

Facilitation of Direct Conditional Knockout of Essential Genes in *Bacillus licheniformis* DSM13 by Comparative Genetic Analysis and Manipulation of Genetic Competence^{∇†}

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The genetic manageability of the biotechnologically important *Bacillus licheniformis* is hampered due to its poor transformability, whereas *Bacillus subtilis* efficiently takes up DNA during genetic competence, a quorum-sensing-dependent process. Since the sensor histidine kinase ComP, encoded by a gene of the quorum-sensing module *comQXPA* of *B. licheniformis* DSM13, was found to be inactive due to an insertion element within *comP*, the coding region was exchanged with a functional copy. Quorum sensing was restored, but the already-poor genetic competence dropped further. The inducible expression of the key regulator for the transcription of competence genes, ComK, in *trans* resulted in highly competent strains and facilitated the direct disruption of genes, as well as the conditional knockout of an essential operon. As ComK is inhibited at low cell densities by a proteolytic complex in which MecA binds ComK and such inhibition is antagonized by the interaction of MecA with ComS (the expression of the latter is controlled by cell density in *B. subtilis*), we performed an *in silico* analysis of MecA and the hitherto unidentified ComS, which revealed differences for competent and noncompetent strains, indicating that the reduced competence possibly is due to a nonfunctional coupling of the *comQXPA*-encoded quorum module and ComK. The obtained increased genetic tractability of this industrial workhorse should improve a wide array of scientific investigations.

Strains of the endospore-forming Gram-positive soil bacterium *Bacillus licheniformis* (a generally-regarded-as-safe [GRAS] species) serve as microbial workhorses, as they produce a number of valuable compounds (10), and they possess a high capacity for the secretion of exoenzymes, such as amylases and proteases, with yields of up to 25 g liter⁻¹ (46). The availability of the genome sequences of strain *B. licheniformis* DSM13 (61) and the isogenic ATCC 14580 (44) already facilitated a number of developments aiming at strain improvement and the enhancement of biosafety (38, 63, 64).

However, the low frequency of transformation regularly observed with those strains is a drawback. Although a derivative (*B. licheniformis* MW3) was obtained by the deletion of the genes encoding type I restriction enzymes, allowing the routine creation of transformants by protoplast transformation, it still has a rather low transformation efficiency (65). Hence, the generation of mutants by homologous recombination is a rather time-consuming and cumbersome process, as is the case for other members of the genus (60).

During the part of their life cycle when they develop natural genetic competence, representatives of the genus *Bacillus* gen-

erally are capable of taking up exogenously supplied DNA. Such a DNA uptake mechanism was first described for *B. subtilis* (49) and frequently has been used to obtain transformants and generate deletions and conditional mutants with inducible gene expression (58). The establishment of such a system in *B. licheniformis* DSM13 would considerably improve genetic handling in this industrial workhorse.

The key regulator responsible for the development of genetic competence is ComK, controlling the transcription of all genes involved in DNA binding, processing, uptake, and homologous recombination between the incoming and the host DNA (16, 20). ComK expression is turned down during exponential growth, and a number of stimuli must be integrated to control *comK* expression (Fig. 1) (14, 18, 23, 40). In *B. subtilis* 168 (27) and *Bacillus amyloliquefaciens* FZB42 (7), the quorum-sensing-dependent escape from ComK proteolysis depends on a regulatory operon containing four genes, *comQ*, *comX*, *comP*, and *comA* (20). ComQ is responsible for processing pre-ComX to generate the active ComX peptide pheromone (1). The accumulation of extracellular ComX is sensed by sensor histidine kinase ComP, which is capable of phosphorylating the cognate response regulator ComA. Phosphorylated ComA (ComA~P) induces the transcription of the *srf* operon, which codes for the biosynthetic pathway of the biotenside surfactin, and which includes the 47 amino acids spanning ComS peptide (34). ComS prevents the proteolysis of ComK by competitively binding to MecA, thereby blocking the proteolytic MecA/ClpCP complex (13, 17, 31, 43, 57). Furthermore, ComA~P also facilitates the transcription of *degQ* by

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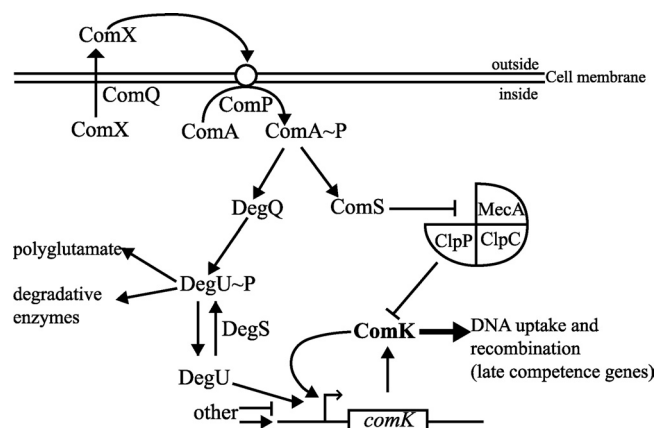


FIG. 1. Regulation of *comK* expression (only proteins and loci addressed in this work are mentioned). ComK activates the transcription of its own gene, forming a positive autoregulatory loop (30, 47). The DegS/DegU two-component system influences competence development in a way that dephosphorylated DegU facilitates the binding of ComK to its cognate promoter, thus leading to the onset of the autoregulatory loop; phosphorylated DegU promotes the synthesis of extracellular enzymes and polyglutamate (19) for biofilm formation. ComK levels are reduced posttranscriptionally at low cell density when MecaA recruits ComK to the proteolytic MecaA-ClpCP complex, making competence development quorum-sensing dependent (for details, see the text). Arrows and T bars indicate positive and negative regulation, respectively. ComX, peptide pheromone; ComQ, modification and secretion of ComX; ComP, sensor histidine kinase; ComA, response regulator; ComS, inhibition of MecaA/ClpCP; DegQ, facilitator of the phosphorylation of DegU; DegU, response regulator; DegS, sensor histidine kinase; MecaA, adaptor-protein; ClpCP, protease; ComK, main transcriptional activator of natural competence.

promoting the phosphorylation of DegQ and increases the amount of DegU~P, which facilitates the synthesis of extracellular enzymes and polyglutamate production (Fig. 1) (8, 35, 39, 50).

However, *B. licheniformis* ATCC 14580 and the isogenic DSM13 carry an insertion element within the *comP* locus. Hence, poor genetic competence in *B. licheniformis* DSM13 may be due to the lack of a functional ComP. Since natural genetic competence has been reported to occur in *B. licheniformis* 9945A (32, 54), it was considered that understanding the lack of natural genetic competence in strain DSM13 and derivatives could lead to the development of competent strains and thus make efficient genetic tools available.

MATERIALS AND METHODS

Bioinformatic sequence analysis. Sequence alignments were done with CLUSTALW (53). Visual alignments of GC contents were performed with Artemis (5).

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. Cultivation was done at 37°C in Luria-Bertani (LB) broth unless otherwise stated. Plasmid-carrying *Escherichia coli* strains were grown with ampicillin (100 µg/ml), and *Bacillus* transformants were grown with erythromycin (0.3 or 5 µg/ml) or tetracycline (12.5 to 37.5 µg/ml); the respective higher concentrations were applied for freely replicating plasmids (multicopy), and the lower concentrations were used for the selection of strains carrying an integrated plasmid (single copy).

Molecular biological techniques. Cloning in *E. coli* was performed essentially as described previously (45). Genomic DNA from *B. licheniformis* strains was isolated as previously described (9) or by using a commercial kit (MasterPure Gram-positive DNA purification kit; Epicentre Biotechnologies, Madison, WI). PCR and Southern blot analysis (48) were performed as previously described

(37). Primers were deduced on the basis of the published *B. licheniformis* DSM13 (isogenic to ATCC 14580) genome sequence (44, 61) and are listed in Table S1 in the supplemental material. PCR mixtures (100 µl) consisted of 200 µM deoxynucleotides, 100 ng of template DNA, 1 pmol of each primer, and 1 U of *Taq* DNA polymerase or *Vent* DNA polymerase (New England Biolabs, Frankfurt, Germany). The purification of amplified fragments after gel electrophoresis was performed with a Qiaquick gel extraction kit (Qiagen, Hilden, Germany). Sequencing was done with fluorescence-labeled dideoxynucleotides of the Big-Dye Terminator v3.1 sequencing kit (Applied Biosystems, CA) and an ABI Prism capillary sequencer (model 3730). Sequences of *B. licheniformis* 9945A are part of an ongoing genome-sequencing project produced by the 454 technology (Roche, Brandford, CT). All fragments have been sequenced to a quality of phred45 or better.

Vector construction and gene deletion. Vectors and plasmids used in this study are compiled in Table 1. For the deletion of the insertion sequence (IS) element in *comP* in *B. licheniformis* MW3, a fragment of an intact *comP* of *B. licheniformis* strain F11 was amplified by PCR with the primer pair comQXPA3/comQXPA4 and cloned into pE007, resulting in vector pcomP. For obtaining a stable uracil auxotrophic mutant, a small deletion of 129 bp comprising the α -D-5-P-ribose-pyrophosphate (PRPP) binding site of the *pyrE* gene product, which is responsible for the conversion of orotate and phosphoribosylpyrophosphate to orotidin-5-phosphate and PPi, was used. Respective recombination flanks were amplified by PCR; flank A was created with the primer pair BalipyrE1/BaliPyrESOE1, and flank B was obtained by using the primer pair BalipyrE2/BaliPyrESOE2 (see Table S1 and Fig. S2 in the supplemental material). The deletion cassette was constructed using splicing by overlap extensions (SOE) (22) and cloned into pUCBM20. The deletion cassette was excised from the pUCBM20 derivative by PstI restriction and cloned into the PstI-linearized pSKE194. XbaI restriction removed the *E. coli* part of the vector, and the remaining *Bacillus* part comprising the temperature-sensitive origin of replication, erythromycin resistance, and *pyrE* deletion cassette was religated; the resulting plasmid was termed p Δ pyrE. For deletion, the vector p Δ pga flank A was amplified with the primer pair pgaFLAfl/pgaFLAr, flank B was constructed by using the primer pair pgaFIBfl/pgaFIBr, and these were successively ligated into pUCBM20. Further cloning was the same as that for p Δ pyrE. In PCRs for identifying mutants and for mutant screening, chromosomal DNA and the primers comQXPA1/comQXPA2 specific for the *comXQPA* operon, the primers hsdR1a/hsdR1b specific for *hsdR1*, the primers hsdR2c/hsdR2d specific for *hsdR2*, the primers pga3/pgas20 specific for the *pga* region, and the primers pyrEscreenfor/pyrEscreenres specific for the *pyrE* region were used. ComK expression vectors were cloned by amplifying *comK* of *B. licheniformis* MW3 using the primer pair comKf_BsrGI/comKr_SphI and cutting with BsrGI and SphI, followed by cloning into the likewise-cut pMM1522 (Mobitec, Göttingen, Germany), a xylose-inducible expression vector (60). The ComK expression vector was designated pMMcomK.

For the direct disruption of the *uvrBA* genes, the suicide vector pUCuvrBAEm was constructed by amplifying the puc21uvrBABali2 vector (63) with the primer pair uvrABali1/uvrBBali2 and the subsequent ligation of the erythromycin resistance gene with the uvrABali1/uvrBBali2 amplicon. The erythromycin resistance gene fragment was obtained by the PciI/ClaI restriction of pSKE194 by filling in the overlaps with Klenow polymerase (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions. The pMutpgk vector for the conditional inactivation of the *pgk* gene was constructed by the NotI/BamHI restriction of pMutin2 (58) and ligation with the NotI/BamHI-restricted pgkforNotI/pgkrevBamHI amplicon. The plasmids (pcomP, p Δ pga, p Δ pyrE, and pMMcomK) were transformed into *Bacillus subtilis* DB104 by the polyethylene glycol (PEG)-mediated transformation of protoplasts essentially as previously described (6). After reiso-lation, these were introduced into *B. licheniformis* MW3, and a PCR screening for deletion mutants was conducted as previously described (65). Mutants obtained are compiled in Table 1. Suicide vectors pUCuvrBAEm and pMutpgk were introduced into *B. licheniformis* MW3.1 carrying pMMcomK by transformation via induced competence.

Transformation with natural and induced competence. A two-step transformation protocol was performed essentially as described in reference 21. Cells were grown overnight on LB agar plates, and single colonies were inoculated into 3 ml HS medium, which contained 5 ml S base [2% (NH₄)₂SO₄, 14% K₂HPO₄, 6% KH₂PO₄, 1% Na₃ citrate × 2H₂O, 0.2% MgSO₄ × 7H₂O (wt/vol)], 0.5 ml yeast extract (10% [wt/vol]), 0.5 ml Casamino Acids (2% [wt/vol]), 2 ml uracil solution (2 mg/ml), 5 ml arginin-histidine solution (8 and 0.4% [wt/vol], respectively), and 1.25 ml glucose (20% [wt/vol]). After overnight incubation at 37°C with vigorous shaking, 20 ml of prewarmed LS medium, containing 5 ml 10× S

TABLE 1. Bacterial strains and plasmids^a

Strain or plasmid	Description	Source or reference
Strains		
<i>Escherichia coli</i> DH5 α F'	<i>endA1 hsdR17</i> (r _K ⁻ , m _K ⁺) <i>supE44 thi-1 gyrA96 relA1</i> Δ (<i>lacIZYA-argF</i>)U169 <i>deoR</i> F' Φ 80d <i>lacZ</i> Δ M15)	67
<i>Bacillus subtilis</i> DB104	<i>his npr2 nprE18 apr</i> Δ 3	24
<i>Bacillus licheniformis</i> DSM13	Wild type	DSMZ
<i>Bacillus licheniformis</i> DSM13 Δ spoIV	Sporulation-deficient DSM13 derivative	S. Wemhoff, this laboratory
<i>Bacillus licheniformis</i> MW3	Δ <i>hsdR1</i> , Δ <i>hsdR2</i>	65
<i>Bacillus licheniformis</i> MW3.1	Δ <i>hsdR1</i> , Δ <i>hsdR2</i> , Δ <i>pyrE</i>	This work
<i>Bacillus licheniformis</i> MW4.1	Δ <i>hsdR1</i> , Δ <i>hsdR2</i> , Δ <i>pyrE</i> , Δ <i>ppa</i>	This work
<i>Bacillus licheniformis</i> MK1.1	Δ <i>hsdR1</i> , Δ <i>hsdR2</i> , <i>comP</i> active, Δ <i>pyrE</i>	This work
<i>Bacillus licheniformis</i> MK2.1	Δ <i>hsdR1</i> , Δ <i>hsdR2</i> , <i>comP</i> active, Δ <i>pyrE</i> , Δ <i>ppa</i>	This work
<i>Bacillus licheniformis</i> F11	Wild type (Δ <i>chiA</i>)	62
Plasmids		
pUCBM20	<i>E. coli</i> cloning vector; Ap ^r	Boehringer Mannheim, Germany
pSKE194	<i>E. coli</i> / <i>Bacillus</i> shuttle vector, Ap ^r , Em ^r , ori _{<i>E. coli</i>} /ori ^{ts}	38
pE007	pSKE194 derivative deprived of its <i>E. coli</i> fraction by XbaI restriction and religation of the <i>Bacillus</i> part and with mcs of pUCBM20; Em ^r , ori ^{ts}	C. Borgmeier, this laboratory
pcomP	<i>Bacillus</i> vector Em ^r with comQXPA3/comQXPA4 amplicon of <i>Bacillus licheniformis</i> F11; Em ^r , ori ^{ts}	This work
p Δ pyrE	pSKE194 derivative with deleted copy for <i>Bacillus licheniformis pyrE</i> ; Em ^r , ori ^{ts}	This work
p Δ ppa	pSKE194 derivative with deleted copy for <i>Bacillus licheniformis MW3</i> polyglutamate-operon; Em ^r , ori ^{ts}	This work
pMM1522	Xylose-inducible expression vector for <i>Bacillus</i>	60
puc21uvrBABali2	pUCBM20 derivative with deletion flanks for <i>uvrBA</i>	63
pUCuvrBAEm	pUCuvrBA derivative with pSKE194 erythromycin resistance cloned between the deletion flanks	This work
pMutpgk	pMutin2 derivative with <i>pgk</i> insertion flank	This work
pMMcomK	pMM1522 derivative, <i>Bacillus licheniformis</i> MW3 ComK expression vector	This work

^a Ap^r, ampicillin resistance; Em^r, erythromycin resistance; ori^{ts}, temperature-sensitive *Bacillus* origin of replication; mcs, multiple cloning site; DSMZ, deutsche Sammlung für Mikroorganismen und Zellkulturen.

base, 0.5 ml yeast extract (10% [wt/vol]), 0.25 ml Casamino Acids (2% [wt/vol]), 2 ml uracil (2 mg/ml), 1.25 ml glucose (20% [wt/vol]), 0.125 ml 1 M MgCl₂ was inoculated with 1 ml of the starter culture and cultivated at 37°C with vigorous shaking. Upon reaching an optical density at 546 nm (OD₅₄₆) of 0.9 to 1 or at cultivation times of 1, 2, 3, or 4 h, 1 ml of competent cells was transferred to an Eppendorf cup containing 1 μ g chromosomal DSM13 Δ spoIV DNA for transformation and incubated for 2 to 4 h in a Thermomixer comfort (Eppendorf, Hamburg, Germany) at 37°C and 600 rpm. Cells were harvested (2 min at 16,000 \times g) and washed three times with 1 ml of 15 mM NaCl to remove residual uracil and plated out on M9 minimal medium (45) supplemented with 0.1 mM CaCl₂, 0.01% (wt/vol) yeast extract, 0.02% (wt/vol) Casamino Acids, and 0.2% (wt/vol) glucose as the carbon source without uracil to select for prototrophs.

The one-step transformation protocol was carried out as described previously (3). Cells were grown to the late stationary phase in competence medium. Transformation with chromosomal DSM13 Δ spoIV-DNA was performed as described for *B. subtilis* 2, 3, and 4 h after the onset of stationary growth.

Induced competence with pMMcomK expression vectors. A 10-ml LB-tet (12.5 μ g/ml tetracycline [Tet], 8 μ g/ml uracil) starter culture was inoculated with a single colony from a fresh LB plate (12.5 μ g/ml Tet, 8 μ g/ml uracil) and grown overnight at 37°C and 170 rpm in a New Brunswick Scientific shaker innova 4320 (Edison, NY). A 50-ml main culture (LB, 12.5 μ g/ml Tet, 8.5 μ g/ml uracil) was inoculated with 500 μ l of the starter culture and grown in a four-baffle 300-ml Erlenmeyer flask at 37°C and 155 rpm until an OD₅₄₆ of 0.5 was reached. The main culture was divided into two 20-ml cultures, and each was transferred into separate 100-ml Erlenmeyer flasks. In culture A, ComK expression was induced with 0.125 to 0.25% xylose, whereas culture B served as the uninduced negative control. Both of the cultures (A and B) were grown for 1 to 3 h. For the transformation, 0.5 ml culture (of A and B) was transferred to an Eppendorf cup containing 1 to 5 μ g DNA. Transformation mixtures were incubated for 30 min in a Thermomixer comfort (Eppendorf, Hamburg, Germany) at 37°C and 700 rpm, after which the cells were harvested by centrifugation, resuspended in 500 μ l M9 minimal medium supple-

mented with uracil (8 μ g/ml), and incubated for another 30 min at 37°C and 700 rpm to stop *comK* induction. For the complementation of uracil auxotrophy, cells were washed three times with M9 minimal medium without uracil to remove residual uracil and finally plated on M9 minimal agar plates for the selection of prototrophs. For obtaining *uvrBA*-EmR mutants, washing was not necessary, as cells were directly plated on LB containing erythromycin (0.5 μ g/ml). The construction and selection of conditional *pgk* mutant strains was done essentially as described for the *uvrBA*-EmR mutants, except that 1 mM isopropyl- β -D-thiogalactopyranoside (IPTG) was added to growth media and selection plates.

RNA isolation. For RNA isolation, cells were cultivated as described for the two-step transformation protocol; 1 ml was harvested after 1 h of cultivation in LS medium. The pellet was resuspended in 400 μ l 10 mM Tris, transferred into Nalgene cryotubes (Nalgene Labware, Hereford, United Kingdom) filled with approximately 300 mg of glass beads (diameter, 150 to 212 μ m; Sigma-Aldrich, Munich, Germany), and frozen in liquid nitrogen. Cell disruption was accomplished by using the Mikro Dismembrator S (Satorius AG, Goettingen, Germany) at 2,700 rpm for 4 min. RNA isolation was done according to the manufacturer's instructions with a high pure RNA isolation kit (Roche Diagnostics GmbH, Mannheim, Germany). The quality and the amount of the RNA samples were controlled using agarose gel electrophoresis and spectrophotometric analysis with a NanoDrop photometer (Peqlab Biotechnologie GmbH, Erlangen, Germany).

Reverse transcription-PCR (RT-PCR). For cDNA synthesis, 0.5 μ g of isolated RNA and a mixture of primers (*rpsJ2*, *rpsE2*, *recA2*, *comK2*, *comGB1*, and *comA2*) was employed in combination with a RevertAid H-minus first-strand cDNA synthesis kit (Fermentas, Leon-Rot, Germany) according to the manufacturer's instructions. The quantification of cDNA synthesis was accomplished by PCR with *Taq* polymerase from 18 to 25 cycles depending on the transcript. For the analysis of the genes, the following primer pairs were used: *comK* cDNA, primer pair *comK1/comK2*; *comA* cDNA, primer pair *comA1/comA2*; *rpsE*

cDNA, primer pair rpsE1/rpsE2; *recA* cDNA, primer pair recA1/recA2; and *comGB* cDNA, primer pair comGB1/comGB2.

Enzyme assays. Extracellular protease activities were determined using minimal medium agar plates as previously described (62). Extracellular glucanase activity was determined using LB plates containing 0.02% lichenin and subsequently stacked with Congo red. Clearing halos around the colony indicate enzyme activity.

UV survival measurements. Cells of wild-type and randomly chosen *uvrBA* mutants (designated 1 to 4) were cultivated to the mid-exponential phase, and in several dilutions equal numbers of cells were spread on LB plates and UV irradiated (20 J/m²) or left nonirradiated as a control. Subsequently the plates were incubated overnight at 37°C.

Nucleotide sequence accession numbers. Sequences determined in the course of this work have been submitted to GenBank under accession numbers GQ499198, GQ505078, GQ505079, GQ505080, GQ505081, and GQ505082.

RESULTS

Comparative analysis of genes involved in quorum sensing.

The only difference between the genome sequences of *B. licheniformis* DSM13 and ATCC14580 (except a negligible 370-bp difference within the 4.3-Mbp genome) is the orientation of the IS element accompanied by a breakdown in the GC content at the site of insertion in *comP*, possibly suggesting a flexible position within the genome (see Fig. S1 in the supplemental material). We have sequenced the *comQXPA* cluster of strain 9945A, which is known to be naturally competent, and also of the F11 strain for comparison. The corresponding genomic loci of the *comQXPA* clusters were aligned and compared to the genetic competent *B. subtilis* 168 and *B. amyloliquefaciens* FZB42. Differences from the sequence of DSM13 are observed in the first two large extracellular loops of the multimembrane-spanning protein ComP of the competent *B. licheniformis* 9945A. These loops are responsible for ComX recognition; however, as the sequence comparison revealed only 33% identities and 54% identities, respectively (42, 66), a replacement may not lead to the desired result. The GC plot of the sequence of *comP* of F11 almost perfectly matches that of DSM13 and ATCC 14580 (see Fig. S1 in the supplemental material), and the first two extracellular loops of ComP, which are responsible for ComX recognition, are almost identical (only one amino acid differs). The IS element is missing, making it a very promising candidate for the replacement of the interrupted gene. Furthermore, the functionality of ComP in *B. licheniformis* F11 can be derived from the polyglutamate formation of F11 (not shown), as *B. subtilis* strains with insertions in *comP* likewise lack polyglutamate synthesis (36, 55, 56), as is the case for *B. licheniformis* DSM13 (Fig. 2). Since different *B. subtilis* strains display genetic polymorphisms concerning the sensor histidine kinase ComP, the peptide pheromone ComX, and ComQ (55), the complete *comQXPA* cluster of *Bacillus licheniformis* F11 was compared to the respective *B. licheniformis* DSM13 sequence. ComQ and ComX of *B. licheniformis* F11 are identical to *B. licheniformis* DSM13. ComP of *B. licheniformis* F11 has few sequence deviations. These are composed of three silent mutations and two amino acid substitutions, one of which is at position 230 from serine in DSM13 to glycine in F11 (triplet change from AGT to GGT), and the other is at position 578 from aspartate in

DSM13 to alanine in F11 (triplet change from GAT to GCT).

Reconstitution of ComP in *B. licheniformis* MW3. The disrupted *comP* of *B. licheniformis* MW3, the restriction-negative DSM13 derivative (65), was replaced by *comP* of *B. licheniformis* F11 (see Materials and Methods and Fig. S1 in the supplemental material). The generated strain *B. licheniformis* MK1 (ComP⁺) displayed polyglutamate synthesis, which was clearly discernible by slime formation (Fig. 2B). Furthermore, cells of the ComP-inactive strains *B. licheniformis* MW3.1 and MW4.1, as well as those of the ComP-active derivatives *B. licheniformis* MK1.1 and MK2.1, were spotted onto lichenin and skim milk agar plates for monitoring their extracellular glucanase and protease activity, respectively (Fig. 2B). *B. licheniformis* MK1.1 and MK2.1 displayed enhanced extracellular glucanase and protease activities compared to those of the ComP-inactive MW3.1 and MW4.1. The strains also were analyzed with respect to their genetic background (Fig. 2A) via PCR to ensure that all carried the deletions in the two loci encoding HsdR1/HsdR2, the restriction endonucleases (65). Based on the knowledge that ComX-dependent quorum sensing enhances polyglutamate slime formation and extracellular enzyme synthesis in *B. subtilis* (35, 50), ComP of *B. licheniformis* F11 appears to be functional when inserted into *B. licheniformis* MW3, as ComX-mediated quorum sensing apparently is reactivated. Additionally, we have added a His₆ tag to ComP to verify the expression of ComP from the xylose-inducible promoter. Signals obtained by Western blotting proved expression (data not shown).

To analyze genetic competence, uracil auxotrophs of MW3 and MK1 were generated by deleting sequences encoding the phosphoribosyl-binding site of PyrE, the orotate phosphoribosyltransferase, which is essential for pyrimidine synthesis (see Fig. S2B in the supplemental material). The respective deletion and concomitant uracil auxotrophy is documented in Fig. 2A and B. Since a negative effect on transformation by excessive polyglutamate production cannot be excluded, mutants with a defect in PGA synthesis were constructed by the almost-complete deletion of the polyglutamate (*pga*) synthetase operon (see Fig. S2A in the supplemental material) in both MW3.1 and MK1.1 (ComP inactive and active, respectively). In the *pga* deletion mutant *B. licheniformis* MK2.1 (ComP active, Δ *pga*, Δ *pyrE*), slime formation was completely abolished (Fig. 2B), providing further evidence that the slime formation of MK1.1 is indeed due to polyglutamate synthesis. As expected, no change in colony morphology was observed after *pga* deletion in strain MW4.1 (ComP inactive, Δ *pga*, Δ *pyrE*), as the disruption of *comP* already sufficed to prevent polyglutamate synthesis (36, 56). The successful removal of the IS element was demonstrated by PCR. *B. licheniformis* MK2.1 and MK1.1 (not shown) display the same *comP* amplicon size of 2,965 bp as that of *B. licheniformis* F11. MK2.1 also carries the deletions of the genes for PGA synthesis and uracil auxotrophy (PyrE) (amplicons of 2,646 and 938 bp, respectively), whereas *B. licheniformis* MW4.1 is characterized by the 4,253-bp *comP* amplicon, which includes the IS element.

Transformation via natural competence. For the first experiments, *B. licheniformis* MW3.1 and MK1.1 were transformed to prototrophy by applying chromosomal DNA of a sporulation-negative but uracil-prototrophic *B. licheniformis* strain

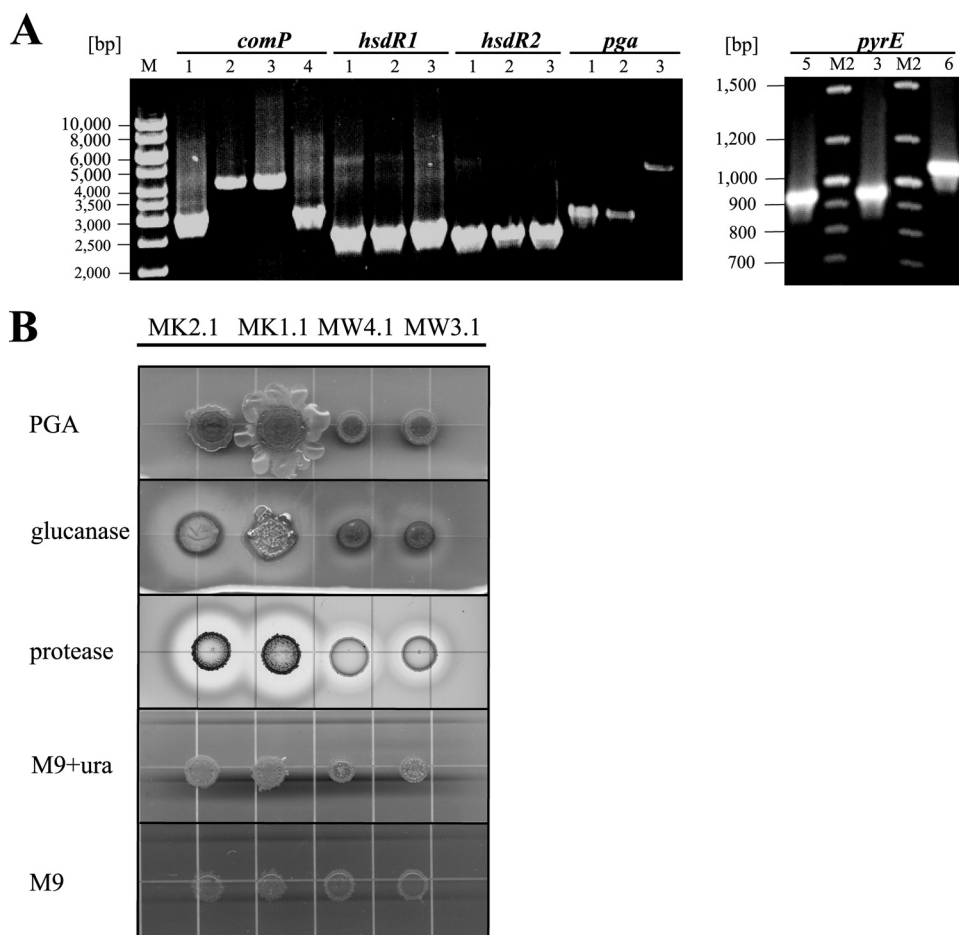


FIG. 2. Genotypical and phenotypical examination of generated mutants. (A) PCR analysis of chromosomal DNA from *B. licheniformis* MK2.1 (Δ *hsdR1*, Δ *hsdR2*, *comP* active, Δ *pyrE*, Δ *pga*) (1), MW4.1 (Δ *hsdR1*, Δ *hsdR2*, Δ *pyrE*, Δ *pga*) (2), MW3.1 (Δ *hsdR1*, Δ *hsdR2*, Δ *pyrE*) (3), F11 (4), MK1.1 (Δ *hsdR1*, Δ *hsdR2*, *comP* active, Δ *pyrE*) (5), and MW3 (Δ *hsdR1*, Δ *hsdR2*) (6). As anticipated, mutants display smaller amplicons than the parental strain. (B) Equal amounts of cells of *B. licheniformis* MW3.1, MW4.1, MK1.1, and MK2.1 cultures were spotted onto agar plates containing LB only (PGA), LB plus lichenin (glucanase), and M9 plus skim milk (protease), respectively. Additionally, cells were dropped on M9 minimal medium supplemented with uracil (M9+ura) and on M9 minimal medium lacking uracil (M9). Note that the ComP-positive strain displays increased polyglutamate formation in either case.

(DSM13 Δ *spoIV*) via a two-step transformation protocol (Fig. 3A). Experiments with DNA from uracil-auxotrophic *B. licheniformis* MW3.1 and MK1.1 served as negative controls and, as expected, no uracil prototrophic transformants could be obtained. *B. licheniformis* MW3.1 carrying the inactive *comP* was only poorly transformable via natural competence with approximately 340 transformants/ μ g DNA. However, MK1.1, encoding an active ComP, exhibited a more-than-hundredfold decrease in transformation efficiency, i.e., 2 and 1.6 transformants/ μ g DNA, respectively. Transformants were routinely checked by PCR analysis for their genetic background (data not shown). To rule out that the polyglutamate slime formation of the ComP-active strain negatively influenced transformation efficiencies, such experiments again were carried out with the Δ *pga* mutant strains *B. licheniformis* MW4.1 and MK2.1. Furthermore, possible effects due to extended cultivation times (1 to 4 h within the exponential phase) on transformation efficiencies after the change from the HS to the LS medium were investigated for both strains (Fig. 3B). In general, transformation efficiencies were higher immediately

after medium change from the starter culture to the main culture, with maximum transformation efficiencies of 3,250 transformants/ μ g DNA for MW4.1 cultivation for 1 h. The *pga* deletion did not enhance the transformation of the ComP active strain MK2.1, as efficiencies of *B. licheniformis* MK2.1 remained low, with 1 and 2 transformants/ μ g DNA after 1 and 2 h of cultivation. Thus, poor transformation efficiencies are not due to polyglutamate synthesis. To further investigate the reason for the poor transformation efficiency in the ComP-active strain *B. licheniformis* MK2.1 in contrast to that of MW4.1, a quantitative PCR (qPCR) analysis was conducted with RNA isolated from cultures after 1 h of cultivation (Fig. 3C). Negative controls without reverse transcriptase showed no amplicon. The mRNA of a ribosomal protein (RpsE) served as an internal control. In the ComP-active strain MK2.1, the transcription levels of the genes for the main activator of natural competence, ComK, and also for ComGB, a protein of the DNA uptake machinery, were significantly lower than that in the ComP-inactive strain MW4.1. Furthermore, the transcription of *comA* and *recA* are enhanced in *B. licheniformis*

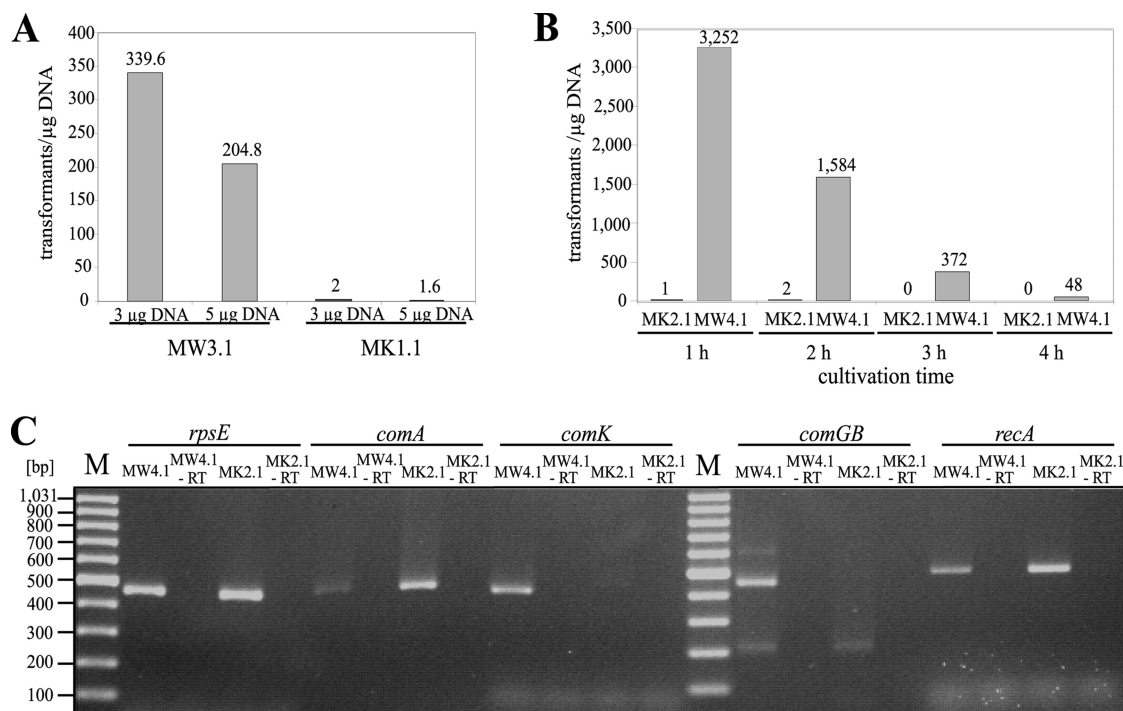


FIG. 3. Influence of the ComP status (active/inactive) on the transformation efficiencies of *B. licheniformis* MW3 derivatives and on gene expression during transformation. (A) Transformation efficiencies of *B. licheniformis* MW3.1 (ComP⁻, Δ pyrE) and of MK1.1 (ComP⁺, Δ pyrE) using different amounts (3 or 5 μ g) of chromosomal DSM13 Δ spoIV-DNA to regain uracil prototrophy. (B) Transformation efficiencies of polyglutamate-negative strains *B. licheniformis* MW4.1 (ComP⁻, Δ pyrE, Δ pga) and *B. licheniformis* MK2.1 (ComP⁺, Δ pyrE, Δ pga) after different cultivation times after medium exchange. (C) RT-PCR analyses of isolated RNA from *B. licheniformis* MW4.1 (ComP⁻, Δ pyrE, Δ pga) and *B. licheniformis* MK2.1 (ComP⁺, Δ pyrE, Δ pga). -RT, negative controls without reverse transcriptase; M, 100-bp DNA ladder (gene ruler; Fermentes, St-Leon-Roth, France); RpsE, ribosomal protein; ComA, response regulator; ComK, main transcriptional activator of natural competence; ComGB, DNA transport protein; RecA, multifunctional protein involved in homologous recombination and DNA repair.

MK2.1 (Fig. 3C). Hence, the reactivation of the sensor histidine kinase ComP in *B. licheniformis* MW3 diminished transformation efficiencies during natural competence due to reduced *comK* expression in the ComP-active strain MK2.1, as shown here with a two-step transformation protocol. A one-step procedure for transformation during natural competence (3, 54) also was applied for *B. licheniformis* MW4.1 and MK2.1, but no prototrophic transformants could be obtained. As the reactivation of ComP did not enhance transformation efficiencies via natural competence, another strategy that focused on inducible ComK expression was tested.

Inducible genetic competence. The *comK* gene of *B. licheniformis* DSM13 was placed under the control of a xylose-inducible promoter in the pMM1522 vector to give pMMcomK (60). Strain *B. licheniformis* MW3.1 (ComP⁻, Δ pyrE) transformed with the ComK expression vector (pMMcomK) subsequently was used in transformation experiments (Fig. 4A). For the determination of the optimal transformation conditions, the strain described above was transformed to prototrophy by using chromosomal DSM13 Δ spoIV DNA and applying different xylose concentrations (0.125 and 0.25%) for *comK* induction along with several cultivation times after induction (1, 2, and 3 h). The highest transformation efficiency, 2.97×10^5 CFU/ μ g DNA, was obtained with 0.25% xylose and a 3-h cultivation time postinduction; induction with 0.125% xylose and a 3-h cultivation time yielded 2.61×10^5 prototrophic transfor-

mants/ μ g DNA (Fig. 4C). Shorter induction times (for example, 2 h) lowered the transformation efficiency to 1.38×10^5 CFU/ μ g DNA for induction with 0.125% xylose and 1.07×10^5 CFU/ μ g DNA with 0.25% xylose. As artificial ComK induction in *B. licheniformis* MW3 resulted in high transformation efficiencies, the regulatory pathway downstream of ComK activation apparently is functional. Growth retardation observed in the induced cultures provided the basis for the curing of the pMMcomK vector (Fig. 4B). The obtained prototrophic strains were grown overnight in LB supplemented with 0.5% xylose but without tetracycline, which are conditions that favor the growth of transformants that have lost the vector. Cells subsequently were plated out on LB medium with 0.5% xylose, and single colonies were used to inoculate LB medium with 0.5% xylose. Such cultures plated on LB medium with 0.5% xylose containing 12.5 μ g/ml tetracycline revealed no growth, indicating the loss of the tetracycline resistance carried on the vector pMMcomK. In a control experiment in which the overnight culture was supplemented with tetracycline, up to 1.14×10^8 colonies were observed on LB medium with 0.5% xylose containing 12.5 μ g/ml tetracycline.

Direct gene disruption. As the target for direct gene disruption experiments, the *uvrBA* operon was chosen, as such mutants previously were shown to be UV sensitive, rendering phenotypic identification possible (63). A suicide vector comprising the erythromycin resistance gene flanked by the recom-

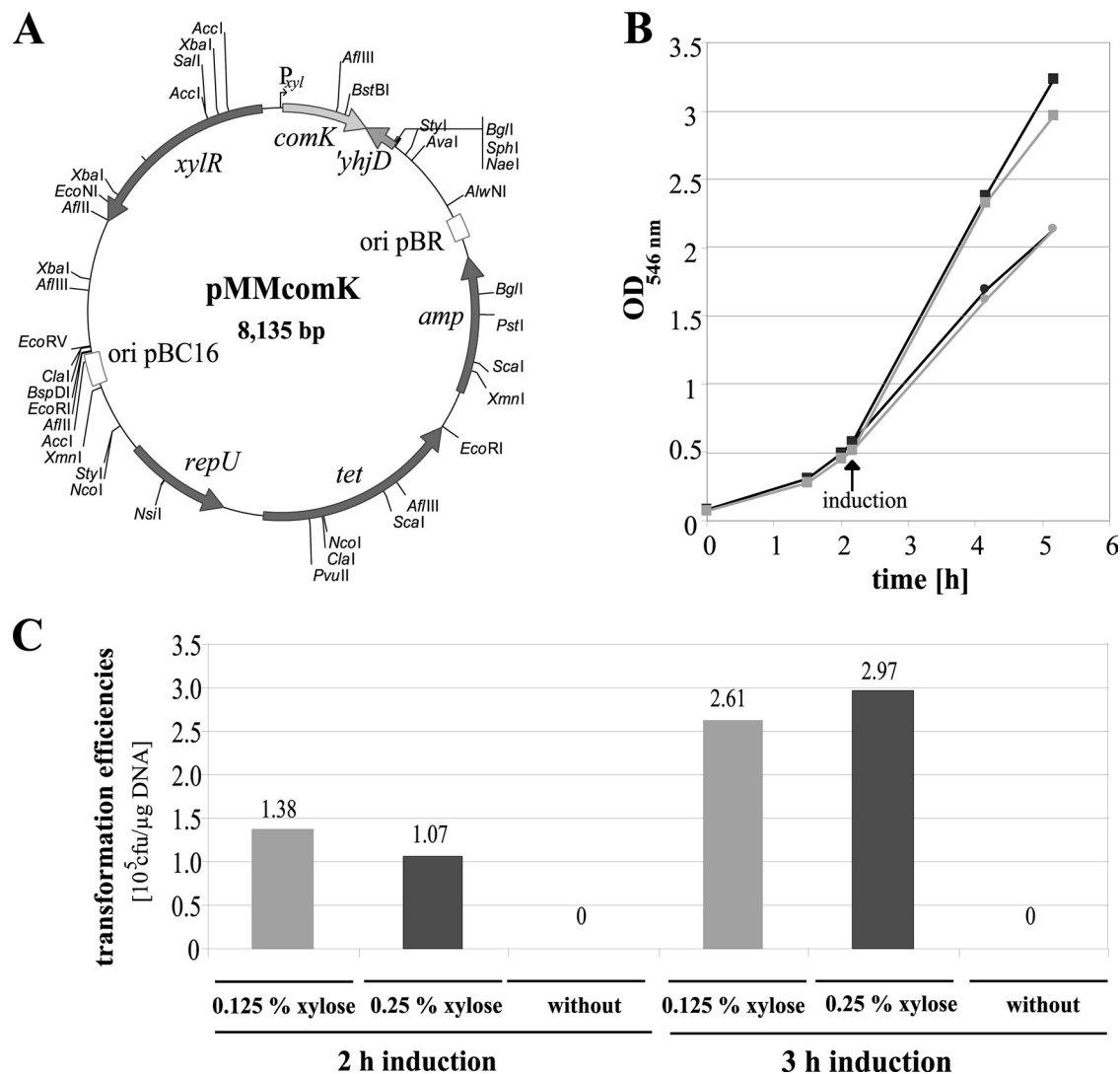


FIG. 4. Induced natural competence by xylose-driven ComK expression. (A) pMMcomK, vector for xylose-inducible ComK expression. Open reading frames are depicted as arrows. Restriction enzymes are abbreviated. ComK, the main activator for natural competence; 'YhjD, gene downstream of *comK*, part of a hypothetical protein; XylR, xylose repressor; RepU, replication protein; Tet, tetracycline resistance (*Bacillus*); Amp, ampicillin resistance (*E. coli*); ori pBC16, *Bacillus* origin of replication; ori pBR, ColE1 origin of replication. (B) Growth curves of *B. licheniformis* MW3.1 pMMcomK with or without the induction of ComK expression. Black circles, induction with 0.25% xylose; gray circles, induction with 0.125% xylose; black and gray squares correspond to uninduced controls. (C) Prototrophic transformants per microgram of DNA of induced (0.125% xylose and 0.25% xylose) and not induced (without) cultures for 2- and 3-h induction times.

bination sequences was used (Fig. 5A). Approximately 3.3×10^3 erythromycin-resistant colonies per μg of DNA were obtained (Fig. 5B). Thirty-four to 43% of the latter were *uvrBA* mutants with the expected genotype (PCR data not shown). The remaining erythromycin-resistant transformants had ectopic integrations of the erythromycin resistance gene (revealed by Southern blot analysis; data not shown). The highest transformation efficiencies were obtained with $1 \mu\text{g}$ of pUCuvr BAEmR, which yielded 1.3×10^3 *uvrBA* mutants/ μg DNA (Fig. 5B). Increasing the amount of DNA from $1 \mu\text{g}$ pUCuvr BAEmR to 3 or $5 \mu\text{g}$ did not improve the observed efficiency. Randomly chosen *uvrBA* mutants were tested for UV sensitivity in a drop dilution assay as shown in Fig. 5C. Hence, by applying the induced competence method, it could be shown

that the direct gene disruption of the *uvrBA* operon was successful and rapidly achievable in *B. licheniformis* MW3.

Conditional knockout of essential genes. A vector based on pMutin2 was used (58) to determine whether transformation efficiencies suffice for the conditional inactivation of an essential gene. The vector (pMutin2) allows the expression of the target gene with the IPTG-inducible P_{spac} promoter by integrating pMutin at the position of the gene in the genome via homologous recombination. Due to promoter exchange, the target gene is transcribed only in the presence IPTG. Thus, the growth of the culture is IPTG dependent, given that the target gene is essential for the organism. For the conditional gene inactivation approach, an operon was chosen that includes four putative essential genes: *pgk*, *tpiA*, *pgm*, and *eno*. The products

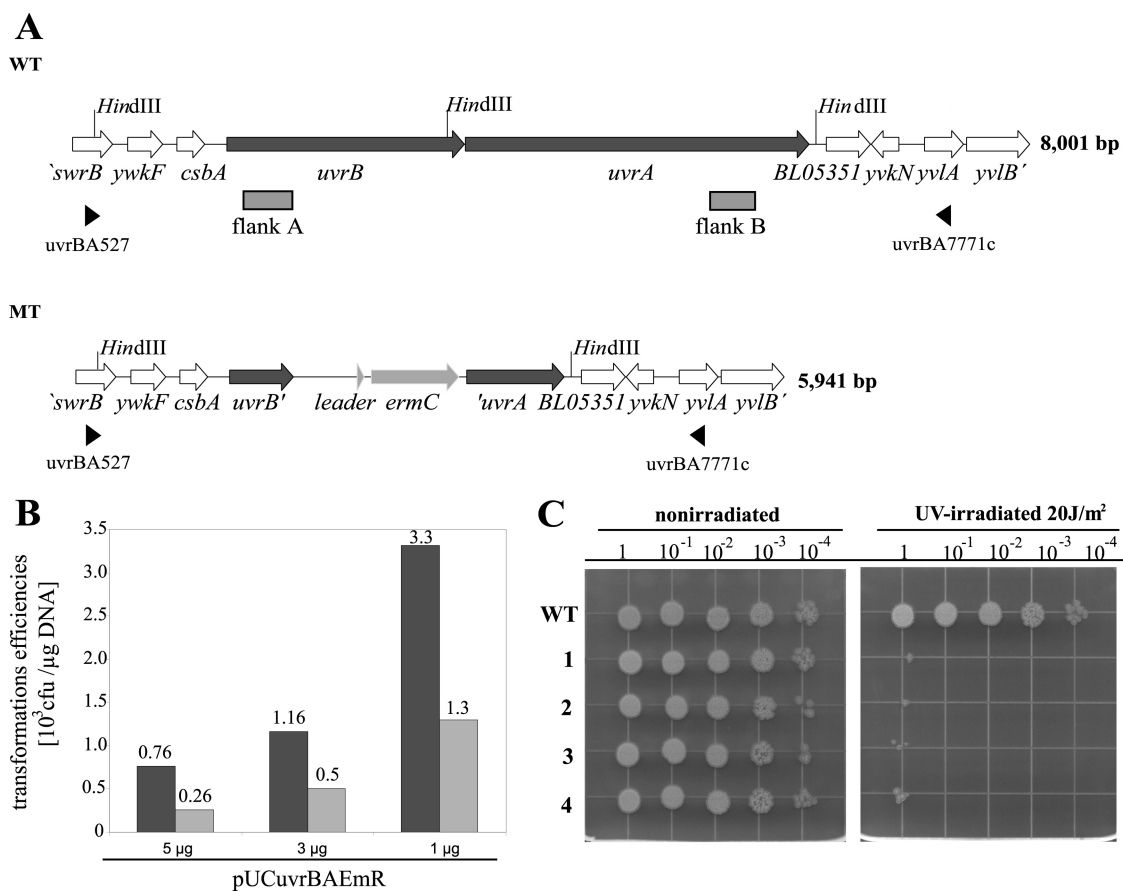


FIG. 5. Direct disruption of the *uvrBA* operon by induced competence. (A) Schematic representation of the *uvrBA* operon from wild-type *B. licheniformis* (WT) and the *uvrBA* mutant (MT). SwrB, motility/swarming protein; YwkF, hypothetical protein; CsbA, conserved uncharacterized protein; UvrB, excinuclease ABC subunit B; UvrA, excinuclease ABC subunit A; BL05351, pseudogene; YvkN and YvlA, hypothetical proteins; YvlB, uncharacterized conserved protein; ErmC, erythromycin resistance. (B) Transformation efficiencies via induced competence with suicide vector pUCuvrBAEmR. Black bars, total amounts of obtained erythromycin-resistant transformants; gray bars, fraction of the *uvrBA* mutants. (C) UV sensitivity of the *uvrBA* mutant strains (1, 2, 3, and 4) compared to that of the wild type.

of these genes are thought to be instrumental in the conversion of trioses to pyruvate (26). Transformants having integrated the respective suicide vector were obtained (Fig. 6A), as shown by PCR with the primer pair pgk_screen_for/pgk_screen_rev (Fig. 6B). It also was shown that the growth of the transformants with an integrated vector (pMutpgk) is IPTG dependent (see Fig. 6C), showing that this operon comprises at least one essential gene.

DISCUSSION

The surmised negative impact on the development of genetic competence due to the insertion of an IS element into the *comP* locus is not unique to *B. licheniformis* DSM13/ATCC 14580 (44, 61). In *B. subtilis natto* NAF4, an element belonging to the IS4 family (IS4Bsu1) frequently integrates into *comP* (36, 56), thereby not only rendering the strain less competent but also causing the complete loss of PGA formation and a decrease in extracellular protease secretion (36, 52, 66), as shown here for *B. licheniformis* DSM13.

Surprisingly, the replacement of the IS3-interrupted *comP* with an intact copy did not restore full genetic competence but

did indeed effect the opposite. PGA formation, known to be detrimental for competence (12, 29, 54), was excluded as the cause of the observed impairment. As the transcription of *comK* as well as that of downstream genes, such as *comGB*, was significantly lowered, no direct link between ComP and ComK via ComS, as observed in *B. subtilis*, could be shown for *B. licheniformis*. The quantitative conversion of DegU into its phosphorylated state, DegU~P, via the ComP/ComA-activated DegQ may additionally provide a clue to the lowered competence development, as unphosphorylated DegU is crucial to start the autoregulatory *comK* loop (19, 20, 25, 35). The enhanced expression of *recA* and, in particular, *comA* again probably is due to the lack of the insertion element, as *comA* transcription is possible not only from its cognate promoter but also from the promoter of *comP* by a readthrough mechanism (66). When ComK was inducibly expressed on a plasmid, the achieved transformation frequencies are in good agreement with those of the known naturally competent strain *B. licheniformis* 9945A (54), demonstrating not only a functional DNA uptake machinery but also its regulation by ComK.

The link between ComP and competence development via ComS may not exist in *B. licheniformis* DSM13, as an open

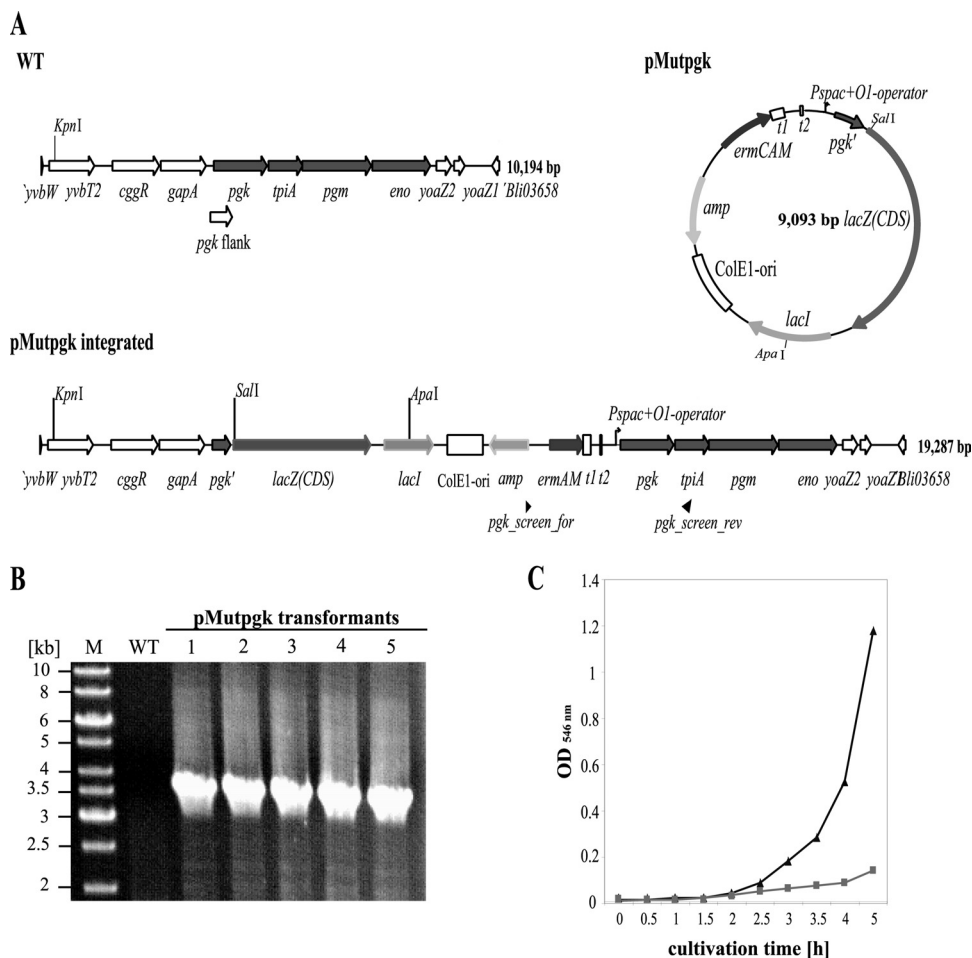


FIG. 6. Conditional inactivation of essential genes in *B. licheniformis* MW3 by induced competence. (A) Schematic representation of the genetic organization of the *pgk* operon of the wild type (WT), conditional mutant (pMutpgk integrated), and vector for inactivation (pMutpgk). P_{gk}, phosphoglyceratkinase; T_{piA}, triosephosphatidomerase; P_{gm}, phosphoglyceratmutase; E_{no}, enolase. P_{spac+O1}, IPTG-inducible promoter with operator 1; t₁ and t₂, strong transcriptional terminators. For detailed information, see reference 58). (B) Verification of obtained transformants by PCRs. (C) IPTG growth of transformants. Black curve marked with triangles, transformants grown in LB with IPTG; gray curve with boxes and curve with triangles, transformants grown without IPTG.

reading frame encoding a potential ComS could not be identified due to poor sequence conservation along with the short sequence length of ComS (28). Thus, the identification was attempted by comparing the N-terminal parts of the *B. subtilis* 168 surfactin synthetase B with the corresponding protein lichenysin synthetase B of the *B. licheniformis* strains, followed by a search for conserved peptides of ComS. A significant C-terminal conservation of the *B. subtilis* ComS compared to that of the considered *B. licheniformis* ComS was identified (Fig. 7A).

The transcription of the lichenysin operon is known to be activated by *B. subtilis* ComA~P; consistently with this finding, a putative ComA Box in the promoter region of the lichenysin operon has been proposed (68, 69). However, ComS_{9945A} differs in several aspects from its counterparts of *B. subtilis* and *B. amyloliquefaciens*: (i) there is an N-terminal extension of 16 amino acids, (ii) there are four amino acids inserted at position 27, and (iii) the potential core sequence for MecA binding (ITRFRP) in *B. licheniformis* 9945A differs from those of *B. subtilis* (ILLYPR) and *B. amyloliquefaciens* (IILFPQ) (7, 40,

43). To prove the functionality of the putative ComS_{9945A} further, experiments are being performed and will be reported later.

Interestingly, the putative ComS proteins of the *B. licheniformis* strains F11, DSM13, and ATCC 14580 are identical, whereas ComS of the only known naturally competent *B. licheniformis* 9945A has some differences (Fig. 7). The C-terminal extension and the differences in the putative binding site of MecA could be responsible for the lack of function in *B. licheniformis* DSM13 and thus also will be the focus of further experiments.

Upon ComK induction, very high transformation efficiencies were achieved. As the growth rate of cultures with induced ComK expression was reduced compared to that of the uninduced control, an elegant means for vector curing is provided, as competent cells of *B. subtilis* are known to block chromosome replication and cell division via ComGA, which is governed by ComK (13, 15, 51). Additionally, genes encoding inhibitors of cell division such as *maf*, *minC*, and *minD* are upregulated by ComK (2, 4, 11).

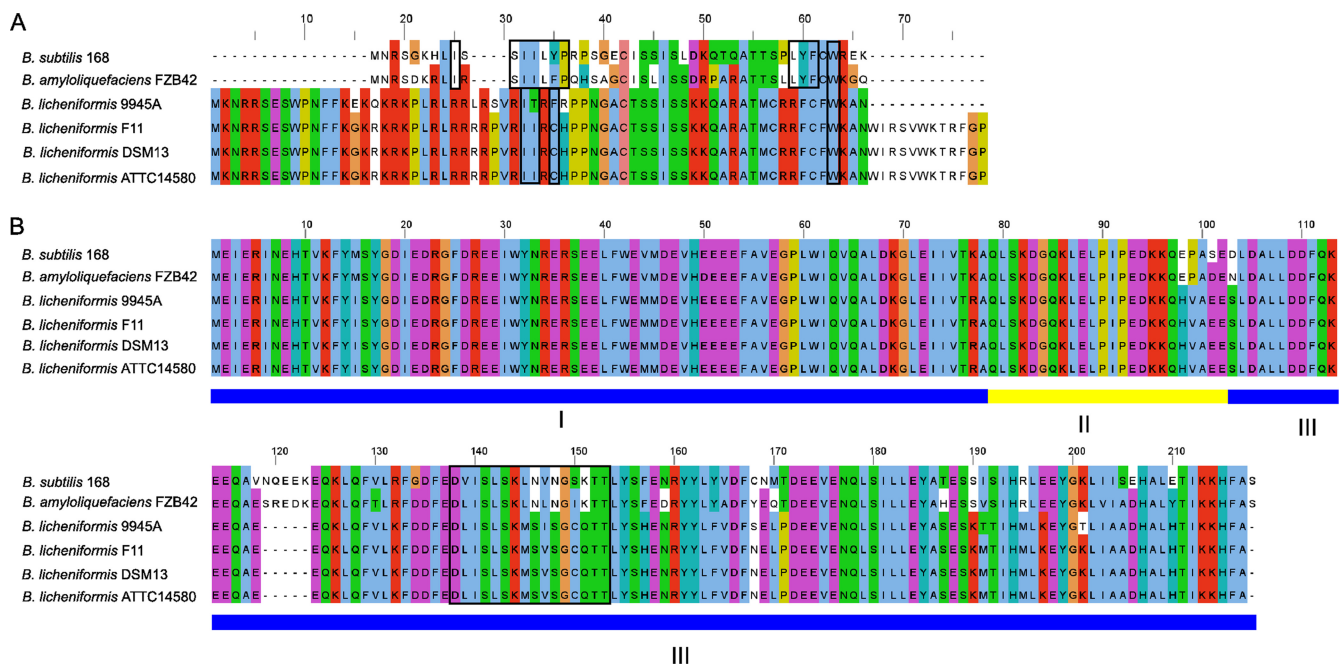


FIG. 7. ClustalW alignments of ComS (A) and MecA (B) colored with JalView. The same colors in all rows mark conserved amino acids. (A) Black boxes show ComS amino acids essential for the competence development (40, 43). (B) The domain structure of MecA is highlighted. The roman numeral I corresponds to the ComK and ComS binding domain, II indicates a predicted loop region, and III indicates the ClpC binding domain (41). The black box marks a second ComS binding domain (43).

For *Bacillus cereus*, a vector-based *comK* induction was used to obtain mutants by natural competence; however, efficiencies were rather poor: the use of chromosomal DNA of *B. cereus* FM1400, which contains an erythromycin resistance gene (*ermC*) in *sigB*, yielded 1 to 9 CFU/ μ g DNA (33). Possibly, the heterologous ComK of *B. subtilis* or detrimental effects of permanent ComK induction by IPTG (13, 14, 59) caused the low number of obtainable transformants, which are three orders of magnitude less than that for *B. licheniformis*. As the high transformation efficiencies facilitated not just an easy direct disruption of the *uvrBA* genes but also the conditional inactivation of essential genes by using the pMutin system (58), the developed method is suitable for the alteration of any chromosomal locus, irrespective of function, making further studies of regulators and essential genes possible.

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