

# Targeted deletion of genes encoding extracellular enzymes in *Bacillus licheniformis* and the impact on the secretion capability

Jens Waldeck<sup>a</sup>, Heike Meyer-Rammes<sup>a</sup>, Susanne Wieland<sup>b</sup>,  
Jörg Feesche<sup>b</sup>, Karl-Heinz Maurer<sup>b</sup>, Friedhelm Meinhardt<sup>a,\*</sup>

<sup>a</sup> Westfälische Wilhelms-Universität Münster, Institut für Molekulare Mikrobiologie und Biotechnologie,  
Corrensstrasse 3, 48149 Münster, Germany

<sup>b</sup> Henkel KGaA, VTB-Enzymtechnologie, Henkelstrasse 67, 40191 Düsseldorf, Germany

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## Abstract

The general secretory pathway is routinely concerned with a multitude of extracellular enzymes. By eliminating obstructive competitors the export machinery may transport larger quantities of remaining proteins under circumstances in which the secretion machinery is fully loaded. Hence, in this study, genes encoding efficiently expressed but dispensable exoenzymes were knocked out in *Bacillus licheniformis* MD1. Single, double, and triple mutants with deletions of *celA*, *chiA*, and *amyB*, respectively, were generated via *in vivo* recombination by making use of a vector with a temperature sensitive origin of replication.

Overexpression of a heterologous amylase gene on a multi-copy plasmid, a common scenario in biotechnological processes, resulted in an articulate reduction of chromosomally encoded extracellular enzyme activities indicating that the secretion machinery works to capacity in such transformants. Deletion mutants with the expression plasmid displayed enhanced amylase activities compared to the strain with the wild type genetic background. In addition, the chromosomally encoded protease activity was clearly higher in transformants with deletions.

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## 1. Introduction

Genetic improvement of bacterial extracellular enzyme production was and still is achieved by applying a panoply of strategies, such as amplification of gene copy number (Gupta et al., 2002), modulation of signal peptides (Quax et al., 1993), or fusions to heterologous signal peptides for efficient targeting to the translocase (Bolhuis et al., 1999; Li et al., 2004). As for many other applications strong promoters are being used to boost extracellular enzyme formation. To further improve productivity, secretion can be enhanced by increasing the copy number of signal peptidase genes (Pummi et al., 2002; Nahrstedt et al., 2004), and by preventing degradation of extracellular enzymes using protease deficient strains (Wittchen and Meinhardt, 1995; Malten et al., 2005). Deregulation and/or co-expression of chaperon encoding genes may also have

positive influences, as efficient protein folding is ensured (Li et al., 2004). Almost all strategies aiming at optimization of extracellular enzyme production in bacteria focused on widening of bottlenecks of secretion and/or on strong expression and enhanced stability of the enzyme concerned. However, overproduction of secreted proteins has turned out to be a rather complex process sometimes severely affecting the secretory system (Wong, 1995), eventually resulting in the so-called secretion stress response, that may limit secretion (Tjalsma et al., 2000; Antelmann et al., 2003). To counteract possible losses or reductions in yield we followed a different approach, i.e. targeted deletion of genes encoding extracellular enzymes secreted via the general secretory pathway (Tjalsma et al., 2004) in order to reduce the burden of the secretion machinery. Such strategy, though previously suggested to bear the potential for improving production (Vehmaanperä et al., 1991; Simonen and Palva, 1993), was mainly applied for generating biosafety strains (Wittchen et al., 1998; Nahrstedt and Meinhardt, 2004; Nahrstedt et al., 2005a,b; Waldeck et al., 2007). Though the knock out of protease encoding genes was found to have

\* Corresponding author. Tel.: +49 251 83 39825; fax: +49 251 83 38388.  
E-mail address: [meinhar@uni-muenster.de](mailto:meinhar@uni-muenster.de) (F. Meinhardt).

positive effects on extracellular enzyme activities in *Bacilli* (Malten et al., 2005), it has not been applied in such a concerted way in order to enhance extracellular enzyme production.

Representatives of the generally regarded as safe (GRAS) bacterium *Bacillus licheniformis* are extensively employed for industrial production of extracellular enzymes (such as proteases and amylases) with yields up to 25 g l<sup>-1</sup> (Schallmeyer et al., 2004). The availability of the genome sequence from *B. licheniformis* DSM13 which is isogenic to ATCC 14580 (Rey et al., 2004; Veith et al., 2004) facilitates direct genetic manipulations and, thus, maximum exploitation.

By targeted deletions of *celA*, *chiA*, and *amyB*, encoding cellulase, chitinase, and  $\alpha$ -amylase, respectively, a set of *B. licheniformis* MD1 mutant strains was generated, and vegetative growth as well as production of exoenzymes was monitored. To our knowledge this study constitutes the first systematic approach to check the impact of such defined deletions on a bacterium's secretion capability.

## 2. Materials and methods

### 2.1. Growth conditions

Strains and plasmids used in this study are listed in Table 1. *Escherichia coli* and *B. licheniformis* cells were routinely cultivated at 37 °C in Luria-Bertani (LB) broth unless otherwise stated. Plasmid-carrying *E. coli* and *B. licheniformis* strains were grown with 100  $\mu$ g ampicillin, 5  $\mu$ g erythromycin, and 50  $\mu$ g kanamycin ml<sup>-1</sup>, respectively.

### 2.2. Cloning and sequencing

Molecular cloning procedures were carried out essentially as described in Sambrook and Russell (2001). Genomic DNA from *B. licheniformis* was isolated as described in Gärtner et al. (1988). PCR reactions of 100  $\mu$ l contained 200  $\mu$ M deoxynucleotides, 100 ng of template DNA, 1 pmol of each primer, and 1 U of *Taq* DNA polymerase (Eppendorf AG, Hamburg, Germany) or *Vent*<sup>®</sup> DNA polymerase (New England Biolabs, Frankfurt a. M., Germany). PCR primers, listed in Table 2, were designed on the basis of the tentative *B. licheniformis* DSM13 genome sequence (Veith et al., 2004). Purification of DNA fragments following gel electrophoresis was performed with the Perfectprep<sup>®</sup> Gel Cleanup Kit (Eppendorf AG). Nucleotide sequences were determined with fluorescent-labeled dideoxynucleotides using the BigDye<sup>®</sup> Terminator v3.1 sequencing kit (Applied Biosystems, CA, USA) and an ABI Prism<sup>®</sup> capillary sequencer (model 3700). For sequence analyses, tools of the Lasergene<sup>®</sup> sequence analysis software (DNASTAR Inc., Madison, WI, USA), Clone Manager Professional Suite 7.01 (Scientific & Educational Software, Cary, NC, USA), NCBI server (Bethesda, MD, USA) and the HUSAR program package (EMBL, Heidelberg, Germany) were applied.

### 2.3. Isolation and sequencing of *amyB*, *celA*, and *chiA*

The coding region as well as parts of the up- and downstream regions of the *amyB*, *celA*, and *chiA* genes were isolated from chromosomal DNA of *B. licheniformis* MD1 via PCR using

Table 1  
Bacterial strains and plasmids

Strain or plasmid	Description	Source/reference
<b>Strains</b>		
<i>Escherichia coli</i> JM109	F <sup>-</sup> , <i>traD36</i> , <i>proAB</i> <sup>+</sup> , <i>lacI</i> <sup>q</sup> , $\Delta$ ( <i>lacZ</i> ), M15/ $\Delta$ ( <i>lac-proAB</i> ), <i>glnV44</i> , <i>e14</i> <sup>-</sup> , <i>gyrA96</i> , <i>recA1</i> , <i>relA1</i> , <i>endA1</i> , <i>thi</i> , <i>hsdR17</i>	Yanisch-Perron et al. (1985)
<i>Bacillus subtilis</i> DB104	<i>his</i> , <i>nprR2</i> , <i>nprE18</i> , <i>aprD3</i>	Kawamura and Doi (1984)
<i>Bacillus licheniformis</i> MD1	wild type	Henkel KGaA
<i>B. licheniformis</i> MD1.3	$\Delta$ <i>celA</i>	This work
<i>B. licheniformis</i> MD1.4	$\Delta$ <i>celA</i> , $\Delta$ <i>amyB</i>	This work
<i>B. licheniformis</i> MD1.5	$\Delta$ <i>amyB</i>	This work
<i>B. licheniformis</i> MD1.6	$\Delta$ <i>chiA</i>	This work
<i>B. licheniformis</i> MD1.7	$\Delta$ <i>chiA</i> , $\Delta$ <i>celA</i>	This work
<i>B. licheniformis</i> MD1.8	$\Delta$ <i>chiA</i> , $\Delta$ <i>celA</i> , $\Delta$ <i>amyB</i>	This work
<i>B. licheniformis</i> MD1.9	$\Delta$ <i>chiA</i> , $\Delta$ <i>amyB</i>	This work
<b>Plasmids</b>		
p $\Delta$ <i>amyB</i>	pE194-derivative with deletion fragment for <i>B. licheniformis amyB</i>	This work
p $\Delta$ <i>celA</i>	pE194-derivative with deletion fragment for <i>B. licheniformis celA</i>	This work
p $\Delta$ <i>chiA</i>	pE194-derivative with deletion fragment for <i>B. licheniformis chiA</i>	This work
pKTH10	Contains the <i>amyE</i> gene of <i>B. amyloliquefaciens</i> ; ori <sub>pUB110</sub> ; Km <sup>R</sup>	Palva (1982)
pSKE194	<i>E. coli/Bacillus</i> -shuttle vector, Ap <sup>R</sup> /Em <sup>R</sup> , ori <sub><i>E. coli</i></sub> /ori <sup>ts</sup>	Nahrstedt et al. (2005b)
pSKE194 <i>amyB</i>	pSKE194-derivative with deletion fragment for <i>B. licheniformis amyB</i>	This work
pSKE194 <i>celA</i>	pSKE194-derivative with deletion fragment for <i>B. licheniformis celA</i>	This work
pSKE194 <i>chiA</i>	pSKE194-derivative with deletion fragment for <i>B. licheniformis chiA</i>	This work
pUCBM20	<i>E. coli</i> cloning vector; Ap <sup>R</sup>	Boehringer Mannheim, Germany
pUC <i>amyB</i>	pUCBM20-derivative deletion fragment for <i>B. licheniformis amyB</i>	This work
pUC <i>celA</i>	pUCBM20-derivative deletion fragment for <i>B. licheniformis celA</i>	This work
pUC <i>chiA</i>	pUCBM20-derivative deletion fragment for <i>B. licheniformis chiA</i>	This work

Ap<sup>R</sup>, ampicillin resistance; Em<sup>R</sup>, erythromycin resistance; ori<sup>ts</sup>, temperature-sensitive *Bacillus* origin of replication.

Table 2  
Oligonucleotides used as primers

Oligonucleotides	5' → 3'-sequence
amy1	ACGAGCCCTCAGGAAGAAC
amy2	AGCGATCCGGTCGAGAAAC
amy3	GGGGTCTCCCGCATGGCTCATGAC
amy4	CGCGGGTGATCAATCATCC
amy10	CGGAATCCGTCTGCTGACATG
celA1	<u>AACTGCAGGGATATGGTGC</u> CGGTTG
celA2	AATG <u>CCCCGGG</u> GACTTTGCACATCC
celA3	TGACGGACACCGGATTTG
celA4	TTTCGGCGTCATCATGCCTC
cel4	ATGCTGATTCTCGCCAGTTACC
cel10	CTGCCGGGTCAGAAAAGATTTCG
chi1	TCCAAACGCTGCAGACCTAC
chi2	TTTATCGCCTGGGTAGCC
chi3	GGGGCCTTTTCGTCACCAATTC
chi4	CATCGCCTCATACTCTG
chi12	GAAACTACACGCTGCTCCTG
chi13	CGAGTTTGATACAGGTCTCATTGG

Generated restriction sites and 5'-extensions are underlined.

primers amy1/amy2, celA1/celA4, and chi1/chi4, respectively. The upstream region of *celA* was isolated from chromosomal DNA of *B. licheniformis* MD1 using vectorette PCR (Ross-Macdonald et al., 1998). All fragments obtained were sequenced on both strands and submitted to the EMBL database under accession no. AJ786636 (*amyB*), AJ616005 (*celA*), and AJ786637 (*chiA*).

#### 2.4. Disruption vectors and gene knock outs

For targeted gene disruption, the *E. coli/Bacillus* shuttle vector pSKE194 (Nahrstedt et al., 2005b) was used. To create an 1781 bp *celA*-deletion spanning the entire coding region, two PCR products obtained from chromosomal DNA of strain MD1 were applied: flank A (position 1057–1425) was amplified with primer pair celA1/celA2, and flank B (position 3206–3646) was generated using primers celA3 and celA4 (nucleotide positions correspond to the sequence deposited in the EMBL database, see also Fig. 1A). By cloning such fragments into pUCBM20 a *celA* deletion cassette was constructed and subsequently ligated into the single *PstI* site of shuttle vector pSKE194, resulting in pSKE194celA (see Table 1). An *XbaI* fragment from pSK194celA comprising the *Bacillus* origin of replication, the erythromycin resistance gene and the deletion cassette was excised and religated, resulting eventually in the *Bacillus* deletion vector pΔcelA. Similarly, for *amyB*-deletion, a 2026 bp fragment was chosen and flank A (amplified with amy1/amy3) from nucleotide 1 to 640 and flank B (amy2/amy4) from 2686 to 3430 were cloned accordingly (primer pair positions are also depicted in Fig. 1B). Following construction in pUCBM20, the respective deletion cassette was ligated into the *PstI* site of pSKE194 and from the obtained vector pSKE194amyB, again an *XbaI* fragment was excised and religated, resulting in disruption vector pΔamyB. As for *celA* and *amyB*, a *chiA*-deletion spanning 2552 bp was created by applying the constructed pΔchiA vector. Two PCR products serving as recombination flanks were generated using primer pair chi1/chi3 and

Table 3  
Predicted gene products of sequenced loci of *B. licheniformis* MD1

Predicted protein	Identity		
	%	aa-overlap	Protein, organism [accession no.]
'YjeA	64	279/279	YjeA, Bs [G69849]
AmyB	100	512/512	AmyS, Bl [P06278]
YvdE'	87	248/248	YvdE, Bs [O06987]
'ScdA	54	225/229	ScdA, Ba Ames [AAP26031]
FenH	73	115/123	FenH, Bs F29-3 [AAF32333]
CelA	99	517/517	Cel5A, Bl GXN151 [AAP51020]
YnfE	65	85/85	YnfE, Bs [NP_389696]
ChiB	78	303/314	Chi, Bs [AAC23715]
ChiA	99	693/693	ChiA, Bl DSM13 [AAU39297]
Mpr'	44	296/308	Mpr, Bs 168 [A35122]

Identity to homologous polypeptide in %, the aminoacid (aa)-overlap and the homologous protein name of respective strain as well as the corresponding accession number (in square brackets) are also stated. Ba, *Bacillus anthracis*; Bl, *Bacillus licheniformis*; Bs, *Bacillus subtilis*.

chi2/chi4 (see Table 3) and cloned into pUCBM20. Such deletion cassette was inserted into the single *PstI* site of pSKE194 resulting in pSKE194chiA. Elimination of the smaller *XbaI*-fragments by restriction and religation (see also Fig. 1) revealed pΔchiA. Respective disruption vectors were introduced into *B. licheniformis* by PEG-mediated transformation of protoplasts essentially as described by Chang and Cohen (1979). Selection was performed with 5 μg erythromycin ml<sup>-1</sup> at 30 °C. Subsequently, clones with an integrated vector were identified by growth on agar plates with 0.3 μg erythromycin ml<sup>-1</sup> at 42 °C but lacking growth with 5 μg ml<sup>-1</sup>. Cultivation without the antibiotic at 42 °C yielded plasmid-free clones, which were screened via PCR for deletions.

#### 2.5. Enzyme assays

*B. licheniformis* was cultivated for 36 h at 37 °C in 20 ml or 120 ml M9 minimal medium (Sambrook and Russell, 2001) supplemented with 0.1 mM CaCl<sub>2</sub>, 0.01% yeast extract, 0.02% casamino acids, and 0.2% glucose or 0.3% succinate as carbon source. It is noteworthy to state that enzyme activities were routinely higher in small volume cultures (20 ml) in Erlenmeyer flasks without a baffle than in the 120 ml cultures in baffle flasks (see Figs. 3B and 4B). Aliquots were centrifuged at 7000 g, and supernatants used for assaying enzymatic activities. Glucose concentration was determined according to FitzGerald and Vermerris (2005) with Accu-Chek Sensor Comfort strips (Roche Diagnostics GmbH, Mannheim, Germany). Protein concentration was measured according to Bradford (1976).

For checking enzyme production qualitatively, amylase-, protease-, chitinase-, and cellulase-activities were determined on minimal medium agar plates containing 0.5% (w/v) soluble starch, 2.0% (w/v) skim milk, 1.0% (w/v) swollen chitin (Gomez Ramirez et al., 2004), and 0.5% (w/v) carboxymethylcellulose, respectively. Following incubation at 37 °C for 24–48 h, amylase-plates were overlaid with Lugol's solution and cellulose-plates with congo-red (0.1%, w/v). Chitinase-plates were incubated at 37 °C for 4 days and clearing halos were determined.

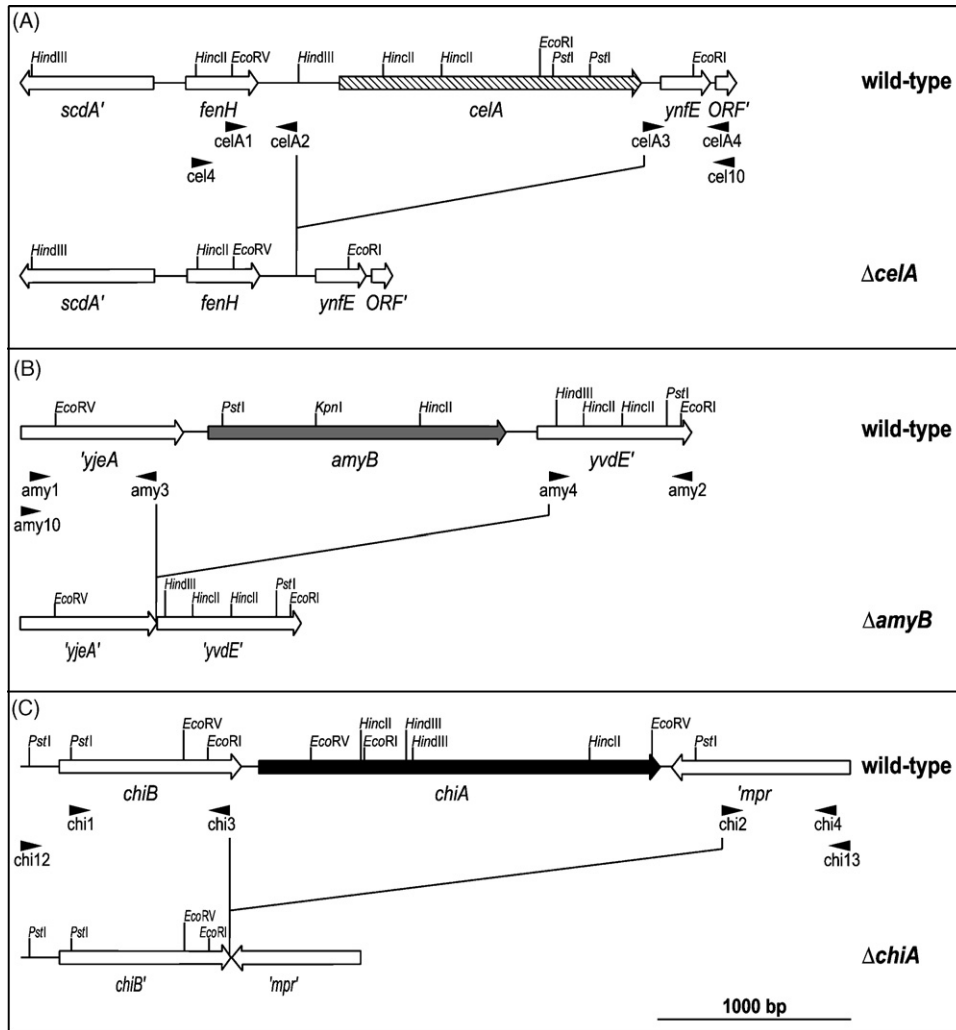


Fig. 1. *B. licheniformis* MD1: schematic representation of the *celA*, *amyB*, and *chiA* encoding genomic regions and respective deletions. Open reading frames (ORFs) are depicted as arrows, the direction of which corresponds to the transcriptional orientation. Genes were designated based on similarities to loci from *Bacillus* spp. as a result of BLAST searches in the NCBI database (see also Table 3). PCR primers are marked as solid arrows (for details see Table 2). (A) The *celA* encoding region comprises of 2793 bp amplified with the primer pair *celA1* and *celA4*: '*scdA*', partial ORF coding for a putative cell division protein; '*fenH*', complete ORF probably involved in fengycin resistance; '*celA*', encoding a putative endo-beta-1,4-glucanase; '*ynfE*', complete ORF coding for a hypothetical protein; '*ORF*', partial putative ORF of unknown function;  $\Delta*celA*$ , has a 1781 bp-deletion. (B) The 3470 bp spanning *amyB* genomic region comprises: '*yjeA*', partial ORF, homologous to an endo-1,4-beta-xylanase; '*amyB*', putative alpha-amylase gene; '*yvdE*', partial ORF encoding a putative HTH-type transcriptional regulator;  $\Delta*amyB*$ , has a 2026 bp-deletion. (C) The *chiA* genomic region consists of 4303 bp with *chiA* and *chiB*, coding for putative chitinases; '*mpr*', partial ORF encoding an extracellular metalloprotease;  $\Delta*chiA*$ , has a 2552 bp-deletion.

2.6. Protease

Protease activity was calculated according to enzymatic hydrolysis of succinyl-Ala-Ala-Pro-Phe-*p*-nitroanilide (AAPF), purchased from Bachem AG (Weil am Rhein, Germany), dissolved in dimethylsulfoxide and finally diluted in buffer to a final concentration of 1.1 mM. Assays were performed in 0.1 M Tris-HCl (pH 8.6) and 1% Brij<sup>®</sup> 35 at 25 °C. The amount of *p*-nitroaniline released was measured at 410 nm (molar absorption coefficient value of 8480/M × cm as described by DelMar et al. (1979)). One unit is defined as the activity releasing 1 μmol of *p*-nitroaniline per minute. The specific activity was calculated as activity per milligram of protein (Kannan et al., 2001).

2.7. Amylase

Amylase activities were assayed applying the Phadebas<sup>®</sup> test (Pharmacia Diagnostics, Freiburg, Germany) by measuring the release of soluble blue colored fragments into the supernatant from an insoluble blue polymer (Hall et al., 1970). One Phadebas<sup>®</sup> pill was suspended in 10 ml A. dest. and aliquots of 500 μl were used to assay 50 μl of supernatant. The reaction was terminated after 15 min by adding 150 μl of 0.5 M NaOH. Controls were treated the same way, but adding the supernatant after stopping with NaOH. The soluble blue hydrolysis products were separated from the insoluble blue substrates by centrifugation (14,000 g) and absorption against the blank was measured at 620 nm. Enzyme activities (U l<sup>-1</sup>) were calculated by making

use of a calibration curve which was obtained with a commercial  $\alpha$ -amylase.

### 3. Results

#### 3.1. Generation of deletion mutants

Targeted deletion of genes encoding dispensable exoenzymes may reduce the burden of the secretion machinery. Thus, we selected polypeptides known to be efficiently produced (Veith et al., 2004; J. Feesche, unpublished results). After checking appropriate extracellular enzymatic activities in plate assays (see also Fig. 2) we introduced defined deletions in MD1, comprising three different loci, i.e. *celA*, *chiA*, and *amyB*, which code for a cellulase, chitinase and  $\alpha$ -amylase, respectively. Since genomic data of *B. licheniformis* was not completely available at that time we isolated and sequenced the corresponding regions on both strands and submitted our data to EMBL (see Section 2 for accession numbers, Table 3). The genetic organization of each locus (depicted in Fig. 1) clearly resembles that of *B. licheniformis* DSM13, displaying only minor deviations at the nucleotide level. As outlined in Section 2 and the legend to Fig. 1, deletion cartridges for each locus were constructed and introduced into recipient strains on temperature sensitive (ts) shuttle-plasmids eventually resulting in the projected deletions (Fig. 1). Respective mutants were screened and characterized via PCR (Fig. 2A). Sequencing of the mutated regions – an overview of the constructed single-, double- and triple-mutants is given in Table 1 – revealed the deletion(s) being located precisely at the intended sites (data not shown).

Furthermore, cells of the *B. licheniformis* wild type strain (MD1) and deletion mutants were spotted onto carboxymethylcellulose- (cmc-), chitin-, or starch-agar plates to verify qualitatively their (lacking) enzyme production. The  $\Delta celA$  as well as the  $\Delta chiA$  mutants, lost most of their enzymatic activities; mutants carrying the  $\alpha$ -amylase gene deletion were totally devoid of respective enzymatic activities (Fig. 2B), which corresponds to genotypic investigations summarized in Fig. 1.

#### 3.2. Growth and secretion capability of deletion mutants

Mutant strains were checked for possible negative side effects on vegetative growth and extracellular enzyme synthesis in 120 ml batch fermentations in baffled flasks. Initial studies in complex liquid media such as LB, based on measurements of the optical density at 546 nm, revealed no differences for any of the mutant strains compared to the wild type. Likewise, no deviations for the mutants' vegetative growth in minimal media were observed, neither in the case of glucose as the single carbon source (Fig. 3A, solid lines) nor when succinate was used (not shown). Also, carbon source consumption during growth in minimal medium with glucose did not differ, as depicted in Fig. 3A (dashed lines).

To scrutinize the strains' abilities to synthesize and secrete extracellular enzymes we chose the well-studied protease activity of *B. licheniformis* as an intrinsic reporter; furthermore, the mutants were checked for  $\alpha$ -amylase expression (BLA) whenever possible. Initial protease tests on skim milk-plates revealed clearing halos similar in size to that of the wild type (not shown). As shown for amylase activity in a former study (Nahrstedt et al.,

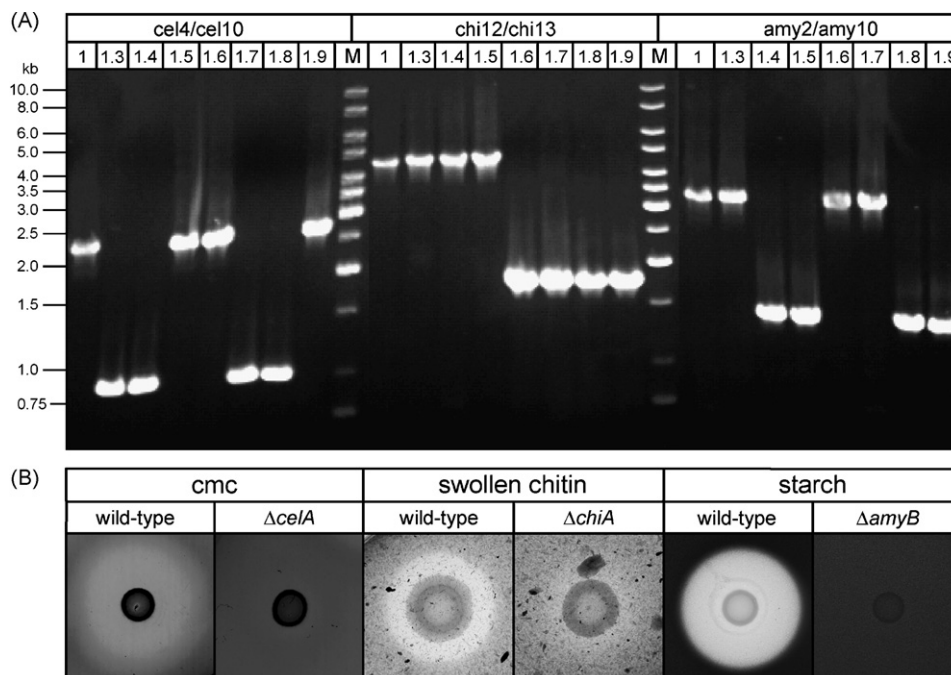


Fig. 2. Genotypic and phenotypic characterization of *B. licheniformis* mutants (MD1.1–MD1.9) in comparison to *B. licheniformis* wild type (MD1). (A) PCR analyses. Chromosomal DNA served as the template for amplification with primers specific for *celA* (*cel4/cel10*), *chiA* (*chi12/chi13*), and *amyB* (*amy2/amy10*), respectively. For positions of primers see Fig. 1. M, 1 kb DNA ladder; lanes 1–1.9 display amplicons of the corresponding *B. licheniformis* MD strains (see also Table 1). (B) *B. licheniformis* wild type (MD1) and single-mutants were spotted on agar plates containing carboxymethylcellulose (cmc), swollen chitin, and starch, respectively. Clearing halos depict enzyme activities.

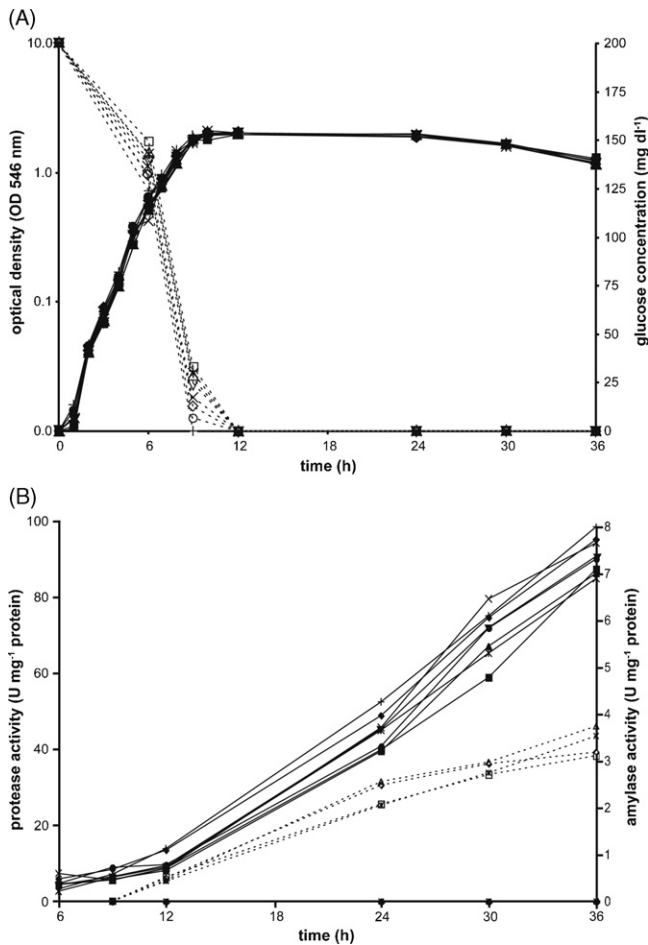


Fig. 3. Batch fermentation as well as protease and amylase activities of wild type *B. licheniformis* MD1 and the *B. licheniformis* mutants MD1.3–MD1.9. (A) Cells were cultivated in 120 ml M9 medium with 200 mg dl<sup>-1</sup> glucose as the sole carbon source at 37 °C for 36 h in Erlenmeyer flasks with a baffle. Growth was monitored by measuring the optical density (OD) at 546 nm. Glucose consumption was determined as outlined in Section 2. Mean values were calculated from two independent experiments. The OD values are depicted as solid lines, glucose concentrations correspond dashed lines. (B) Protease activities (determined with AAPF) and amylase activities (applying the Phadebas® test). Mean values calculated from triplicates of two independent experiments are given. Protein concentrations according to Bradford (1976). (■) and (□), *B. licheniformis* MD1 (wild type); (◆) and (◇), *B. licheniformis* MD1.3 ( $\Delta celA$ ); (⊕) and (+), *B. licheniformis* MD1.4 ( $\Delta amyB$ ); (●) and (○), *B. licheniformis* MD1.5 ( $\Delta celA$ ,  $\Delta amyB$ ); (▲) and (△), *B. licheniformis* MD1.6 ( $\Delta chiA$ ); (✱) and (✶), *B. licheniformis* MD1.7 ( $\Delta celA$ ,  $\Delta chiA$ ); (▼) and (▽), *B. licheniformis* MD1.8 ( $\Delta celA$ ,  $\Delta chiA$ ,  $\Delta amyB$ ); (×) and (×), *B. licheniformis* MD1.9 ( $\Delta chiA$ ,  $\Delta amyB$ ).

2005b), protease and amylase activities were lower in LB than in minimal medium with glucose as the sole carbon source, presumably due to high concentrations of free amino acids in LB, and that is why, we employed minimal medium (MM) for monitoring BLA and protease activities quantitatively. All strains, cultivated up to 36 h, reached the stationary phase after ~10 h at an OD<sub>(546 nm)</sub> of approximately 2.0 (Fig. 3A). Samples were taken at medial (6 h) and late (9 h) exponential growth-phase, and at early (12 h) and late stationary phase (24 h) as well as at the incipient decay phase (30 and 36 h); undiluted supernatants were used for determining specific enzyme activities. As expected,

values constantly increased at every reading point (Fig. 3B); amylase activities were not seen in mutants MD1.4, MD1.5, MD1.8, and MD1.9, due to the deletion of *amyB* and the absence of other genes encoding starch-degrading polypeptides. Protease and  $\alpha$ -amylase values of the mutants and the wild type displayed no discernable differences at all (Fig. 3B). Enzyme assays of the supernatants of M9-cultures grown with succinate (instead of glucose) as sole carbon source revealed congruent results (data not shown). Thus, under the experimental conditions applied, synthesis and secretion of chromosomally encoded proteases and (if available) amylases were neither positively nor negatively affected in the deletion mutants.

Overexpression of a heterologous enzyme was expected to overload the secretion machinery. Hence, plasmid pKTH10 carrying the  $\alpha$ -amylase encoding gene of *Bacillus amyloliquefaciens* (Palva, 1982) was transformed into the  $\Delta amyB$  mutants as well as the wild type aiming at hyperproduction. Indeed, for the transformants, amylase activity was found to be remarkably high (see Fig. 4A and B, upper part). Since we obtained enhanced extracellular enzyme activities in small volume fermentations performed in Erlenmeyer flasks without a baffle, these experiments were performed applying such conditions. The values in Fig. 3B have been obtained in fermentations carried out with baffle-flasks in 120 ml M9 medium. Though standard deviations did not permit explicit conclusions, it is noteworthy that – at the average – amylase activities were enhanced on a mutant background in either case. To check whether these results express a firm tendency we used the triple deletion transformant MD1.8/pKTH10 in twelve independent batch fermentations and determined amylase activities in comparison to the respective wild type transformant MD1/pKTH10. The mean values obtained were 292.1 U mg<sup>-1</sup> ( $\pm 13.5$ ) protein for MD1.8/pKTH10 and 269.6 U mg<sup>-1</sup> ( $\pm 32.9$ ) protein for MD1/pKTH10, which agrees with our findings outlined in Fig. 4. In addition, the protease activity was quantified and compared to that of the wild type as an intrinsic reporter for extracellular enzyme activity (see Fig. 4B). Specific protease activities in pKTH10 carrying strains were always considerably lower, but – as for the amylase activity – the mean values for protease activity of the pKTH10 carrying deletion mutants were in either case higher than that of the transformant with the wild type genetic background.

#### 4. Discussion

Three efficiently expressed but dispensable genes for growth in standard media were chosen as targets for genetic knock outs aiming at a reduction of the burden of the secretion machinery. Deletions comprised the Shine/Dalgarno sequences (ribosomal binding sites) as well as the entire respective coding regions including the predicted signal peptides. The faint residual cellulase/glucanase activities of  $\Delta celA$  mutants are presumably due to additional glucan-hydrolysing enzymes such as that encoded by *celB* (accession no AJ786638) or others identified in the *B. licheniformis* genome (Rey et al., 2004; Veith et al., 2004). Similarly, the residual faint chitinase activity seen in  $\Delta chiA$  mutants may be due to the presence of an intact *chiB* gene product,

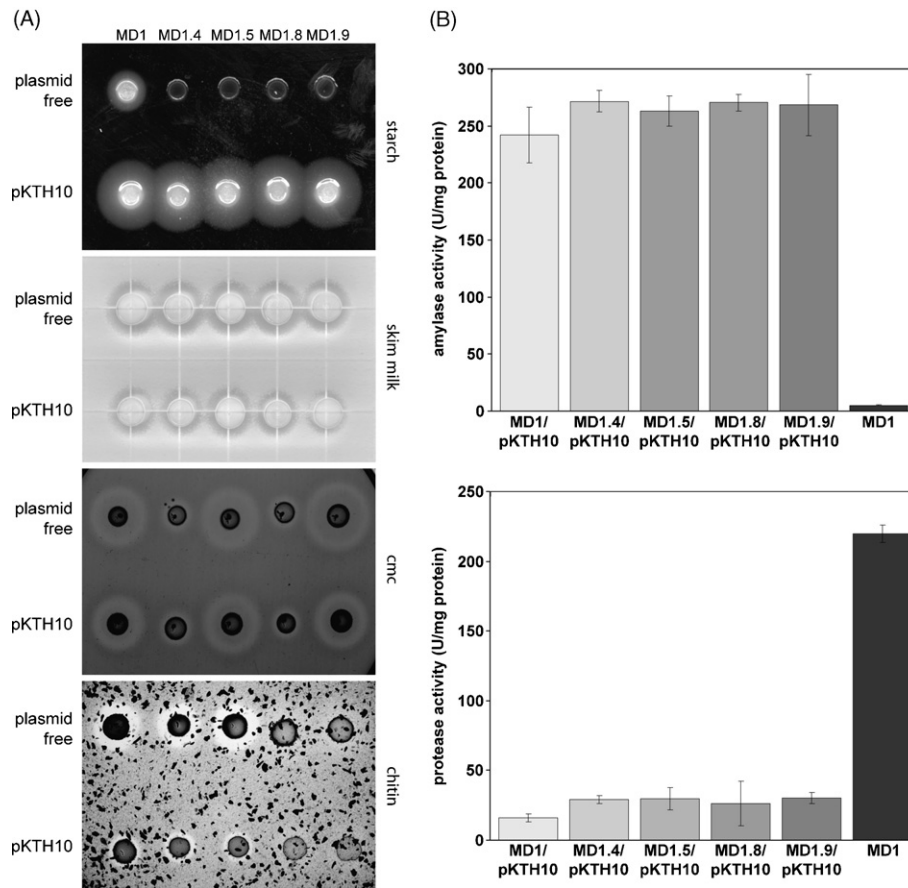


Fig. 4. Extracellular enzyme activities of pKTH10 carrying transformants in comparison to the plasmid free recipients. (A) Equal numbers of cells were spotted onto M9 agar containing starch, skim milk, cmc, and swollen chitin, respectively and incubated as outlined in Section 2. Clearing halos denote enzyme activities. (B) Enzyme assays of the supernatant from 36 h cultures in 20 ml M9 with 200 mg dl<sup>-1</sup> glucose as the sole carbon source at 37 °C; mean values from duplicates calculated from five independent experiments.

though in a previous study it was shown that a frame shift mutation in *chiA* resulted in loss of chitinase activity below a detectable level even though *chiB* was not affected (Waldeck et al., 2006). Anyway, *chiA* appears to be the major chitinase encoding gene in different *B. licheniformis* strains. At present it remains obscure, however, why the  $\Delta amyB$  did not display any corresponding hydrolysing activities although at least one alternative glucan-1,4- $\alpha$ -maltohydrolase ( $\alpha$ -amylase) is encoded in *B. licheniformis* (Veith et al., 2004). Amylase activities could neither be seen in plate agar nor the Phadebas<sup>®</sup> tests, the latter applied to define  $\alpha$ -amylase expression in deletion mutants precisely. Remaining enzyme activities (such as protease) did not differ between mutant and wild type strains, indicating that the strain's secretion capacity suffices to translocate all of the encoded extracellular enzymes. In other words, deleting 1–3 genes does not enhance secretion of other enzymes because the machinery is suited to secrete larger amounts of enzymes than being produced by the cell, i.e. under normal conditions secretion is not a limiting factor for such an industrial strain. Since growth in different media was not affected and secretion of chromosomally encoded enzymes was not negatively influenced in any of the mutants obtained, such strains appeared to be equally suited for enzyme production.

When a plasmid (pKTH10) harboring the  $\alpha$ -amylase gene of *B. amyloliquefaciens* was introduced the situation changed completely, presumably due to the overload of the secretion machinery. Secretion of (chromosomally encoded) proteases, cellulases, and chitinases was clearly impaired under these conditions, indicating that the secretion machinery was working to capacity, in other words, the mutants' extracellular, signal peptide possessing polypeptides compete for secretion.

Amylase production was on the average higher in mutants than in the wild type. Though standard deviations in fermentations aiming at overexpression of the amylase may at first sight detract from an augmentation, such finding was confirmed by a number of additional experiments. When extracellular intrinsic protease activity was measured, enhanced protein secretion in the deletion mutants became clearly evident. However, a systematic differentiation between single, double, and triple mutants was not possible.

From the *B. licheniformis* DSM13 genome sequence 296 predicted proteins were reported to have an N-terminal signal peptide, most of which (220) being presumably exported via the general secretory pathway since they display recognition sites for cleavage by a type I signal peptidase (Voigt et al., 2006). Thus, deletion of three genes theoretically may result in

approximately 1–2% relief but only if all of them are equally considered. Such influence on secretion is hardly measurable at all, thus, the choice of the genes (*celA*, *chiA*, *amyB*), which code for efficiently secreted enzymes, presumably contributed considerably. Consequently, the elimination of additional highly expressed and efficiently secreted but dispensable extracellular enzymes by deleting the respective genes will potentially reduce the burden of the strain's secretion machinery further. Other knock out targets, such as *yheN* coding for a putative endo-1,4-beta-xylanase, can be identified by extracellular proteome analysis (Voigt et al., 2006).

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