Unravelling the genetic basis for competence development of auxotrophic *Bacillus licheniformis* 9945A strains

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Bacterial natural genetic competence – well studied in *Bacillus subtilis* – enables cells to take up and integrate extracellularly supplied DNA into their own genome. However, little is known about competence development and its regulation in other members of the genus, although DNA uptake machineries are routinely encoded. Auxotrophic *B. licheniformis* 9945A derivatives, obtained from repeated rounds of random mutagenesis, were long known to develop natural competence. Inspection of the colony morphology and extracellular enzyme secretion of two of these derivatives, M28 and M18, suggested that regulator genes are collaterally hit. M28 emerged as a 14 bp deletion mutant concomitantly displaying a shift in the reading frame of *degS* that encodes the sensor histidine kinase, which is part of the molecular switch that directs cells to genetic competence, the synthesis of extracellular enzymes or biofilm formation, while for M18, sequencing of the suspected gene revealed a 375 bp deletion in *abrB*, encoding the major transition state regulator. With respect to colony morphology, enzyme secretion and competence development, both of the mutations, when newly generated on the wild-type *B. licheniformis* 9945A genetic background, resulted in phenotypes resembling M28 and M18, respectively. All of the known naturally competent *B. licheniformis* representatives, hitherto thoroughly investigated in this regard, carry mutations in regulator genes, and hence genetic competence observed in domesticated strains supposedly results from deregulation.

**INTRODUCTION**

Natural genetic competence, a physiological state enabling bacteria to take up and chromosomally establish DNA from the environment, is widespread (Dubnau, 1999) and is currently known for more than 60 prokaryotic species (Johnsbrorg et al., 2007). Inferred from studies with *Streptococcus pneumoniae, Bacillus subtilis, Haemophilus influenzae* and *Neisseria gonorrhoeae*, environmental conditions triggering competence development vary greatly (Hamoen et al., 2003a; Johnsbrorg & Håvarstein, 2009; Koomey, 1998; Redfield et al., 2005).

In *B. subtilis*, the generation of genetically competent cells is subject to a complex regulatory network that integrates a number of stimuli which include nutritional stress and cell density (Hamoen et al., 2003a). ComK, the key transcriptional regulator for competence development, positively influences the expression of all so-called late competence genes, which are instrumental in DNA binding, DNA uptake, and homologous recombination of the incoming and the host DNA (Haijema et al., 1996; Hamoen et al., 2003a; van Sinderen et al., 1995). Expression of *comK* is strictly governed by a number of cellular regulators (Fig. 1). ComK has the capacity to bind to the promoter of its own gene, thereby auto-stimulating transcription (van Sinderen & Venema, 1994). For the successful development of genetic competence, ComK must reach a critical threshold level. It is the DegS/DegU two-component regulatory system that functions as the molecular switch, eventually directing the cells to genetic competence: The unphosphorylated response regulator DegU provokes binding of ComK dimers to its own promoter, thus priming the auto-stimulatory ComK expression loop (Hamoen et al.,...
2000, 2003a). Mutually exclusive, phosphorylated DegU triggers – depending on the degree of phosphorylation – the production of polyglutamate (PGA) (Ohsawa et al., 2009), motility, biofilm formation (Kobayashi, 2007; Verhamme et al., 2007) or the synthesis of extracellular, degradative enzymes (Dahl et al., 1992). Efficient phosphorylation of DegU, mediated by its cognate kinase DegS, needs DegQ as a cofactor (Kobayashi, 2007).

AbrB, the transition state regulator, which in general represses stationary phase genes during exponential growth (Strauch & Hoch, 1993; Strauch et al., 1989), influences competence development in *B. subtilis* in a way that high levels of AbrB prevent ComK expression by directly repressing transcription from the comK promoter, whereas low AbrB levels promote competence development by inhibiting expression of rok, which encodes a repressor of comK transcription (Hahn et al., 1995a; Hamoen et al., 2003b; Hoa et al., 2002). However, should ComK emerge during exponential growth, it is degraded by the proteolytic ClpCP/MecA complex, keeping the ComK level low (Turgay et al., 1997, 1998; van Sinderen & Venema, 1994). Recruitment of ComK to the proteolytic complex is brought about by the adaptor protein MecA (Hahn et al., 1995b; Persuh et al., 1999; Prepiak & Dubnau, 2007). To ensure competence development, the mandatory escape from ComK proteolysis is mediated by the small peptide ComS, which prevents ComK degradation by inactivating MecA (Hamoen et al., 1995; Ogura et al., 1999). Expression of ComS relies on the ComP/ComA two-component regulatory system (Comella & Grossman, 2005; Hahn & Dubnau, 1991), which is activated upon extracellular accumulation of the ComX pheromone, thus constituting one mechanism to couple competence development to cell density in *B. subtilis* (Bacon Schneider et al., 2002; Magnuson et al., 1994). In addition to ComX, the competence and sporulation factor, an extracellular pentapeptide originating from the product of *phrC* that is internalized by the Opp oligopeptide permease facilitates quorum sensing by modulating ComA-dependent gene expression in a concentration-dependent manner (Lazazzera et al., 1999; Solomon et al., 1996).

Although genetic competency is known for other species of the genus *Bacillus* (Gwinn & Thorne, 1964; Koumoutsi et al., 2004; Mirończuk et al., 2008), and the capability for DNA uptake is common (Kovács et al., 2009; Lorenz & Wackernagel, 1994), the regulation of competence development has only sparsely been addressed experimentally except for *B. subtilis*; indeed, the prevalent model conceptions refer to the latter.

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**Fig. 1.** Competence development in *B. subtilis* is strictly regulated (modified from Hoffmann et al., 2010). Arrows and T bars indicate positive and negative regulation, respectively. Asterisks and grey colouring refer to deviations in *B. licheniformis* discovered in this contribution (see Results and Discussion for details). ComX, peptide pheromone; ComQ, modification and secretion of ComX; ComP, histidine sensor kinase sensing ComX; ComA, related response regulator; ComS, inhibition of the proteolytic MecA/ClpCP; DegQ, facilitator of DegU phosphorylation; DegU, response regulator; DegS, sensor histidine kinase activating genes among other degradative enzymes; MecA, adaptor protein; ClpCP, protease; ComK, key positive transcriptional regulator of natural competence; AbrB, negative transition state regulator, repressor of stationary phase genes during exponential growth; Rok, repressor of ComK.
Certain mutated strains of the common soil bacterium *Bacillus licheniformis* – representatives of which are being industrially exploited for the production of extracellular enzymes (Schallmey et al., 2004) – were reported to efficiently develop natural genetic competence: auxotropic UV-irradiation mutants of *B. licheniformis* 9945A, additionally lacking PGA formation, could be transformed at high rates (Gwinn & Thorne, 1964; McCuen & Thorne, 1971; Thorne & Stull, 1966).

Here we elucidate by molecular characterization and directed mutagenesis the genetic basis for the enhanced natural genetic competence of the two *B. licheniformis* 9945A auxotrophic strains M28 and M18 (Gwinn & Thorne, 1964; Leonard & Mattheis, 1965).

**METHODS**

**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⁻¹) or tetracycline (20 μg ml⁻¹) and *Bacillus* transformants with tetracycline (12.5–25 μg ml⁻¹) or kanamycin (5–15 μg ml⁻¹) when required.

**Molecular biological techniques.** Cloning in *E. coli* was performed essentially as described by Sambrook & Russell (2001). Plasmid DNA was isolated using the GeneJET Plasmid Miniprep kit (Fermentas). Genomic DNA from *B. licheniformis* strains was isolated as previously described (Nahrstedt et al., 2004) or by applying the MasterPure Gram-positive DNA Purification kit (Biozym Scientific). Southern analysis (Southern, 1975) and in vitro amplification of DNA were

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<td><strong>Strain or plasmid</strong></td>
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<td><em>E. coli</em> DH5αF’</td>
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<td><em>E. coli</em> S17-1</td>
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<td><em>B. licheniformis</em> DSM 13</td>
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<td><strong>Plasmid</strong></td>
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<td>pKVM2</td>
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<td>pKVM2ΔRM</td>
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<td>pKVM2ΔGlyA</td>
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<td>pKVM2degSΔ22–35</td>
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<td>pJR7Kan</td>
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<td>pJR7KandegS</td>
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<td>pUCBM20</td>
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*ApR, ampicillin resistance; DSMZ, Deutsche Sammlung für Mikroorganismen und Zellkulturen; KanR, kanamycin resistance; ori, origin of replication for *E. coli*; oriR, temperature-sensitive origin of replication for *Bacillus*; oriR, origin for conjugative transfer; RM, restriction modification system (hsdR–hsdM–hsdS); TetR, tetracycline resistance.*
performed as previously described (Nahrstedt & Meinhardt, 2004). Primers were initially designed on the basis of the B. licheniformis DSM 13 genome sequence (isogenic to ATCC 14580) (Rey et al., 2004; Veith et al., 2004) and more recently on the basis of the B. licheniformis 9945A genome sequence (Rachinger et al., 2013a).

PCRs (100 µl) consisted of 200 µM deoxynucleotides, 100 ng template DNA, 1 pmol of each primer and 1 U Taq DNA polymerase or Phusion DNA polymerase (Finnzymes). Purification of amplified fragments or restriction fragments after gel electrophoresis was performed with the GeneJET Gel Extraction kit (Fermentas). Sequencing was accomplished with fluorescence-labelled deoxynucleotides of the Big Dye Terminator v3.1 sequencing kit (Applied Biosystems) and an ABI Prism capillary sequencer (model 3730).

**Vector construction, subsequent gene deletion and complementation.** Strains and plasmids used in this study are detailed in Table 1, and the corresponding primers in Table S1 (available in the online Supplementary Material). For deletion of the B. licheniformis 9945A restriction modification (RM) system, gyaA, and the partial deletion of degS (positions 22–35 from the start codon), a marker-less deletion protocol (Rachinger et al., 2013b), was used. The flanking regions of the deletion targets were amplified by PCR; flank A was constructed using the respective primer pairs P1/P2; for flank B, P3/ P4 were used. The flanks were fused by splice overlap extension (SOE) PCR (Heckman & Pease, 2007), cut and cloned in the likewise cut vector pKVM2. The RM system deletion construct was cut with NcoI/BamHI, the gyaA construct with Bgill/Mdal and the degS construct with NcoI/MdhI. For gene deletion in B. licheniformis 9945A, the constructed vectors were transferred by conjugation from E. coli S17-1. Vector-bearing B. licheniformis 9945A cells were selected on nutrient broth (NB) plates containing polymyxin B (20 µg ml⁻¹) and tetracycline (20 µg ml⁻¹); subsequent recombination of homologous flanks was facilitated by incubation at 42 °C based on the temperature-sensitive origin of replication (ori) of pKVM2.

For complementation of the degS mutation in B. licheniformis M28, the corresponding intact 9945A sequence and its flanking regions were amplified using the primer pair degSFor and degSRev. The purified PCR product was subcloned into the HinclI-cut pUCBM20, from which the Xhol and Xnot fragment was cloned into the likewise cut pJR7Kan vector, which carries a temperature-sensitive ori for Bacilli, resulting in pJR7KandegS. The vector was introduced into B. licheniformis M28 by natural competence transformation and selected on kanamycin-containing LB agar.

For disruption of abrB in B. licheniformis 9945A and M18, combination flanks were amplified using primer pairs FLA_abrB_for/FLA_abrB_rev for flank A and FB_abrB_for/FB_abrB_rev for flank B. The flanks were fused via splicing by SOE PCR (Heckman & Pease, 2007) and cloned into EcoRV-cut pUCBM20. The vector was reopened by PCR using the primer pair FLA_abrB_rev/FB_abrB_for. The tetracycline resistance gene was amplified from vector pMM1522 using primer pair tetkofor/tetkorev, phosphorylated and cloned into the plasmid ampiclone to obtain the disruption vector pUCabriBTet. The latter was introduced into B. licheniformis GM3 carrying pMMcomKKan by transformation via induced competence (Harwood & Cutting, 1990; Hoffmann et al., 2010) and into B. licheniformis M18 by natural competence transformation.

For restoration of the truncated abrB in B. licheniformis M18, flanks were amplified from chromosomal B. licheniformis 9945A DNA using the primer pair abrBseq10 and FLA_abrB_rev_tet for flank A and the primer pair abrBseq6 and abrBseq11 for flank B, which comprise an intact abrB. The tetracycline resistance gene was amplified from pMM1522 with the primer pair Tetfor and Tetrevsoe and subsequently fused between the flanks using SOE (Heckman & Pease, 2007). The resulting complementation cassette was directly transformed into B. licheniformis M18 by natural competence transformation, and the selection of transformants was carried out on LB agar supplemented with tetracycline.

**Transformation via natural competence.** Transformations of B. licheniformis 9945A, M28 and M18 derivatives to obtain prototrophy were performed (in triplicate unless otherwise stated) with 1 µg chromosomal DNA of two prototrophic strains, B. licheniformis 9945A or B. licheniformis DSM 13AspoIV DNA. Chromosomal DNA of the strain to be transformed served as the negative control.

In transformation experiments of the marker-less B. licheniformis 9945A and the co-transformants T1+/T2+ of M18 and M28 (glycine/arginine prototroph and PGA synthesis), 30 µg of pMM1522 vector was applied, which provides – in contrast to chromosomal DNA – the tetracycline resistance gene as a selective marker.

Transformation of B. licheniformis M18 derivatives was performed using a modified two-step transformation protocol based on the

Fig. 2. B. licheniformis 9945A-derived auxotrophic mutants M28 and M18 are affected in different pathways. Phenotypic inspection of B. licheniformis 9945A, M28 and M18. (a) Transformation efficiencies via natural competence using vector pMM1522 to obtain tetracycline resistance. (b) Extracellular enzyme secretion. Equal amounts of cells were spotted on LB agar plates (colony morphology), M9 + skimmed milk (protease) and LB-lichenin (glucanase).
Fig. 3. *B. licheniformis* M28 carries a frame shift mutation in *degS* leading to altered enzyme secretion and competence development. (a) Comparison of the *degSU* genomic region of *B. licheniformis* 9945A (WT) and its derivative M28 as well as the corresponding amino acid sequence with domain indication predicted by InterProScan (v4.7, Hunter et al., 2012). yvyE, encoding an uncharacterized protein; *degS*, encoding the sensor histidine kinase DegS; *degU*, encoding the response regulator DegU; yviA, encoding an uncharacterized protein. (b) Phenotypic analysis of *B. licheniformis* GM1 (*ΔglyA*), GM1 *degS*– (*ΔglyA, degSΔ22–35*), M28 (Gly–, PGA–), GM3 (*ΔglyA, ΔRM*) and GM3 *degS*– (*ΔglyA, ΔRM, degSΔ22–35*). (c) Phenotypic analysis of *B. licheniformis* GM1 (*ΔglyA*), *B. licheniformis* M28 (PGA–, Gly–) compared with the complemented strains *B.*
**Genetic competence in B. licheniformis**

**licheniformis M28 degS+** (Gly−, intact degS). In (b) and (c), for analysis of extracellular enzyme secretion, equal amounts of cells were spotted onto LB agar plates (colony morphology), M9+ skimmed milk (protease) and LB-lichenin (glucanase). Relative transformation efficiencies via natural competence were obtained using chromosomal 9945A wild-type DNA to regain prototrophy. Mean transformation efficiency of M28 is set as 100 %. WT, wild-type; PGA−, polyglutamate deficient; Gly−, glycine auxotroph.

Transformation of *B. licheniformis* M28 derivatives was performed using a modified competence protocol, again based on the procedures described by Thorne & Stull (1966). Briefly, NB and NBSG-X media were supplemented with 0.1 μg thiamine ml−1 or 5 μg glycine ml−1. NB overnight cultures were used to inoculate 25 ml NBSG-X (containing 12.5 g glucose l−1) in 500 ml baffled flasks with a starting point OD600 of 0.01–0.02. The NBSG-X cultures were incubated on a Multitron shaker (Infors) at 37 °C and 200 r.p.m., for 18–20 h. Subsequently, the transformation was performed for 3 h (37 °C, 200 r.p.m.) in a test tube containing 0.8 ml TM media, 0.1 ml NBSG-X culture (OD diluted according to the smallest measured OD), 0.1 ml 2 M NaCl solution and DNA. Subsequently, cells were plated on tetracycline-containing MB medium or minimal agar [40 mM Na–K-phosphate buffer (pH 7.5), 10 mM NH₄Cl, 0.1 mM CaCl₂, 0.5 mM MgSO₄ and SL9 trace element solution (Veith et al., 2004)] and incubated for 2–5 days at 37 °C. Transformation was quantified as c.f.u. (μg DNA)−1.

**Enzyme assays.** Extracellular protease activities were determined using minimal medium agar plates supplemented with skimmed milk as previously described (Waldeck et al., 2006). Extracellular glucanase activity was determined using LB plates containing 0.02 % lichenin, which, after growth, were stained with Congo red. Clearing haloes around the colonies correspond to enzyme activities.

**Quantitative real-time PCR (qPCR).** For RNA isolation, LB cultures of *B. licheniformis* 9945A and M18 were incubated until they reached the early and late exponential growth phase, respectively. A total of 1 ml was harvested, and the pellet was resuspended in 400 μl 10 mM Tris. The resuspended cells were transferred into Nalgene Cryo Tubes (Nalgene Labware) filled with approximately 300 mg of glass beads (diameter 150–212 μm; Sigma Aldrich) and frozen in liquid nitrogen. The cells were disrupted by using the Mikro Dismembrator S (Sartorius) at 2700 r.p.m. for 3 min. RNA was isolated with a High Pure RNA Isolation Kit (Roche Diagnostics) according to the manufacturer’s instructions. The quality and the amount of RNA samples were controlled using agarose gel electrophoresis and spectrophotometric analysis with a NanoDrop photometer (Peglab Biotechnologie).

For cDNA synthesis, 1 μg of isolated RNA and 0.2 μg random hexamer primer were employed in combination with a RevertAid Hminus first-strand cDNA synthesis kit (Fermentas) following the manufacturer’s instructions. Primers applied for qPCR analyses were designed with the Primer Express 3.0 software (Applied Biosystems). Each reaction well contained 10 μl of 2 × Power SYBR Green PCR Master Mix (Applied Biosystems), 20 ng of cDNA as a template and primers in a final volume of 20 μl. The primer pair RT_abrB_for/RT_abrB_rev was used to estimate abrB transcription; primer pair RT_rpsE_for/RT_rpsE_rev served to determine transcription of the housekeeping gene *rpsE*, encoding the ribosomal protein RpsE as the endogenous control. Samples were quantified in triplicate, including non-template controls as negative controls. The procedure was repeated with DNA isolated from two independent samples. Obtained data were analysed with the StepOne Software v2.0.2 (Applied Biosystems). Relative copy numbers were calculated as previously described (Lee et al., 2006).

**RESULTS**

**M28 and M18 derivatives of 9945A display different phenotypes**

Two of the long known auxotrophic mutants of *B. licheniformis* 9945A that concomitantly lost the ability for PGA formation displayed high transformation efficiencies via natural competence: *B. licheniformis* M28 (Gly−, PGA−) and *B. licheniformis* M18 (Arg−, PGA−) (Gwinn & Thorne, 1964; Leonard & Matthes, 1965) (Fig. 2a). Our additional inspection with respect to colony morphology and the production of extracellular enzymes (protease and glucanase) revealed clearly discriminate phenotypes for the mutants. The wild-type *B. licheniformis* 9945A formed widespread colonies, whereas M28 colonies were small and flat; M18 colonies were small and wrinkled. With respect to extracellular enzyme activities, *B. licheniformis* M28 exhibited lower protease and glucanase activities than the wild-type; *B. licheniformis* M18 displayed similar glucanase activities as for the wild-type but clearly increased protease activities (Fig. 2b).

**M28 reveals a frame shift in degS**

The PGA-negative phenotype and the reduced enzyme activities of M28 suggested that the regulatory switch encoded by *degSU* was hit, because *B. subtilis* degU mutants display low extracellular enzyme activities and lack PGA formation (Dahl et al., 1991; Ohsawa et al., 2009). Indeed, transductional mapping of the locus responsible for the M28 phenotype positioned the competence-promoting mutation to the region between the gly-42 and his-6 auxotrophic markers (30 and 60 % co-transduction, respectively; McCuen & Thorne, 1971), where DegS and DegU are encoded. Eventually, sequencing of the corresponding M28 genomic region revealed a 14 bp deletion

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Fig. 4. *B. licheniformis* M18 displays a deletion in *abrB*, affecting extracellular enzyme secretion and natural competence development. (a) The *abrB* genomic region of *B. licheniformis* 9945A (WT), its derivative M18 and the corresponding amino acid sequences with domain indications (Neubauer et al., 2014; Phillips & Strauch, 2001; Xu et al., 1996). ORFs are depicted as arrows; dashed lines refer to the deletion in M18. *metS*, encoding methionyl-tRNA synthetase; *abrB*, encoding the general transcriptional repressor of stationary phase processes; *yabC*, encoding tetrapyrrole (corrin/porphyrin) methylases. Grey shadings refer to non-matching amino acids; ( ), missing amino acid; underlined amino acids are indispensable for repressor activity of AbrB. (b) Phenotypic analysis of *B. licheniformis* M18 (Arg−, PGA−), *B. licheniformis* M18Δ*abrB* (Arg−, PGA−, Δ*abrB*::tet), *B. licheniformis* GM3Δ*abrB* (ΔglyA, ΔRM, Δ*abrB*::tet) and *B. licheniformis* GM3 (ΔglyA, ΔRM). (c) Phenotypic analysis of *B. licheniformis* GM1 (ΔglyA) and *B. licheniformis* M18 (PGA−, Arg−) compared with the complemented strains *B. licheniformis* M18abrB+ (Arg−, intact *abrB*). In (b) and (c), for analysis of extracellular enzyme secretion, equal amounts of cells
leading to a frame shift mutation in \textit{degS}, which results in a premature translational stop (Fig. 3a).

Randomly chosen co-transformants T1 + and T2 + of M28 (Gly +, PGA +) obtained with chromosomal DNA of \textit{B. licheniformis} DSM 13ΔspoIV displayed extracellular enzyme activities and transformation efficiencies as for the wild-type 9945A (Fig. S1A, B), and in fact, sequencing of \textit{degS} (data not shown) of \textit{B. licheniformis} M28 T1 + and T2 + revealed in both cases intact genes, emphasizing that the \textit{degS} mutation was responsible for the enhanced transformation efficiency of M28.

\textbf{Mutating \textit{degS} in 9945A increases competence}

To determine whether the frame shift mutation in \textit{degS} accounts for the M28 phenotype, the latter was compared with a glycine auxotrophic 9945A derivative, into which the M28 \textit{degS}− mutation was introduced (strain GM1 \textit{degS}− in Fig. 3b). With respect to extracellular enzyme activities, introduction of the mutation resulted in the expected phenotypes. The transformation efficiency obtained for strain GM1 \textit{degS}− accounts for approximately one-fifth (17.89 \%) of the M28 transformation efficiency (set as 100 \%), highlighting a clear impact of the \textit{degS} mutation on competence development. As we were particularly interested in speeding up strain development for industrial purposes, i.e. in the enhancement of transformability, the \textit{degS} mutation was also introduced into an auxotrophic 9945A derivative in which the RM system was additionally deleted (strain GM3 \textit{degS}− in Fig. 3b). Indeed, a further increase of the transformation efficiency to 206.87 \% (relative to M28) was obtained. For absolute numbers [transformants (\textmu g DNA)−1] obtained in the distinct experiments, see Table S2.

\textbf{Complementation of the M28 \textit{degS} mutation reduces transformability}

Chromosomal integration of the \textit{degS} complementation vector in M28 is able to generate an intact gene copy, but the formation of depends on the flank first used for homologous recombination. From the four integrants, which were randomly chosen for plasmid curing, three displayed the wild-type colony morphology after successful plasmid elimination; the remaining one resembled M28. Sequencing confirmed the restoration of the \textit{degS} loci in clones displaying the \textit{B. licheniformis} 9945A colony type (sequence data are provided in Fig. S2A). Protease and glucanase activities of two of the randomly chosen \textit{degS}− complemented \textit{B. licheniformis} M28 strains (M28 \textit{degS}+) compared with \textit{B. licheniformis} 9945A are shown in Fig. 3(c); PGA production on minimal agar was restored as well (Fig. S2B). In comparative transformation experiments, the \textit{B. licheniformis} M28 \textit{degS}+ strains behaved similarly (2.92 and 2.52 \% of M28, respectively, Fig. 3c) to wild-type 9945A (5.50 \%), emphasizing that the \textit{degS} mutation in \textit{B. licheniformis} M28 accounts for the enhanced competence.

\textbf{M18 has a deletion in the \textit{abrB}- locus}

Genetic mapping by co-transduction positioned the mutation responsible for the M18 competence phenotype between the \textit{purA} (ade−) and \textit{str-1} (streptomycin resistance) genetic markers (30 and 42 \% co-transduction, respectively; McCuen & Thorne, 1971). The high extracellular protease activity, the wrinkled colony phenotype and the adjustment of the transduction data to the presently available \textit{B. licheniformis} genome sequence (Rachinger et al., 2013a; Rey et al., 2004; Veith et al., 2004) suggested that the general transition state regulator AbrB was possibly hit. Subsequent PCR analysis (included in Fig. S5B) and sequencing revealed a 375 bp deletion comprising the promoter region, an N-terminal part of the AbrB encoding region as well as an additional 6 bp insertion, resulting in an amino acid sequence variation in the predicted truncated AbrB sequence (see Fig. 4a).

Randomly chosen M18 transformants obtained with chromosomal DNA of \textit{B. licheniformis} DSM 13ΔspoIV, in which both the auxotrophic marker and PGA formation were restored (co-transformants M18 T1 + and T2 +), exhibited enzyme activities and transformation capabilities as for the wild-type 9945A (Fig. S1A, B). PCR analysis of the above M18 co-transformants revealed reversion of the deletion to the complete \textit{abrB} (Fig. S1C), supporting the assumption that the \textit{abrB} mutation accounts at least partially for the enhanced transformation efficiency of M18.

Fusion of the \textit{metS−abrB} intergenic region with the partially deleted \textit{abrB} may result in an altered hybrid protein, but in which the DNA-binding domain is lacking. As the mutation in M18 comprises the promoter region, transcript formation is unlikely to occur; however, comparative real-time qPCR analyses were performed to check M18 \textit{abrB} transcription during early and late exponential growth. The value obtained for the mutant was arbitrarily set as 1 to allow for comparison with wild-type 9945A. In contrast to M18, the transcripts of the latter were readily seen. While in \textit{B. licheniformis} 9945A \textit{abrB} transcription increased during exponential growth, values for M18 were constantly low, emphasizing that the truncated \textit{abrB} in M18 is not transcribed. The qPCR data are depicted in Fig. S3.
**abraB mutants of 9945A show increased competence**

Comparative transformation experiments for estimating the effect of the M18 abraB mutation on natural competence development were performed. For this, both the truncated M18 abraB sequence and the wild-type gene in a glycine auxotrophic and – due to improved genetic handling – restriction negative mutant (ΔglyA, ΔRM) were substituted by a tetracycline resistance cassette, eventually resulting in M18ΔabraB (Arg–, PGA–, ΔabraB::tet) and GM3ΔabraB (ΔglyA, ΔRM, ΔabraB::tet), respectively, shown in Fig. 4(b) (for genotypic verification, see Fig. S4). As anticipated, colony morphology and enzyme secretion of GM3ΔabraB correspond to the M18 phenotype. Subsequently, natural genetic competence was checked using 9945A chromosomal DNA to select prototrophs in transformation experiments. The transformation efficiency of GM3ΔabraB (ΔglyA, ΔabraB::tet, ΔRM) of 21.30% clearly exceeds the value of 0.01% for the corresponding wild-type GM3 (ΔglyA, ΔRM), showing that the abraB deletion contributes to natural competence. The efficiency observed for M18ΔabraB (Arg–, PGA–, ΔabraB::tet) (91.14%) equals that of M18. For absolute numbers [transformants (μg DNA)^−1] obtained in each experiment, see Table S3.

**Re-establishment of abraB reduces transformability**

For restoring abraB in B. licheniformis M18, the intact gene was integrated as part of a complementation cassette (Fig. S5A) and the replacement was verified by PCR, which delivered the expected amplicon sizes for B. licheniformis M18, 9945A and two randomly chosen abraB restored strains (referred to as M18 abraB+ in Fig. S5B). Sequencing revealed that both of the M18 abraB+ strains carry an intact copy of the gene (Fig. S6A). Plate assays to check the secretion capability for extracellular enzymes protected protease activities of the M18 abraB+ strains as for the wild-type (Fig. 4c); PGA production on minimal agar was likewise restored (Fig. S6B). In line with such findings is the fact that the complemented strains are hardly transformable (i.e. 0.01 and 0.00 % of M18, respectively), which agrees with the wild-type 9945A transformation frequency (0.00 %) (Fig. 4c).

**DISCUSSION**

In B. licheniformis, natural genetic competence was only reported in regulator mutants: due to the is3bla1-mediated inactivation of comP, B. licheniformis DSM13 develops – although poorly – natural genetic competence (Hoffmann et al., 2010) and, as shown here, the highly transformable B. licheniformis 9945A derivatives M28 and M18 (Thorne & Stull, 1966) carry mutations in degS and abraB, respectively. As the M28 degS deletion created in the parental strain 9945A increased transformability, a key role of the mutation for competence development of M28 was clear. Although removal of the RM system (ΔRM) on the degSΔ22–35 background enhanced transformability to 200%, such deletion is not causal for transformability, as it manifests solely in the degSΔ22–35 background. The ability to develop genetic competence is thus due to the impaired degS; supposedly, as for B. subtilis, the lack of the protein results in a high level of unphosphorylated DegU, which primes the auto-stimulatory ComK expression loop. The further tremendous increase in transformability caused by the additional deletion of the RM system (degSΔ22–35, ΔRM) in the 9945A background is in accordance with reports for B. licheniformis DSM 13 which – after deletion of both RM systems present in the strain – displayed a clear increase in transformability (Waschkau et al., 2008). Such enhancement is probably due to the lack of DNA degradation rather than the additional increase in natural genetic competence. The increase in transformability caused by ΔRM in the 9945A background might, however, be due to the lack of collateral mutations being detrimental for competence development in M28. Consistent with the comP::is3bla1-mediated phenotype of B. licheniformis DSM 13 is that M28 lost its high genetic competency and regained efficient extracellular enzyme secretion when degS was restored. In B. subtilis 168, degS mutants display decreased levels of degradative enzymes as well, but in contrast to B. licheniformis, competence development is not affected (Msdalek et al., 1990).

As M18, like M28, emerged from several rounds of random mutagenesis (Leonard & Mattheis, 1965), additional competence affecting mutations cannot be excluded. As deletion of the remaining part of abraB in M18 delivered transformation efficiencies equal to M18, and the data obtained by qPCR additionally proved that the truncated abraB is not transcribed, AbraB is not needed for competence development in B. licheniformis. This finding is different from B. subtilis, in which low levels are required (Hahn et al., 1995a), presumably by repressing rok transcription (Hoa et al., 2002). Although there is a Rok homologue in B. licheniformis (Rachinger et al., 2013a; Rey et al., 2004; Veith et al., 2004), its cellular tasks remain obscure. In B. subtilis, Rok functions in addition to regulating comK expression as a repressor of genes encoding membrane-localized and secreted proteins (Albano et al., 2005) and, moreover, the existence of several rok-like genes was recently reported (Singh et al., 2012). As anticipated for a general transition state regulator, repair of the truncated abraB in M18 restored the wild-type colony morphology and extracellular enzyme secretion. As the development of natural genetic competence also dropped to the wild-type level, the comK promoter is apparently repressed by the intact AbraB as for B. subtilis.

The highly competent B. subtilis 168 (Anagnostopoulos & Spizizen, 1961; Spizizen, 1958) was – not least because of this attribute – spread worldwide, becoming the paradigm for the species (Kunst et al., 1997) as well as for the related B. licheniformis. The former descended from derivatives...
of the X-ray mutagenized *B. subtilis* Marburg strain (Burkholder & Giles, 1947; Zeigler et al., 2008), which were, as a result of the mutagenic treatment, collaterally also disturbed in biofilm formation (McLoon et al., 2011). Notably, all the hitherto known *B. subtilis* and *B. licheniformis* strains displaying efficient natural genetic competency turned out to be regulator mutants. Hence, mutagenic treatments during successive domestication steps ought to be kept in mind when rating a strain’s competence potential.

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