Applied and Environmental Microbiology, May 2009, p. 3077–3085 Vol. 75, No. 10
0099-2240/09/$08.00+0 doi:10.1128/AEM.00087-09

Construction of a Gene Knockout System for Application in *Paenibacillus alvei* CCM 2051^T, Exemplified by the S-Layer Glycan Biosynthesis Initiation Enzyme WsfP$^\nabla$

Kristof Zarschler, Bettina Janesch, Sonja Zayni, Christina Schäffer,* and Paul Messner*

Department für NanoBiotechnologie, Universität für Bodenkultur Wien, A-1180 Vienna, Austria

Received 14 January 2009/Accepted 17 March 2009

The gram-positive bacterium *Paenibacillus alvei* CCM 2051^T is covered by an oblique surface layer (S-layer) composed of glycoprotein subunits. The S-layer O-glycan is a polymer of [→3)-β-D-Gal*p*-(1[α-D-Glc*p*-(1→6)]→4)-β-D-Man*p*NAc-(1→] repeating units that is linked by an adaptor of -[GroA-2→OPO_2→4-β-D-Man*p*NAc-(1→4)]→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→ to specific tyrosine residues of the S-layer protein. For elucidation of the mechanism governing S-layer glycan biosynthesis, a gene knockout system using bacterial mobile group II intron-mediated gene disruption was developed. The system is further based on the *sgsE* S-layer gene promoter of *Geobacillus stearothermophilus* NRS 2004/3a and on the *Geobacillus-Bacillus-Escherichia coli* shuttle vector pNW33N. As a target gene, *wsfP*, encoding a putative UDP-Gal:phosphoryl-polyprenol Gal-1-phosphate transferase, representing the predicted initiation enzyme of S-layer glycan biosynthesis, was disrupted. S-layer protein glycosylation was completely abolished in the insertional *P. alvei* CCM 2051^T *wsfP* mutant, according to sodium dodecyl sulfate-polyacrylamide gel electrophoresis evidence and carbohydrate analysis. Glycosylation was fully restored by plasmid-based expression of *wsfP* in the glycan-deficient *P. alvei* mutant, confirming that WsfP initiates S-layer protein glycosylation. This is the first report on the successful genetic manipulation of bacterial S-layer protein glycosylation in vivo, including transformation of and heterologous gene expression and gene disruption in the model organism *P. alvei* CCM 2051^T.

Bacterial cell surface layer (S-layer) glycoproteins provide a unique self-assembly matrix that has been optimized by nature for regular and periodic display of glycans with nanometer scale accuracy (21, 31). Exploitation of this self-assembly system for surface display of functional, tailor-made glycans is an attractive alternative to the use of common cell surface anchors (7), with potential areas of application relating to any biological phenomenon that is based on carbohydrate recognition, such as receptor-substrate interaction, signaling, or cell-cell communication. A prerequisite for this endeavor is the availability of an S-layer glycoprotein-covered bacterium that is amenable to genetic manipulation. Despite the high application potential offered by the S-layer glycan display system, there are so far only two reports in the literature dealing with the genetic manipulation of S-layer glycoprotein-carrying bacteria. Both reports concern the gram-negative periodontal pathogen *Tannerella forsythia* ATCC 43037, but neither of them affects S-layer protein glycosylation (12, 24). In archaea, in contrast, molecular studies of S-layer protein glycosylation are quite advanced (1), but with the archaeal system, S-layer glycoprotein self-assembly, which is a prerequisite for the desired glycan display, has not been manageable in vitro so far.

Our model organisms and, hence, candidates for S-layer-mediated glycan display enabled by carbohydrate engineering techniques are members of the *Bacillaceae* family. Currently, the S-layer glycosylation system of the thermophilic bacterium *Geobacillus stearothermophilus* NRS 2004/3a is best understood (20, 23, 29, 33, 34). However, a drawback of this organism is its resistance to take up foreign DNA. Although described in the literature (13, 14, 37), transformation of thermophilic bacilli seems to be a strain-specific trait. Based on successful transformation experiments in our laboratory, the mesophilic bacterium *Paenibacillus alvei* CCM 2051^T (ATCC 6344; DSM 29) (formerly *Bacillus alvei* [4]) was chosen to set up a system for genetic manipulation. The bacterium is completely covered with an oblique S-layer lattice composed of glycoprotein species. Various aspects of its S-layer, including ultrastructural characterization (27), glycosylation analysis (2, 18), and glycan biosynthesis (11), have been investigated so far. The S-layer O-glycans are polymers of [→3)-β-D-Gal*p*-(1[α-D-Glc*p*-(1→6)]→4)-β-D-Man*p*NAc-(1→] repeating units that are linked via the adaptor -[GroA-2→OPO_2→4-β-D-Man*p*NAc-(1→4)]→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-α-L-Rha*p*-(1→3)-β-D-Gal*p*-(1→ to specific tyrosine residues (2, 18) of the S-layer protein SpaA (GenBank accession number FJ751775).

Due to the presence of an identical adaptor saccharide backbone in the S-layer glycan of *G. stearothermophilus* NRS 2004/3a (29), where its biosynthesis is initiated by the UDP-Gal:phosphoryl-polyprenol Gal-1-phosphate transferase WsaP (33), it was conceivable that a homologous enzyme would initiate S-layer glycosylation in *P. alvei* CCM 2051^T. Considering that the S-layer protein glycosylation machinery has been found to be encoded by S-layer glycosylation (*slg*) gene clusters

* Corresponding author. Mailing address: Department für Nano-Biotechnologie, Universität für Bodenkultur Wien, Gregor-Mendel-Strasse 33, A-1180 Vienna, Austria. Phone: 43-1-47654, ext. 2202. Fax: 43-1-4789112. E-mail for Paul Messner: paul.messner@boku.ac.at. E-mail for Christina Schäffer: christina.schaeffer@boku.ac.at.

$^\nabla$ Published ahead of print on 20 March 2009.

TABLE 1. Oligonucleotide primers used for PCR amplification reactions

Oligonucleotide	Sequence (5′ → 3′)[a]
P(SgsE)_*Hin*dIII_for	AATCAAAGCTTTGTTTTTGCACAAAATGTTTGCC
P(SgsE)_*Sph*I_rev	AATCAGCATGCAGCCTAAAATCCCCCTTCG
P(SgsE)_*Sph*I_for	AATCAGCATGCTGTTTTTGCACAAAATGTTTGCC
P(SgsE)_*Hin*dIII_rev	AATCAAAGCTTAAAGCCTAAAATCCCCCTTCG
Targe_*Sph*I_for	AATCAGCATGCGCTGGCGTAATAGCGAAGA
Targe_*Sph*I_rev	AATCAGCATGCTACCGCACAGATGCGTAAG
KO_*wsfP*_control_for_1	TCTTATCCTTGGTGCCGGTACACTTG
KO_*wsfP*_control_rev_1	AGCCTGTAATTCCAGGACGCACA
*wsfP*_for_*Sph*I	AATCAGCATGCTTCGCAAAAATCAAAGGTTTTTGTCGAAG
*wsfP*_rev_*Kpn*I	AATCAGGTACCTTAATATGCATTTTTATTTATAAACCCATTCC
*wsaP*_for_*Sph*I	AATCAGCATGCTGGTTAAGGTGATTAGAGGAAGAGAGCGG
*wsaP*_rev_*Kpn*I	AATCAGGTACCTTAATATGCATTTTTATTTACCAAACCATTGG
P_555 556s-IBS	AAAAAAGCTTATAATTATCCTTAGCTGACTGGAAAGTGCGCCCAGATAGGGTG
P_555 556s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCTGGAAAACTAACTTACCTTTCTTTGT
P_555 556s-EBS2	TGAACGCAAGTTTCTAATTTCGATTTCAGCTCGATAGAGGAAAGTGTCT
P_654 655s-IBS	AAAAAAGCTTATAATTATCCTTAATTCTCGCACTAGTGCGCCCAGATAGGGTG
P_654 655s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGCACTACCTAACTTACCTTTCTTTGT
P_654 655s-EBS2	TGAACGCAAGTTTCTAATTTCGGTTAGAATCCGATAGAGGAAAGTGTCT
P_1176 1177s-IBS	AAAAAAGCTTATAATTATCCTTAAGACCCGAACGGGTGCGCCCAGATAGGGTG
P_1176 1177s-EBS1d	CAGATTGTACAAATGTGGTGATAACAGATAAGTCGAACGGCCTAACTTACCTTTCTTTGT
P_1176 1177s-EBS2	TGAACGCAAGTTTCTAATTTCGATTGGTCTTCGATAGAGGAAAGTGTCT
Cm_*Kpn*I_for	AATCAGGTACCAAGCCGATGAAGATGGA
Cm_*Kpn*I_rev	AATCAGGTACCACAGTCGGCATTATCTC

[a] Artificial restriction sites are underlined.

(21), degenerate primers for the *rml* genes catalyzing the dTDP-L-Rha biosynthesis required for building up the adaptor saccharide of the *P. alvei* CCM 2051^T S-layer glycan were used to define a point of entry into the glycosylation locus (K. Zarschler, B. Janesch, P. Messner, and C. Schäffer, unpublished data). Chromosome walking revealed the existence of an *slg* gene cluster of about 24 kb, including an open reading frame (ORF) predicted to encode the initiation enzyme of S-layer protein glycosylation. The corresponding gene, named *wsfP*, served as a first target for the gene knockout system developed in the course of the present study. This target was chosen because loss of function would be easily screenable, resulting in an S-layer glycosylation-deficient mutant. The gene knockout system constructed for insertional inactivation of the chromosomal *wsfP* gene of *P. alvei* CCM 2051^T is based on the commercially available bacterial mobile group II intron Ll.LtrB of *Lactococcus lactis*, in combination with further components available in our laboratory, including the broad-host-range S-layer gene promoter of *sgsE* from *G. stearothermophilus* NRS 2004/3a (22) and the *Geobacillus-Bacillus-Escherichia coli* shuttle vector pNW33N. Bacterial mobile group II introns are retroelements inserted into specific DNA target sites at high frequency by use of the retrohoming mechanism, by which the excised intron lariat RNA is inserted directly into a DNA target site and is then reverse transcribed by the associated intron-encoded enzyme protein (6, 8, 17). Since the DNA target site is recognized primarily by base pairing of intron RNA, which can be modified, and a few intron-encoded-enzyme-protein recognition positions, these introns can be inserted efficiently into any specific DNA target (9, 15, 35, 40).

The aim of this study is the development of a genetic tool for manipulation of S-layer protein glycosylation in *P. alvei* CCM 2051^T. For proof of concept, we specifically deal with (i) the construction of a broad-host-range gene knockout system based on the *L. lactis* Ll.LtrB intron; (ii) its modification for specific disruption of the *wsfP* gene on the *P. alvei* CCM 2051^T chromosome, encoding the putative initiation enzyme of S-layer glycan biosynthesis; and (iii) the reconstitution of enzyme activity by plasmid-based expression of *wsfP* and its predicted functional homologue *wsaP* from *G. stearothermophilus* NRS 2004/3a.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *P. alvei* CCM 2051^T (Table 1) was obtained from the Czech Collection of Microorganisms (CCM; Brno, Czech Republic) and was grown at 37°C and 200 rpm in Luria-Bertani (LB) broth or on LB agar plates supplemented with 10 μg/ml chloramphenicol (Cm), when appropriate. *G. stearothermophilus* NRS 2004/3a (Table 1) was obtained from F. Hollaus (19) and grown on modified S-VIII medium at 55°C (29). *Escherichia coli* DH5α (Invitrogen, Lofer, Austria) was grown in LB broth at 37°C supplemented with 30 μg/ml Cm, when appropriate.

General methods. Genomic DNA of *G. stearothermophilus* NRS 2004/3a and of *P. alvei* CCM 2051^T was isolated by using a Genomic Tip 100 kit (Qiagen, Vienna, Austria) according to the manufacturer's instructions, except that for the latter organism, cells were broken by repeated freezing and thawing cycles (10 times), because of its resistance toward lysozyme (27). Restriction enzymes, calf intestinal alkaline phosphatase, and T4 DNA ligase were purchased from Invitrogen. A MinElute gel extraction kit (Qiagen) was used to purify DNA fragments from agarose gels, and a MinElute reaction cleanup kit (Qiagen) was used to purify digested oligonucleotides and plasmids. Plasmid DNA from transformed cells was isolated with a QIAprep Spin Miniprep kit (Qiagen). Agarose gel electrophoresis was performed as described elsewhere (26). PCR (My Cycler; Bio-Rad, Hercules, CA) was performed using an Expand Long Range dNTPack (Roche, Vienna, Austria). PCR conditions were optimized for each primer pair (Table 2), and amplification products were purified using a MinElute PCR purification kit (Qiagen). Transformation of *E. coli* DH5α was done according to the manufacturer's protocol (Invitrogen). Transformants were screened by in situ PCR using RedTaq ReadyMix PCR mix (Sigma-Aldrich, Vienna, Austria); recombinant clones were analyzed by restriction mapping and confirmed by sequencing (Agowa, Berlin, Germany). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was carried out according to a standard protocol (16), using a Protean II electrophoresis apparatus (Bio-Rad). Protein bands were visualized with Coomassie brilliant blue G250 staining reagent. Periodic acid-Schiff (PAS) staining for carbohydrates was performed according to the method of Hart and coworkers (10).

TABLE 2. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant characteristic(s)	Source
Strains		
P. alvei CCM 2051^T	Wild-type isolate; Km^r	CCM
P. alvei CCM 2051 *wsfP*::Ll.LtrB	*P. alvei* CCM 2051^T carrying a targetron insertion at the *wsfP* locus; Km^r	This study
Escherichia coli DH5α	F$^-$ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)*U169 deoR recA1 endA1 hsdR17* (r_K^- m_K^-) *phoA supE44 thi-1 gyrA96 relA1* λ$^-$	Invitrogen
Plasmids		
pNW33N	*Geobacillus-Bacillus-E. coli* shuttle vector; Cm^r	*Bacillus* Genetic Stock Center
pEXALV	pNW33N carrying the *sgsE* S-layer gene promoter of *G. stearothermophilus* NRS 2004/3a; Cm^r	This study
pNW33NΔ*Hin*dIII	pNW33N without its unique HindIII restriction site; Cm^r	This study
pJIR750ai	*Clostridium perfringens-E. coli* shuttle vector carrying alpha-toxin gene (*plc*) targetron; Cm^r	Sigma-Aldrich
pJIR750ai_P(SgsE)	pJIR750ai carrying the *sgsE* S-layer gene promoter of *G. stearothermophilus* NRS 2004/3a; Cm^r	This study
pTT_*plc*	pNW33N without HindIII carrying the *sgsE* S-layer gene promoter of *G. stearothermophilus* NRS 2004/3a in front of the *plc* intron cassette of pJIR750ai; Cm^r	This study
pTT_*wsfP*555	pTT_*plc* targeted for insertion between positions 555 and 556 from the initial ATG codon of *wsfP*; Cm^r	This study
pTT_*wsfP*654	pTT_*plc* targeted for insertion between positions 654 and 655 from the initial ATG codon of *wsfP*; Cm^r	This study
pTT_*wsfP*1176	pTT_*plc* targeted for insertion between positions 1176 and 1177 from the initial ATG codon of *wsfP*; Cm^r	This study
pEXALV_*wsfP*	pEXALV carrying the *wsfP* gene of *P. alvei* CCM 2051^T; Cm^r	This study
pEXALV_*wsaP*	pEXALV carrying the *wsaP* gene of *G. stearothermophilus* NRS 2004/3a; Cm^r	This study

Transformation of *P. alvei* CCM 2051^T. Transformation of *P. alvei* CCM 2051^T followed the protocol of Turgeon et al. (36), with some modifications. Briefly, the organism was grown to an optical density at 600 nm (OD_{600}) of 0.2 to 0.3. Subsequently, the culture was washed five times with ice-cold electroporation buffer (250 mM sucrose-1 mM HEPES-1 mM $MgCl_2$-10% glycerol, pH 7.0), resuspended in 1/500 of a culture volume, and stored in 50-μl aliquots at −70°C. In transformation studies, plasmid DNA originating from the *E. coli-G. stearothermophilus-Bacillus subtilis* shuttle vector pNW33N (*Bacillus* Genetic Stock Center, Columbus, OH) (Table 1) was used, and electroporation was done at a capacitance of 25 μF, using a Gene Pulser II apparatus connected to a pulse controller (Bio-Rad). For optimization of electroporation conditions, voltage was varied between 5.0 and 20 kV/cm, and resistance was set to 100 Ω, 200 Ω, and 400 Ω. Five hundred nanograms of plasmid DNA was added to an aliquot of electrocompetent cells, and the mixture was transferred into a prechilled 1-mm electroporation cuvette (Bio-Rad). Immediately after application of the pulse, the cell suspension was diluted with 4 ml of prewarmed casein-peptone soymeal-peptone broth (Sigma-Aldrich), containing 250 mM sucrose, 5 mM $MgCl_2$, and 5 mM $MgSO_4$, and incubated for 2 h at 37°C, allowing expression of the antibiotic resistance marker. Finally, cells were spread on LB agar supplemented with Cm and incubated overnight at 37°C.

Construction of an expression vector for *P. alvei* CCM 2051^T. A ~400-bp DNA fragment containing the *sgsE* surface layer gene promoter of *G. stearothermophilus* NRS 2004/3a (22) was amplified from *G. stearothermophilus* NRS 2004/3a genomic DNA with primers P(SgsE)_*Hin*dIII_for and P(SgsE)_*Sph*I_rev, digested with HindIII and SphI, and ligated into HindIII/SphI-linearized and dephosphorylated pNW33N plasmid. The resulting plasmid was named pEXALV.

Construction of a *wsfP* gene knockout mutant. A schematic diagram for construction of a shuttle plasmid containing the *wsfP* targetron used to construct the *P. alvei* CCM 2051^T *wsfP* mutant is given in Fig. 1. Plasmids are listed in Table 2.

(i) Deletion of a HindIII restriction site from pNW33N. Plasmid pNW33N was digested with HindIII, and the 5′ overhangs were filled in to form blunt ends by a large (Klenow) fragment of DNA polymerase I. The modified plasmid was self-ligated and transformed into *E. coli* DH5α, and the loss of the unique HindIII restriction site was verified. The resulting plasmid was named pNW33NΔ*Hin*dIII (Fig. 1A).

(ii) Insertion of P(*sgsE*) in front of the intron cassette of pJIR750i. The *sgsE* promoter was amplified from genomic DNA of *G. stearothermophilus* NRS 2004/3a by PCR using primers P(SgsE)_*Sph*I_for and P(SgsE)_*Hin*dIII_rev. The resulting fragment was digested with SphI and HindIII, cloned into SphI/HindIII-linearized and dephosphorylated pJIR750ai plasmid, and transformed into *E. coli* DH5α. Thereby, the promoter region P(*cpb2*) of the β-2 toxin gene (*cpb2*) from *Clostridium perfringens* in front of the α-toxin gene (*plc*) targetron was replaced by the *sgsE* surface layer gene promoter P(SgsE) of *G. stearothermophilus* NRS 2004/3a. The resulting plasmid was named pJIR750ai_P(SgsE) (Fig. 1B).

(iii) Transfer of the promoter-intron cassette construct into pNW33NΔ*Hin*dIII. Purified plasmid DNA of pJIR750ai_P(SgsE) was used as a template for PCR with primers Targe_*Sph*I_for and Targe_*Sph*I_rev. The resulting ~3,900-bp fragment containing P(SgsE), the Ll.LtrA ORF, and the *plc* targetron was digested with SphI, cloned into SphI-linearized and dephosphorylated pNW33NΔ*Hin*dIII plasmid, and transformed into *E. coli* DH5α. The plasmid was named pTT_*plc* (Fig. 1C).

(iv) Modification of the intron cassette for targeting to the putative *wsfP* gene of *P. alvei* CCM 2051^T. The Ll.LtrB targetron was retargeted to be inserted into the putative *wsfP* gene of *P. alvei* CCM 2051^T by using a computer algorithm that identifies potential insertion sites and directly designs PCR primers for modifying the intron RNA to base pair with these sites (TargeTron; Sigma-Aldrich). For gene interruption and stable insertion, the insertion sites with the lowest E-values and, for this reason, with high intron insertion efficiency were used. There are three short sequence elements involved in the base pairing interaction between the DNA target site (IBS1, IBS2, and δ′) and intron RNA (EBS1, EBS2, and δ). Modifications of intron RNA sequences (EBS1, EBS2, and δ) to base pair with the *wsfP* target site sequences were introduced via PCR by primer-mediated mutation with the primer sets comprising P_555|556s-IBS, P_555|556s-EBS1d, P_555|556s-EBS2, and P_654|655s-IBS; P_654|655s-EBS1d, P_654|655s-EBS2, and P_1176|1177s-IBS; and P_1176|1177s-EBS1d and P_1176|1177s-EBS2. The amplified 353-bp fragment was subsequently digested with HindIII and BsrGI and ligated into pTT_*plc* vector digested with the same restriction enzymes (Fig. 1D). The three resulting vectors were named pTT_*wsfP*555, pTT_*wsfP*654, and pTT_*wsfP*1176.

(v) Creation of a *wsfP* gene knockout with the *wsfP* targetron. pTT_*wsfP*555, pTT_*wsfP*654, and pTT_*wsfP*1176 were electroporated into *P. alvei* CCM 2051^T, and the cell suspension was plated on LB supplemented with Cm. Integration of the intron was assayed by colony PCR, using primers KO_*wsfP*_control_for_1 and KO_*wsfP*_control_rev_1, which hybridize to flanking sequences of the insertion sites.

(vi) Confirmation of *wsfP* gene insertion. For proof of insertion of the intron at the correct position, the PCR product obtained from genomic DNA of *P. alvei* CCM 2051^T *wsfP*::Ll.LtrB upon use of the primer pair comprising KO_*wsfP*_control_for_1 and KO_*wsfP*_control_rev_1 was sequenced.

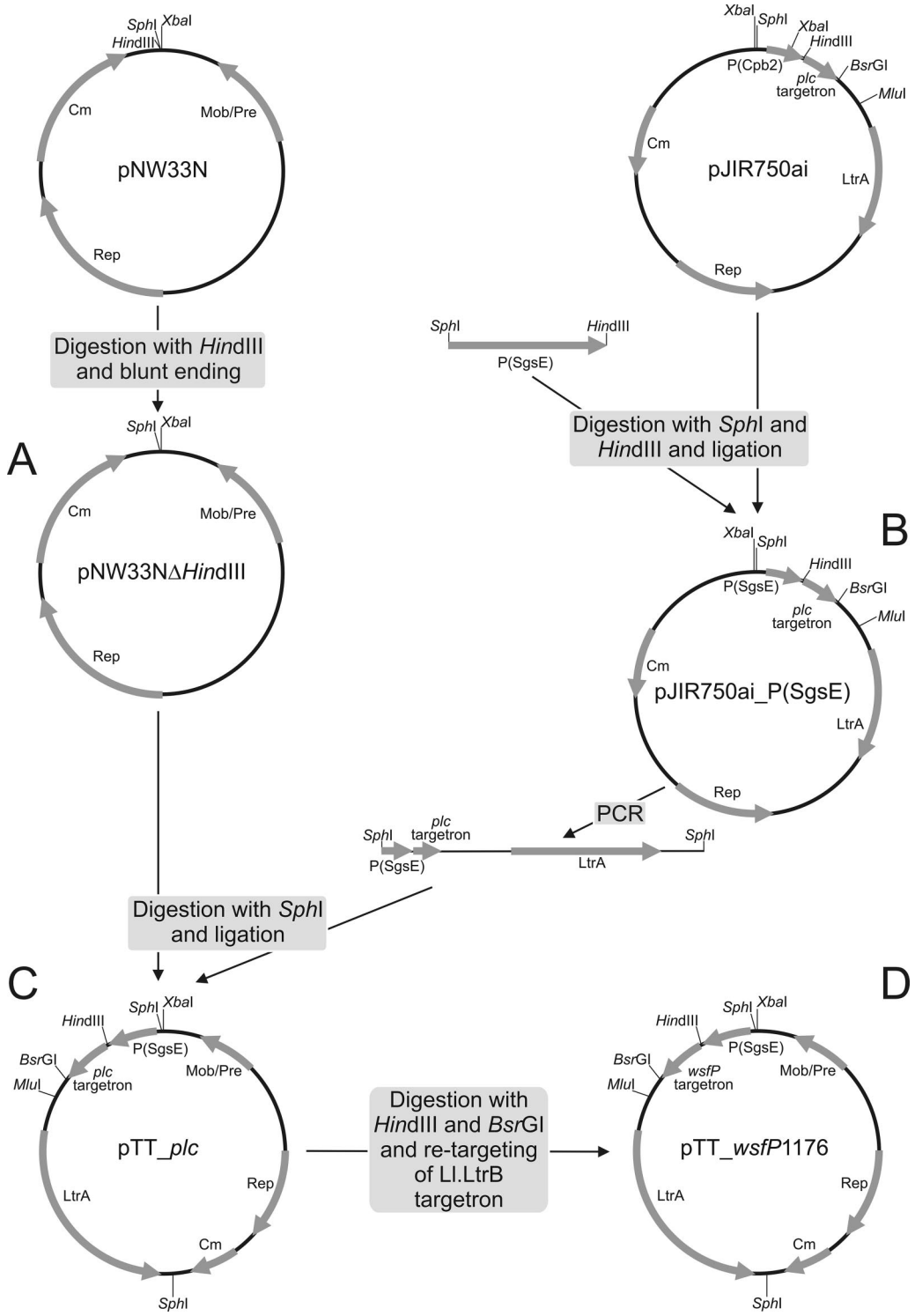

FIG. 1. Schematic drawing of the construction of the shuttle plasmid pTT_*wsfP*1176, containing the *wsfP* targetron.

Analysis of S-layer glycosylation in *P. alvei* CCM 2051^T wild-type cells and in *P. alvei* CCM 2051^T *wsfP*::LI.LtrB. The presence or absence of S-layer protein glycosylation on intact bacterial cells was monitored by SDS-PAGE followed by PAS staining (3) and by high-performance anion-exchange chromatography–pulsed electrochemical detection with a CarboPAc PA-1 column (Dionex, Sunnyvale, CA) after hydrolysis of crude S-layer preparations with trifluoroacetic acid (2, 30).

Reconstitution of enzyme activity in *P. alvei* CCM 2051^T *wsfP*::LI.LtrB by plasmid-based enzyme expression. The coding sequence of *wsfP* was amplified from genomic DNA of *P. alvei* CCM 2051^T by using primers *wsfP*_for_*Sph*I and *wsfP*_rev_*Kpn*I. The ~1,400-bp PCR product was digested with SphI and KpnI and ligated into SphI/KpnI-linearized and dephosphorylated pEXALV plasmid. This construct was named pEXALV_*wsfP*. Similarly, the coding sequence of *wsaP* from *G. stearothermophilus* NRS 2004/3a was cloned into

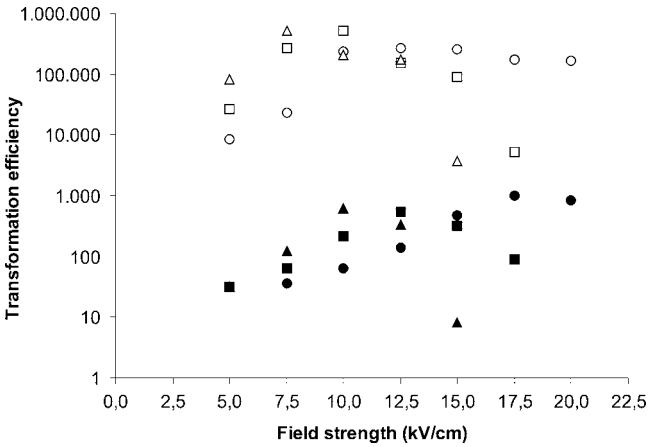

FIG. 2. Determination of optimal electroporation parameters for wild-type cells (●, ■, and ▲) and *wsfP* mutant cells (○, □, and △) of *P. alvei* CCM2051^T. The relationship between the numbers of transformants obtained per μg of DNA (pNW33N) and per 10^6 competent cells and the applied voltage is shown. Electroporation experiments were performed with cultures from the early growth phase (OD_{600}, ~0.2 to 0.3) at voltages ranging from 5 to 20 kV/cm and at resistance levels of 100 Ω (●/○), 200 Ω (■/□), or 400 Ω (▲/△).

pEXALV, using the primer pair comprising *wsaP*_for_*Sph*I and *wsaP*_rev_*Kpn*I and genomic DNA of *G. stearothermophilus* NRS 2004/3a as a template. The resulting construct was named pEXALV_*wsaP*. Each construct was transformed into *P. alvei* CCM 2051^T *wsfP*::Ll.LtrB, and reconstitution of UDP-Gal:phosphoryl-polyprenol Gal-1-phosphate transferase activity was analyzed. As a negative control, *P. alvei* CCM 2051^T transformants harboring pEXALV without *wsfP* were used.

RESULTS

Determination of optimal electroporation conditions for wild-type *P. alvei* CCM 2051^T cells. For transformation studies, *P. alvei* CCM 2051^T cells from the early logarithmic growth phase (OD_{600} of ~0.2 to 0.3) were used. From the different electroporation settings applied, an electric field at 100 Ω/25 μF/17.5 kV · cm^{-1} gave the best result; a transformation efficiency of 1×10^3 transformants per μg of plasmid DNA (pNW33N) and per 10^6 competent cells was obtained (Fig. 2).

Description of the putative initiation enzyme WsfP of S-layer glycan biosynthesis in *P. alvei* CCM 2051^T. On the basis of the structural identity of adaptor saccharide backbones in the S-layer glycans of *P. alvei* CCM 2051^T (18) and *G. stearothermophilus* NRS 2004/3a (29), where its biosynthesis is initiated by the UDP-Gal:phosphoryl-polyprenol Gal-1-phosphate transferase WsaP (33), it was conceivable that a homologous enzyme would initiate S-layer protein glycosylation in *P. alvei* CCM 2051^T. The putative initiation enzyme of SpaA glycosylation was chosen as a first target for the gene knockout system to be developed in the course of the present study, because the glycosylation-deficient phenotype resulting from its disruption would be easily screenable by SDS-PAGE and PAS staining. Disruption of the *wsfP* gene should result in the prevention of the initiation reaction and, thus, in the complete loss of S-layer glycans. For this purpose, the bacterial chromosome of *P. alvei* CCM 2051^T was searched for a putative S-layer glycosylation (*slg*) gene cluster as present in all other S-layer glycoprotein-carrying bacteria investigated so far (21). The chromosome walking strategy leading to the identification of the *slg* gene cluster of *P. alvei* CCM 2051^T will be published elsewhere (K. Zarschler, B. Janesch, P. Messner, and C. Schäffer, unpublished data). Specifically, an ORF of 1,407 bp encoding a putative UDP-Gal:phosphoryl-polyprenol Gal-1-phosphate transferase, named *wsfP* (for nomenclature, see reference 21), was identified. The ORF shows high similarity to WsaP (identity = 60%, similarity = 75%; GenBank accession number FJ751776). Typical of a member of the polyisoprenylphosphate hexose-1-phosphate transferase family, whose representatives transfer hexose-1-P residues from UDP-hexoses to a lipid carrier (33), the topological model of WsfP shows five transmembrane helices, a central loop facing the periplasmic space, and a highly conserved carboxy-terminal cytosolic tail containing the catalytic domain (25) (Fig. 3).

Implementation of the bacterial mobile group II intron Ll.LtrB for *wsfP* gene disruption in *P. alvei* CCM 2051^T. For *wsfP* gene disruption in *P. alvei* CCM 2051^T, a broad-host-range gene knockout system based on the *L. lactis* Ll.LtrB intron was constructed by following the strategy of Chen and coworkers (5). The *sgsE* S-layer gene promoter of *G. stearothermophilus* NRS 2004/3a, known to work also in *Bacillus subtilis* (22), was placed in front of the intron cassette of pJIR750ai, composed of the intron RNA and the ORF coding for the LtrA protein. This promoter-intron cassette construct was finally transferred into the *Geobacillus-Bacillus-E. coli* shuttle vector pNW33N to create a plasmid-borne Ll.LtrB mobile group II intron for gene disruption in *P. alvei* CCM 2051^T. Prior to the retargeting of the Ll.LtrB mobile group II intron for insertion into the putative *wsfP* gene on the *P. alvei* CCM2051^T chromosome, the gene was analyzed by a computer algorithm for identification of potential insertion sites. The algorithm predicted 11 intron insertion sites across the 1,407-bp *wsfP* gene. For gene interruption, the insertion sites with high intron insertion efficiency between positions 555 and 556 (E-value = 0.094), 654 and 655 (E-value = 0.010), and 1176 and 1177 (E-value = 0.202) were selected for intron modification (positions are given relative to the initial ATG codon; lower E-values correspond to higher predicted intron insertion efficiencies; target sites with E-values of <0.5 are predicted to be efficient introns). PCRs using primers designed by the algorithm for retargeting the intron by primer-mediated mutation were performed, and donor plasmids containing the *wsfP* targetrons were constructed. The plasmids containing the targetrons P555, P654, and P1176, named pTT_*wsfP*555, pTT_*wsfP*654, and pTT_*wsfP*1176, respectively, were transformed into *P. alvei* CCM 2051^T by electroporation. Analysis of 28 Cm-resistant *P. alvei* CCM 2051^T colonies for *wsfP* disruption showed that one colony transformed with pTT_*wsfP*1176 contained both wild-type (0.78-kb PCR product) and intron-inserted (1.68-kb PCR product) *wsfP* (Fig. 4). The rest of the colonies also contained the vector, which is the criterion for selection of colonies, but for unknown reasons, no intron insertion has occurred in the *wsfP* gene. The observation of getting both a wild-type gene and an intron-inserted gene by PCR screening of bacterial colonies was also described by Chen et al. (5) when screening for an α-toxin gene (*plc*) knockout in *Clostridium perfringens* ATCC 3624 by using a plasmid-borne Ll.LtrB mobile group II intron. Since intron RNA insertion occurs in some but not all of the progeny cells of a

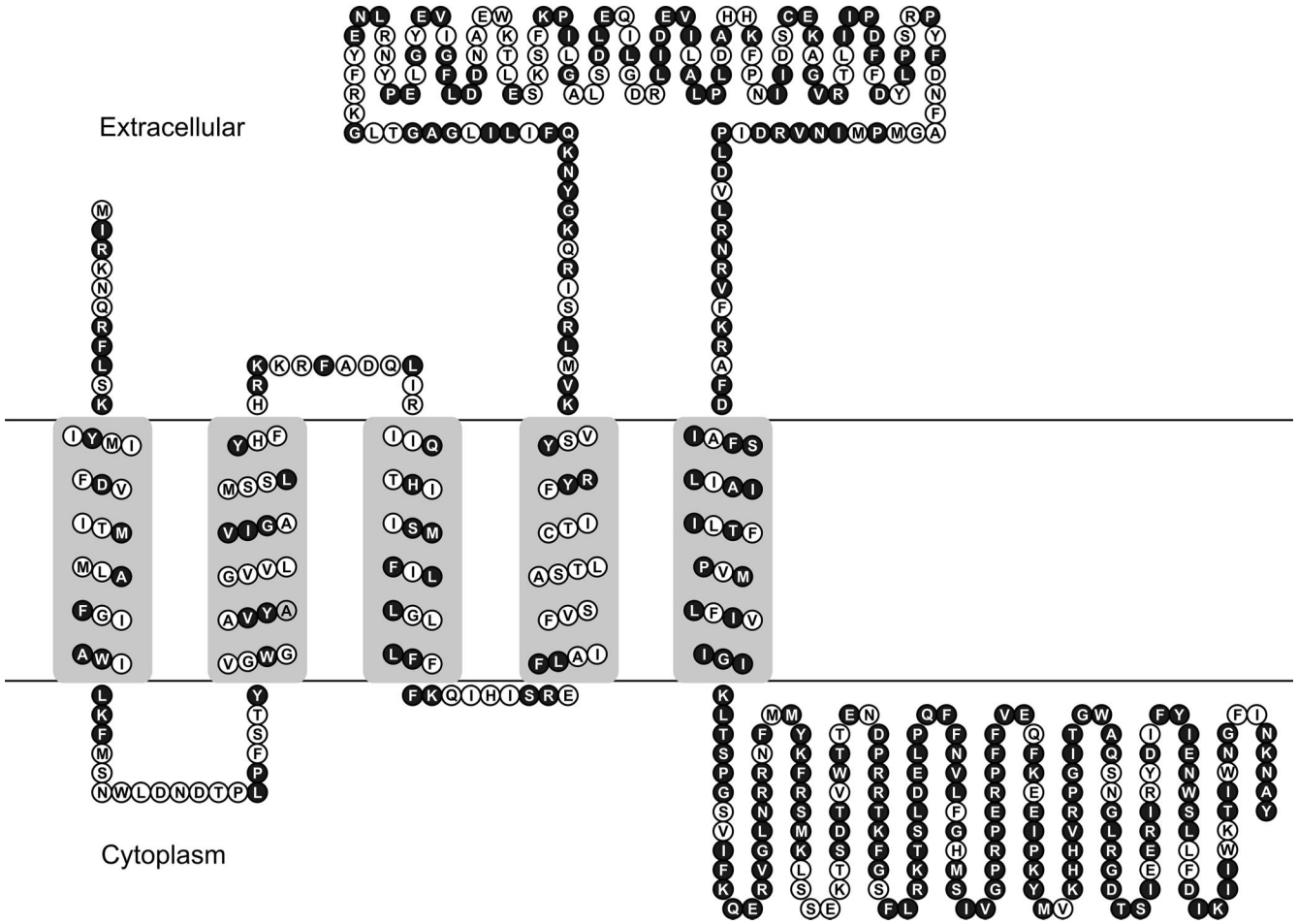

FIG. 3. Predicted topology of the WsfP protein of *P. alvei* CCM 2051^T. Shown are the five transmembrane helices (boxed), the central extracellular loop, and the carboxy-terminal cytosolic tail. Black amino acid residues are identical to corresponding amino acids in the functional WsaP homologue of *G. stearothermophilus* NRS 2004/3a.

single transformed bacterium, an isolated colony contains some cells with the intron-inserted gene and some cells with the wild-type gene. Therefore, bacteria from a colony containing both the wild-type gene and the intron-inserted gene were singularized on LB supplemented with Cm by streaking, and 74 colonies were screened again, using the same primer pair. This time, 36 colonies showed only the intron-inserted *wsfP* gene, 28 colonies only the wild-type *wsfP* gene, and 8 colonies both. A colony possessing only intron-inserted *wsfP*, named *P. alvei* CCM 2051^T *wsfP*::Ll.LtrB, was selected for further analysis.

Proof of functionality of the developed gene knockout system. To confirm the absence of the functional WsfP enzyme and, thus, the loss of S-layer glycoprotein glycan biosynthesis in *P. alvei* CCM 2051^T *wsfP*::Ll.LtrB, SDS-PAGE of intact cells accompanied by PAS staining and carbohydrate analysis of S-layer extract was performed. A clone showing exclusively the intron-inserted *wsfP* gene was cultivated in 5 ml LB medium supplemented with Cm, and an aliquot of biomass was loaded on an SDS-PAGE gel. As shown in Fig. 5, lanes 2 and 6, the *wsfP* mutant shows only a single S-layer protein band at ~105 kDa, representing nonglycosylated SpaA S-layer protein (the molecular mass estimated from the gel is in accordance with the molecular mass of 105.9 kDa, as calculated from the amino acid sequence), while the S-layer protein of *P. alvei* CCM 2051^T wild-type cells possessing an intact *wsfP* gene migrates in three distinct bands, with apparent molecular masses of ~240, ~160, and ~105 kDa, with the upper two bands representing different glycoforms of SpaA (Fig. 5, lanes 1 and 5). This experiment clearly demonstrated that WsfP is the initiation enzyme of SpaA S-layer protein glycosylation.

This finding is further supported by the comparative sugar composition analysis of the crude S-layer fraction derived from *P. alvei* CCM 2051^T wild-type cells and from the *wsfP* mutant (Fig. 6). The results have to be interpreted in the light of a secondary cell wall polymer (SCWP), composed of [(Pyr4,6)-β-D-Man*p*NAc-(1→4)-β-D-Glc*p*NAc-(1→3)]$_{n\sim11}$-(Pyr4,6)-β-D-Man*p*NAc-(1→4)-α-D-Glc*p*NAc-(1→*O*)-P→] repeats, which mediates attachment of the S-layer to the bacterial cell wall, being also contained in the samples (28). Consequently, the wild-type S-layer sample that possesses an overall degree of glycosylation of ~2.5% contains ManNAc-GlcNAc at an approximate molar ratio of 1:1, in addition to the S-layer glycan components Gal-ManNAc-Glc-Rha at an approximate molar ratio of 7:7:7:1. Quantification of sugars indicates an S-layer

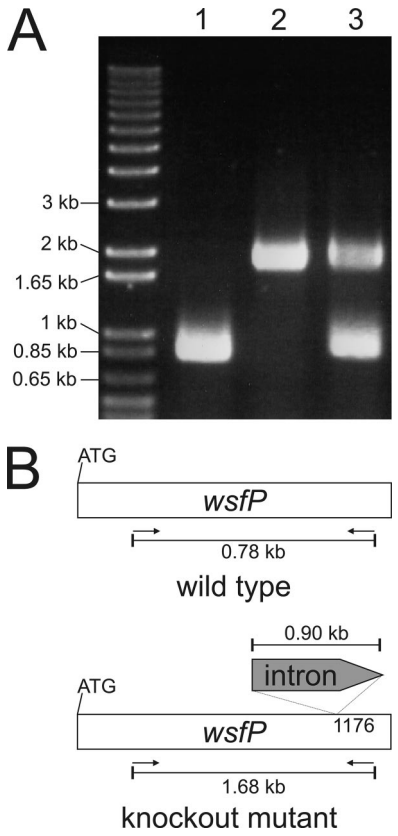

FIG. 4. Bacterial mobile group II intron-mediated gene disruption of *wsfP* in *P. alvei* CCM 2051^T. (A) Screening of Cm-resistant *P. alvei* CCM 2051^T colonies for intron insertion by in situ PCR using primers KO_*wsfP*_control_for_1 (→) and KO_*wsfP*_control_rev_1 (←). A PCR product obtained from a wild-type colony (lane 1), a PCR fragment obtained from a *wsfP* mutant (lane 2), and PCR products obtained from a colony containing both wild-type and intron-inserted *wsfP* (lane 3) are shown. (B) Schematic drawing of the *wsfP* gene with (bottom) and without (top) intron insertion, indicating the positions of primers KO_*wsfP*_control_for_1 (→) and KO_*wsfP*_control_rev_1 (←).

protein that carries, on average, two glycan chains of ~21 repeating units, with an SCWP of ~11 repeating being associated with the protein. The *wsfP* mutant, in contrast, is completely devoid of galactose and rhamnose, while the components of the SCWP can be clearly identified at the correct molar ratio. This comparative analysis clearly demonstrates that the developed gene knockout system is fully functional in abolishing S-layer protein SpaA glycosylation in *P. alvei* CCM 2051^{T}and serves as an additional proof of WsfP function. On the basis of the elucidated S-layer glycan structure (2, 18), it is conceivable that the nonstoichiometrically high glucose content detected in either analysis originates from an impurity present in the crude samples.

After subculturing of *P. alvei* CCM 2051^T *wsfP*::Ll.LtrB containing plasmid pTT_*wsfP*1176 without selective antibiotic for 10 days by replica plating, Cm-sensitive *wsfP* mutant clones lacking plasmid DNA but still showing *wsfP* gene disruption and the loss of S-layer glycans were isolated. The absence of the vector was confirmed by obtaining a negative PCR result using the primer pair comprising Cm_*Kpn*I_for and Cm_*Kpn*I_rev for amplifying the Cm resistance cassette (data not shown); *wsfP* disruption was verified by obtaining a PCR product of 1.68 kb, using the primer pair comprising KO_*wsfP*_control_for_1 and KO_*wsfP*_control_rev_1 (Fig. 4).

Reconstitution of S-layer glycan biosynthesis by plasmid-based expression of *wsfP* and *wsaP*. For the final proof of function of WsfP, reconstitution of S-layer glycosylation was analyzed. Transformation of pEXALV_*wsfP* into the Cm-sensitive *wsfP* mutant resulted in plasmid-based expression of the functional WsfP protein, as demonstrated by reconstitution of S-layer glycoprotein glycan biosynthesis (Fig. 5, lanes 3 and 7). Restoration of S-layer protein glycosylation was observed in *P. alvei* CCM2051^T *wsfP*::Ll.LtrB also after heterologous expression of WsaP from *G. stearothermophilus* NRS 2004/3a, expressed from pEXALV_*wsaP* (Fig. 5, lanes 4 and 8). However, in this experiment, glycosylation was obviously less efficient, with the nonglycosylated SpaA protein band appearing more intense and the glycoform band migrating at ~240 kDa appearing less intense on the gel than in the homologous expression approach. Nevertheless, these data confirm the initial assumption that WsfP and WsaP are functional homologues.

Electrocompetence of *P. alvei* CCM 2051^T *wsfP*::Ll.LtrB cells. Since there is speculation that glycosylation of surface proteins may affect the transformation efficiency of cells, *P. alvei* CCM 2051^T *wsfP*::Ll.LtrB cells were analyzed for their electrocompetence. Following the established procedure (see above), optimal electroporation conditions were determined for the *wsfP* mutant by using pNW33N plasmid DNA. A transformation efficiency up to 5×10^5 transformants per μg of

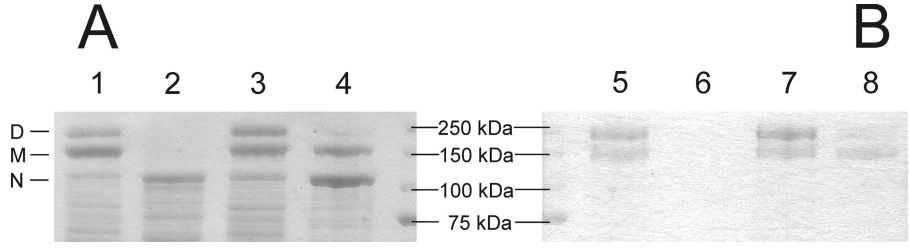

FIG. 5. SDS-PAGE gels showing the S-layer glycosylation profile of *P. alvei* CCM 2051^T wild-type cells (lanes 1 and 5), *wsfP* mutant cells (lanes 2 and 6), and *wsfP* mutant cells after reconstitution with WsfP (lanes 3 and 7) and WsaP (lanes 4 and 8) upon plasmid-based expression. Results are shown for Coomassie brilliant blue G250 staining (A) and PAS staining for carbohydrate (B). Nonglycosylated (N), monoglycosylated (M) and diglycosylated (D) S-layer SpaA proteins are indicated on the left. SDS-PAGE was performed using a 10% gel, and 10 μg and 20 μg of protein were loaded for Coomassie and PAS staining, respectively.

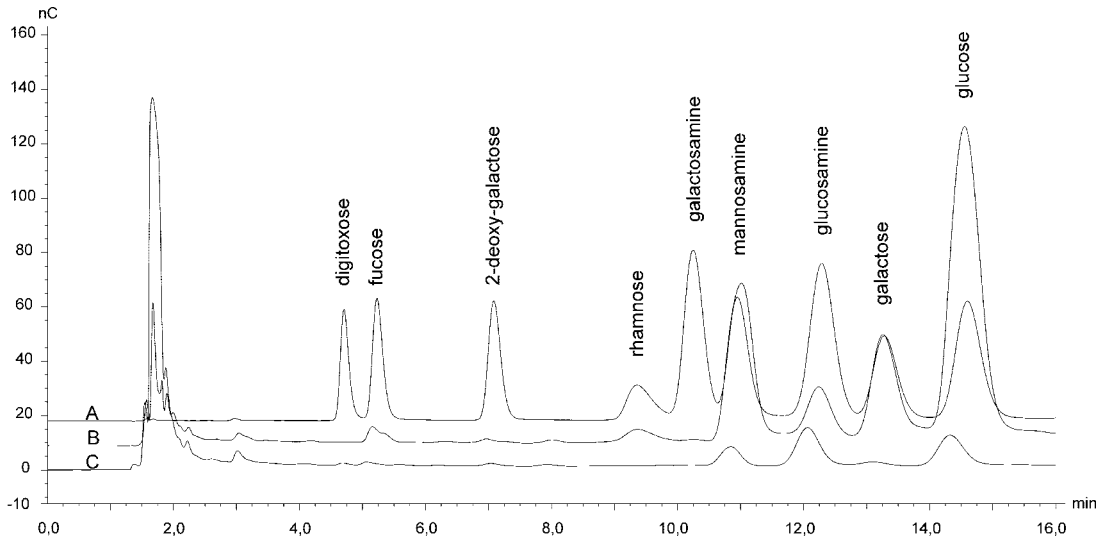

FIG. 6. Dionex carbohydrate analysis of S-layer extracts from *P. alvei* CCM 2051^T wild-type and *wsfP* mutant cells. (A) Standards (1 nmol each); (B) S-layer from wild-type cells (30 μg); (C) S-layer from *wsfP*::Ll.LtrB cells (175 μg).

plasmid DNA and per 10^6 competent cells was obtained by applying an electric field at 200 Ω/25 μF/10 kV · cm^{-1} or 400 Ω/25 μF/7.5 kV · cm^{-1} (Fig. 3). This corresponds to a factor of 500 for improvement of transformation efficiency for mutant cells versus wild-type cells of *P. alvei* CCM 2051^T.

DISCUSSION

Due to the lack of suitable tools for genetic manipulation of bacterial S-layer glycosylation pathways, progress in the elucidation of the glycan biosynthesis mechanism, which is a prerequisite for the desired production of functional S-layer *neo*-glycoproteins, was limited to in vitro testing of individual enzymes from these pathways (34) and to heterologous carbohydrate-engineering approaches in the past (32).

For the envisaged in vivo display of functional glycans via the S-layer anchor, in this work, a reliable and effective tool for the production of gene knockout mutants and for the expression of heterologous genes in the model organism *P. alvei* CCM 2051^T was developed. A targetron gene knockout system was constructed by cloning the Ll.LtrB group II intron, controlled by the *sgsE* surface layer gene promoter of *G. stearothermophilus* NRS 2004/3a, into the shuttle plasmid pNW33N; retargeting the intron; and producing insertional mutants after transformation of the plasmid into *P. alvei* CCM 2051^T. During the past 10 years, bacterial mobile group II introns have become a versatile instrument for site-specific chromosomal insertion in various prokaryotic species (5, 15, 38, 39). The requirements for adapting targetrons to specific needs are their sufficient expression via an inducible or constitutive promoter from a plasmid replicating in the host organism and the possibility of transferring this DNA into the desired host. From the adaptation of a targetron-based gene disruption system to *P. alvei* CCM 2051^T, an essential tool for elucidating molecular details about S-layer protein glycosylation has evolved. In this system, plasmid pNW33N and the *sgsE* S-layer gene promoter of *G. stearothermophilus* NRS 2004/3a are integral components. Since this plasmid replicates in thermophilic and mesophilic *Bacillaceae*, and the *sgsE* S-layer gene promoter drives gene expression in several thermophilic and mesophilic bacterial species (22), the system is likely to be applicable to different organisms within the radiation of *Bacillus* and related taxa.

For proof of functionality of targetron-mediated gene disruption in *P. alvei* CCM 2051^T, the *wsfP* gene was chosen. This gene codes for a putative UDP-Gal:phosphoryl-polyprenol Gal-1-phosphate transferase and shows high similarity to the gene coding for WsaP, the initiation enzyme of S-layer glycan biosynthesis in *G. stearothermophilus* NRS 2004/3a (33). Mutant cells of *P. alvei* CCM 2051^T carrying the Ll.ltrB intron in the chromosomal *wsfP* gene inserted between positions 1176 and 1177 from the initial ATG codon lost the ability to glycosylate their cognate S-layer protein SpaA. This effect was completely restored by the expression of plasmid-encoded WsfP. Heterologously expressed WsaP from *G. stearothermophilus* NRS 2004/3a also reconstituted the S-layer glycosylation process, albeit less efficiently, which might be due to the thermophilic origin of this initiation enzyme. By applying the constructed tool to the *wsfP* target, the first enzyme from the otherwise largely unknown S-layer glycan biosynthesis pathway of *P. alvei* CCM 2051^T (11) could be functionally characterized as initiating UDP-Gal:phosphoryl-polyprenol Gal-1-phosphate transferase.

In summary, in the course of the present study, an effective tool for gene disruption and heterologous gene expression in *P. alvei* CCM 2051^T was established, with *P. alvei* CCM 2051^T being the first gram-positive S-layer glycoprotein-carrying organism amenable to this kind of genetic engineering. The observation that *P. alvei* CCM 2051^T *wsfP*::Ll.LtrB cells show clearly improved transformation efficiency in comparison to wild-type cells, which may be due to spatial hindrance or charge repulsion effects between the DNA molecules and the S-layer glycan on the wild type, hampering the passage through the cell envelope, may have implications for the envisaged utilization of *P. alvei* CCM 2051^T as a means for surface display of functional, recombinant glycans. Thus, this work is opening

up new possibilities for the future design of functional glycans on S-layer proteins for in vivo and in vitro applications.

ACKNOWLEDGMENTS

We thank Andrea Scheberl for excellent technical assistance.

Financial support came from the Austrian Science Fund, project P20745-B11 (to P.M.) and projects P19047-B12 and P20605-12 (to C.S.), and the Hochschuljubiläumsstiftung der Stadt Wien, project H-02229-2007 (to K.Z.).

REFERENCES

1. **Abu-Qarn, M., J. Eichler, and N. Sharon.** 2008. Not just for Eukarya anymore: protein glycosylation in Bacteria and Archaea. Curr. Opin. Struct. Biol. **18:**544–550.
2. **Altman, E., J. R. Brisson, P. Messner, and U. B. Sleytr.** 1991. Structure of the glycan chain from the surface layer glycoprotein of *Bacillus alvei* CCM 2051. Biochem. Cell Biol. **69:**72–78.
3. **Altman, E., C. Schäffer, J.-R. Brisson, and P. Messner.** 1995. Characterization of the glycan structure of a major glycopeptide from the surface layer glycoprotein of *Clostridium thermosaccharolyticum* E207-71. Eur. J. Biochem. **229:**308–315.
4. **Ash, C., F. G. Priest, and M. D. Collins.** 1993. Molecular identification of rRNA group 3 bacilli (Ash, Farrow, Wallbanks and Collins) using a PCR probe test. Proposal for the creation of a new genus *Paenibacillus.* Antonie van Leeuwenhoek **64:**253–260.
5. **Chen, Y., B. A. McClane, D. J. Fisher, J. I. Rood, and P. Gupta.** 2005. Construction of an α toxin gene knockout mutant of *Clostridium perfringens* type A by use of a mobile group II intron. Appl. Environ. Microbiol. **71:** 7542–7547.
6. **Cousineau, B., D. Smith, S. Lawrence-Cavanagh, J. E. Mueller, J. Yang, D. Mills, D. Manias, G. Dunny, A. M. Lambowitz, and M. Belfort.** 1998. Retrohoming of a bacterial group II intron: mobility via complete reverse splicing, independent of homologous DNA recombination. Cell **94:**451–462.
7. **Desvaux, M., E. Dumas, I. Chafsey, and M. Hébraud.** 2006. Protein cell surface display in Gram-positive bacteria: from single protein to macromolecular protein structure. FEMS Microbiol. Lett. **256:**1–15.
8. **Eskes, R., J. Yang, A. M. Lambowitz, and P. S. Perlman.** 1997. Mobility of yeast mitochondrial group II introns: engineering a new site specificity and retrohoming via full reverse splicing. Cell **88:**865–874.
9. **Guo, H., M. Karberg, M. Long, J. P. Jones III, B. Sullenger, and A. M. Lambowitz.** 2000. Group II introns designed to insert into therapeutically relevant DNA target sites in human cells. Science **289:**452–457.
10. **Hart, C., B. Schulenberg, T. H. Steinberg, W. Y. Leung, and W. F. Patton.** 2003. Detection of glycoproteins in polyacrylamide gels and on electroblots using Pro-Q Emerald 488 dye, a fluorescent periodate Schiff-base stain. Electrophoresis **24:**588–598.
11. **Hartmann, E., P. Messner, G. Allmeier, and H. König.** 1993. Proposed pathway for biosynthesis of the S-layer glycoprotein of *Bacillus alvei*. J. Bacteriol. **175:**4515–4519.
12. **Honma, K., H. K. Kuramitsu, R. J. Genco, and A. Sharma.** 2001. Development of a gene inactivation system for *Bacteroides forsythus*: construction and characterization of a BspA mutant. Infect. Immun. **69:**4686–4690.
13. **Imanaka, T., M. Fujii, I. Aramori, and S. Aiba.** 1982. Transformation of *Bacillus stearothermophilus* with plasmid DNA and characterization of shuttle vector plasmids between *Bacillus stearothermophilus* and *Bacillus subtilis*. J. Bacteriol. **149:**824–830.
14. **Isono, K.** 1970. Transformation of amylase producing ability in *Bacillus stearothermophilus*. Jpn. J. Genet. **45:**285–291.
15. **Karberg, M., H. Guo, J. Zhong, R. Coon, J. Perutka, and A. M. Lambowitz.** 2001. Group II introns as controllable gene targeting vectors for genetic manipulation of bacteria. Nat. Biotechnol. **19:**1162–1167.
16. **Laemmli, U. K.** 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature **227:**680–685.
17. **Lambowitz, A. M., and S. Zimmerly.** 2004. Mobile group II introns. Annu. Rev. Genet. **38:**1–35.
18. **Messner, P., R. Christian, C. Neuninger, and G. Schulz.** 1995. Similarity of "core" structures in two different glycans of tyrosine-linked eubacterial S-layer glycoproteins. J. Bacteriol. **177:**2188–2193.
19. **Messner, P., F. Hollaus, and U. B. Sleytr.** 1984. Paracrystalline cell wall surface layers of different *Bacillus stearothermophilus* strains. Int. J. Syst. Bacteriol. **34:**202–210.
20. **Messner, P., D. Pum, and U. B. Sleytr.** 1986. Characterization of the ultrastructure and the self-assembly of the surface layer of *Bacillus stearothermophilus* strain NRS 2004/3a. J. Ultrastruct. Mol. Struct. Res. **97:**73–88.
21. **Messner, P., K. Steiner, K. Zarschler, and C. Schäffer.** 2008. S-layer nanoglycobiology of bacteria. Carbohydr. Res. **343:**1934–1951.
22. **Novotny, R., H. Berger, T. Schinko, P. Messner, C. Schäffer, and J. Strauss.** 2008. A temperature-sensitive expression system based on the *Geobacillus stearothermophilus* NRS 2004/3a *sgsE* surface-layer gene promoter. Biotechnol. Appl. Biochem. **49:**35–40.
23. **Novotny, R., C. Schäffer, J. Strauss, and P. Messner.** 2004. S-layer glycan-specific loci on the chromosome of *Geobacillus stearothermophilus* NRS 2004/3a and dTDP-L-rhamnose biosynthesis potential of *G. stearothermophilus* strains. Microbiology **150:**953–965.
24. **Sakakibara, J., K. Nagano, Y. Murakami, N. Higuchi, H. Nakamura, K. Shimozato, and F. Yoshimura.** 2007. Loss of adherence ability to human gingival epithelial cells in S-layer protein-deficient mutants of *Tannerella forsythensis*. Microbiology **153:**866–876.
25. **Saldias, M. S., K. Patel, C. L. Marolda, M. Bittner, I. Contreras, and M. A. Valvano.** 2008. Distinct functional domains of the *Salmonella enterica* WbaP transferase that is involved in the initiation reaction for synthesis of the O antigen subunit. Microbiology **154:**440–453.
26. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
27. **Sára, M., K. Moser-Thier, U. Kainz, and U. B. Sleytr.** 1990. Characterization of S-layers from mesophilic *Bacillaceae* and studies on their protective role towards muramidases. Arch. Microbiol. **153:**209–214.
28. **Schäffer, C., N. Müller, P. K. Mandal, R. Christian, S. Zayni, and P. Messner.** 2000. A pyrophosphate bridge links the pyruvate-containing secondary cell wall polymer of *Paenibacillus alvei* CCM 2051 to muramic acid. Glycoconj. J. **17:**681–690.
29. **Schäffer, C., T. Wugeditsch, H. Kählig, A. Scheberl, S. Zayni, and P. Messner.** 2002. The surface layer (S-layer) glycoprotein of *Geobacillus stearothermophilus* NRS 2004/3a. Analysis of its glycosylation. J. Biol. Chem. **277:** 6230–6239.
30. **Schäffer, C., T. Wugeditsch, C. Neuninger, and P. Messner.** 1996. Are S-layer glycoproteins and lipopolysaccharides related? Microb. Drug Resist. **2:**17–23.
31. **Sleytr, U. B., C. Huber, N. Ilk, D. Pum, B. Schuster, and E. M. Egelseer.** 2007. S-layers as a tool kit for nanobiotechnological applications. FEMS Microbiol. Lett. **267:**131–144.
32. **Steiner, K., A. Hanreich, B. Kainz, P. G. Hitchen, A. Dell, P. Messner, and C. Schäffer.** 2008. Recombinant glycans on an S-layer self-assembly protein: a new dimension for nanopatterned biomaterials. Small **4:**1728–1740.
33. **Steiner, K., R. Novotny, K. Patel, E. Vinogradov, C. Whitfield, M. A. Valvano, P. Messner, and C. Schäffer.** 2007. Functional characterization of the initiation enzyme of S-layer glycoprotein glycan biosynthesis in *Geobacillus stearothermophilus* NRS 2004/3a. J. Bacteriol. **189:**2590–2598.
34. **Steiner, K., R. Novotny, D. B. Werz, K. Zarschler, P. H. Seeberger, A. Hofinger, P. Kosma, C. Schäffer, and P. Messner.** 2008. Molecular basis of S-layer glycoprotein glycan biosynthesis in *Geobacillus stearothermophilus*. J. Biol. Chem. **283:**21120–21133.
35. **Toro, N., J. I. Jimenez-Zurdo, and F. M. Garcia-Rodriguez.** 2007. Bacterial group II introns: not just splicing. FEMS Microbiol. Rev. **31:**342–358.
36. **Turgeon, N., C. Laflamme, J. Ho, and C. Duchaine.** 2006. Elaboration of an electroporation protocol for *Bacillus cereus* ATCC 14579. J. Microbiol. Methods **67:**543–548.
37. **Welker, N. E.** 1993. The genetic map of *Bacillus stearothermophilus* NUB36, p. 483–487. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology, and molecular genetics. American Society for Microbiology, Washington, DC.
38. **Yao, J., and A. M. Lambowitz.** 2007. Gene targeting in gram-negative bacteria by use of a mobile group II intron ("targetron") expressed from a broad-host-range vector. Appl. Environ. Microbiol. **73:**2735–2743.
39. **Yao, J., J. Zhong, Y. Fang, E. Geisinger, R. P. Novick, and A. M. Lambowitz.** 2006. Use of targetrons to disrupt essential and nonessential genes in *Staphylococcus aureus* reveals temperature sensitivity of Ll.LtrB group II intron splicing. RNA **12:**1271–1281.
40. **Zhong, J., M. Karberg, and A. M. Lambowitz.** 2003. Targeted and random bacterial gene disruption using a group II intron (targetron) vector containing a retrotransposition-activated selectable marker. Nucleic Acids Res. **31:** 1656–1664.