application potential these systems need to be optimized in the respective hosts.

The genetic toolbox described here forecasts future developments in functional genomics of *Paenibacilli*. Gain-of-

function and loss-of-function analyses are important elements of functional genomics and they require genetic systems for gene overexpression and gene deletion. For instance, controlled gene expression is important in gene deletion using CRISPR/Cas9 (Jiang and Marraffini 2015; Makarova 2011) or for CRISPRi/dCas9-mediated gene knockdown (Cleto et al. 2016; Tong et al. 2015). A prerequisite for the use of CRISPR technology as well as for gain-of-function analyses in *Paenibacillus* has been achieved in this study by the two vector system for independent mannitol and/or xylose-inducible gene expression.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The research performed did not involve human participants and/or animals.

References

- Adlakha N, Shams S (2015) Efficient production of (R, R)-2, 3-butanediol from cellulosic hydrolysate using *Paenibacillus polymyxa* ICGEB2008. *J Ind Microbiol Biotechnol* 42:21–28. doi:10.1007/s10295-014-1542-0
- Altschul SF, Gish W, Miller W, Myers EW, Lipman DJ (1990) Basic local alignment search tool. *J Mol Biol* 215:403–410
- Bakhiet N, Stahly DP (1985) Studies on transfection and transformation of protoplasts of *Bacillus larvae*, *Bacillus subtilis*, and *Bacillus popilliae*. *Appl Environ Microbiol* 49:577–581
- Becker J, Schäfer R, Kohlstedt M, Harder BJ, Borchert NS, Stöveken N, Bremer E, Wittmann C (2013) Systems metabolic engineering of *Corynebacterium glutamicum* for production of the chemical chaperone ectoine. *Microb Cell Factories* 12:110. doi:10.1186/1475-2859-12-110
- Beneduzi A, Costa PB, Melo IS, Bodanese-zanettini MH, Passaglia LMP (2010) *Paenibacillus riograndensis* sp. nov., a nitrogen-fixing species isolated from the rhizosphere of *Triticum aestivum*. *Int J Syst Evol Microbiol* 60:128–133. doi:10.1099/ijs.0.011973-0
- Beneduzi A, Moreira F, Costa PB, Vargas LK, Lisboa BB, Favreto R, Ivo J, Maria L, Passaglia P (2013) Diversity and plant growth promoting evaluation abilities of bacteria isolated from sugarcane cultivated in the south of Brazil. *Appl Soil Ecol* 63:94–104. doi:10.1016/j.apsoil.2012.08.010
- Biedendieck R, Borgmeier C, Bunk B, Stammen S, Scherling C, Meinhardt F, Wittmann C, Jahn D (2011) Systems biology of recombinant protein production using *Bacillus megaterium*. *Methods Enzymol* 500:165–195. doi:10.1016/B978-0-12-385118-5.00010-4
- Brito LF, Bach E, Kalinowski J, Rückert C, Wibberg D, Passaglia LM, Wendisch VF (2015) Complete genome sequence of *Paenibacillus riograndensis* SBR5, a gram-positive diazotrophic rhizobacterium. *J Biotechnol* 207:30–31. doi:10.1016/j.jbiotec.2015.04.025
- Choi HA, Lee YC, Lee JY, Shin HJ, Han HK, Kim GJ (2013) A simple bacterial transformation method using magnesium- and calcium-aminoclay. *J Microbiol Methods* 95:97–101. doi:10.1016/j.mimet.2013.07.018
- Cleto S, Jensen JK, Wendisch VF, Lu TK (2016) *Corynebacterium glutamicum* metabolic engineering with CRISPR interference (CRISPRi). *ACS Synth Biol* 5:375–385. doi:10.1021/acssynbio.5b00216
- Datsenko KA, Wanner BL (2000) One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. *Proc Natl Acad Sci* 97:6640–6645. doi:10.1073/pnas.120163297
- Eikmanns BJ, Thum-schmitz N, Eggeling L, Ludtke K, Sahm H (1994) Nucleotide sequence, expression and transcriptional analysis of the *Corynebacterium glutamicum* *gltA* gene encoding citrate synthase. *Microbiology* 140:1817–1828. doi:10.1099/13500872-140-8-1817
- Fernandes GDC, Trarbach LJ, Campos SB De, Beneduzi A, Passaglia LMP (2014) Alternative nitrogenase and pseudogenes: unique features of the *Paenibacillus riograndensis* nitrogen fixation system. *Res Microbiol* 165:571–580. doi:10.1016/j.resmic.2014.06.002
- Gibson DG (2014) Programming biological operating systems: genome design, assembly and activation. *Nat Methods* 11:521–526. doi:10.1038/nmeth.2894
- Han HK, Lee YC, Lee MY, Patil AJ, Shin HJ (2011) Magnesium and calcium organophyllosilicates: synthesis and in vitro cytotoxicity study. *ACS Appl Mater Interfaces* 3:2564–2572. doi:10.1021/am200406k
- Hanahan D, Harbor CS (1983) Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* 166:557–580
- Hanahan BD, Jessee J (1991) Plasmid transformation of *Escherichia coli* and other bacteria. *Methods Enzymol* 204:63–113
- Heggeset TMB, Krog A, Balzer S, Wentzel A, Ellingsen TE, Brautaset T (2012) Genome sequence of thermotolerant *Bacillus methanolicus*: features and regulation related to methylotrophy and production of L-lysine and L-glutamate from methanol. *Appl Environ Microbiol* 78:5170–5181. doi:10.1128/AEM.00703-12
- Heravi KM, Altenbuchner J (2016) Regulation of the *Bacillus subtilis* mannitol utilization genes: promoter structure and transcriptional activation by the wild-type regulator (MtlR) and its mutants. *Microbiology* 160:91–101. doi:10.1099/mic.0.071233-0
- Ikeda M, Miyamoto A, Mutoh S, Kitano Y, Tajima M, Shirakura D, Takasaki M (2013) Development of biotin-prototrophic and hyperauxotrophic *Corynebacterium glutamicum* strains. *Appl Environ Microbiol* 79:4586–4594. doi:10.1128/AEM.00828-13
- Irla M, Neshat A, Brautaset T, Rückert C, Kalinowski J, Wendisch VF (2015) Transcriptome analysis of thermophilic methylotrophic *Bacillus methanolicus* MGA3 using RNA-sequencing provides detailed insights into its previously uncharted transcriptional landscape. *BMC Genomics* 16:1–22. doi:10.1186/s12864-015-1239-4
- Irla M, Heggeset TMB, Nærdal I, Paul L, Tone H, Le SB, Brautaset T, Wendisch VF (2016) Genome-based genetic tool development for *Bacillus methanolicus*: theta- and rolling circle-replicating plasmids for inducible gene expression and application to methanol-based cadaverine production. *Front Microbiol* 7:1481. doi:10.3389/fmicb.2016.01481
- Jakobsen OM, Brautaset T, Degnes KF, Heggeset TMB, Balzer S, Flickinger MC, Valla S, Ellingsen TE (2009) Overexpression of wild-type aspartokinase increases L-lysine production in the thermotolerant methylotrophic bacterium. *Appl Environ Microbiol* 75:652–661. doi:10.1128/AEM.01176-08
- Jiang W, Marraffini LA (2015) CRISPR-Cas: new tools for genetic manipulations from bacterial immunity systems. *Annu Rev Microbiol* 69:209–228. doi:10.1146/annurev-micro-091014-104441

- Jitrapakdee S, Wallace JC (2003) The biotin enzyme family: conserved structural motifs and domain rearrangements. *Curr Protein Pept Sci* 4:217–229
- Jones PD, Graham V, Segal L, Baillie WJ, Briggs MH (1962) Forms of soil biotin. *Life Sci* 1:645–648
- Khlebnikov A, Datsenko KA, Skaug T, Wanner BL, Keasling JD (2016) Homogeneous expression of the P_{BAD} promoter in *Escherichia coli* by constitutive expression of the low-affinity high-capacity AraE transporter. *Microbiology* 147:3241–3247. doi:10.1099/00221287-147-12-3241
- Kim S, Lee YC, Cho DH, Lee HU, Huh YS, Kim GJ, Kim HS (2014) A simple and non-invasive method for nuclear transformation of intact-walled *Chlamydomonas reinhardtii*. *PLoS One* 9:e101018. doi:10.1371/journal.pone.0101018
- Leonhardt H (1990) Identification of a low-copy-number mutation within the pUB II0 replicon and its effect on plasmid stability in *Bacillus subtilis*. *Gene* 94:121–124
- Leonhardt H, Alonso JC (1991) Parameters affecting plasmid stability in *Bacillus subtilis*. *Gene* 103:107–111
- Makarova KS (2011) Evolution and classification of the CRISPR–Cas systems. *Nat Rev Microbiol* 9:467–477. doi:10.1038/nrmicro2577
- Murray KD, Aronstein KA (2008) Transformation of the gram-positive honey bee pathogen, *Paenibacillus larvae*, by electroporation. *J Microbiol Methods* 75:325–328. doi:10.1016/j.mimet.2008.07.007
- Nguyen HD, Nguyen QA, Ferreira RC, Ferreira LCS, Tran LT, Schumann W (2005) Construction of plasmid-based expression vectors for *Bacillus subtilis* exhibiting full structural stability. *Plasmid* 54:241–248. doi:10.1016/j.plasmid.2005.05.001
- Pátek M, Nešvera J (2011) Sigma factors and promoters in *Corynebacterium glutamicum*. *J Biotechnol* 154:101–113. doi:10.1016/j.jbiotec.2011.01.017
- Peters-Wendisch P, Götker S, Heider SAE, Reddy GK, Nguyen AQ, Stansen KC, Wendisch VF (2014) Engineering biotin prototrophic *Corynebacterium glutamicum* strains for amino acid, diamine and carotenoid production. *J Biotechnol* 192:346–354. doi:10.1016/j.jbiotec.2014.01.023
- Rim H, Soo K, Park Y, Bin S, Jeong H, Keun S, Seung C, Park H (2014) Inactivation of the phosphoglucosyltransferase gene *pgm* in *Paenibacillus polymyxa* leads to overproduction of fusaricidin. *J Ind Microbiol Biotechnol* 41:1405–1414. doi:10.1007/s10295-014-1470-z
- Rosado A, Duarte GF, Seldin L (1994) Optimization of electroporation procedure to transform *B. polymyxa* SCE2 and other nitrogen-fixing *Bacillus*. *J Microbiol Methods* 19:1–11. doi:10.1016/0167-7012(94)90020-5
- Sambrook J (2001) *Molecular cloning: a laboratory manual*, 3rd edn. Cold Spring Harbor Laboratory press, New York
- Solovyyev V, Salamov A (2011) Automatic annotation of microbial genomes and metagenomic sequences. In: Li RW (ed) *Metagenomics and its applications in agriculture, biomedicine and environmental studies*. Nova Science Publishers, pp 61–78
- Sperber ER, Tadra-Sfeir MZ, Sperotto RA, Fernandes Gde C, Pedrosa Fde O, de Souza EM, Passaglia LM (2016) Iron deficiency mechanisms enlightened by gene expression analysis in *Paenibacillus riograndensis* SBR5. *Res Microbiol* 167:501–509. doi:10.1016/j.resmic.2016.04.007
- Tong Y, Charusanti P, Zhang L, Weber T, Lee SY (2015) CRISPR-Cas9 based engineering of actinomycetal genomes. *ACS Synth Biol* 4:1020–1029. doi:10.1021/acssynbio.5b00038
- Yoshida N, Sato M (2009) Plasmid uptake by bacteria: a comparison of methods and efficiencies. *Appl Microbiol Biotechnol* 83:791–798. doi:10.1007/s00253-009-2042-4
- Yoshida N, Ikeda T, Yoshida T, Sengoku T, Ogawa K (2001) Chrysotile asbestos fibers mediate transformation of *Escherichia coli* by exogenous plasmid DNA. *FEMS Microbiol Lett* 195:133–137. doi:10.1016/S0378-1097(00)00564-4
- Yu B, Sun J, Bommarreddy RR, Song L, Zeng A (2011) Novel (2R, 3R)-2, 3-butanediol dehydrogenase from potential industrial strain *Paenibacillus polymyxa* ATCC 12321. *Appl Environ Microbiol* 77:4230–4233. doi:10.1128/AEM.02998-10