Isolation and Molecular Characterization of Chitinase-Deficient *Bacillus licheniformis* Strains Capable of Deproteinization of Shrimp Shell Waste To Obtain Highly Viscous Chitin[▽]

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Proteolytic but chitinase-deficient microbial cultures were isolated from shrimp shell waste and characterized. The most efficient isolate was found to be a mixed culture consisting of two *Bacillus licheniformis* strains, which were first determined microscopically and physiologically. Molecular characterization was carried out by sequencing the 16S rRNA gene of both strains. According to the residual protein and ash content, the chitin obtained by fermentation of such a mixed culture was found to be comparable to a commercially available, chemically processed product. However, the strikingly high viscosity (80 versus 10 mPa of the commercially available sample) indicates its superior quality. The two strains differed in colony morphology and in their secretion capabilities for degradative extracellular enzymes. Sequencing of the loci encoding amylase, cellulase, chitinases, and proteases, as well as the *degS/degU* operon, which is instrumental in the regulation of degradative enzymes, and the *pga* operon, which is responsible for polyglutamic acid production, revealed no differences. However, a frameshift mutation in *chiA*, encoding a chitinase, was validated for both strains, providing an explanation for the ascertained absence of chitinolytic activities and the concomitant possibility of producing highly viscous chitin in a fermentational deproteinization process.

Chitin (a polysaccharide consisting of β -1,4-linked *N*-acetyl-D-glucosamine moieties) is the second most abundant biopolymer on earth; thus, it represents a nearly constant source of renewable raw material. Along with its deacetylated derivative chitosan, chitin recently has gained biotechnological significance, not only because of favorable pharmaceutical features, such as antimicrobial, anticholesterol, and antitumor activities, but also because of its potential for wastewater treatment, drug delivery, and wound healing and as a dietary fiber (26).

Approximately 50 to 60% of the total weight of shellfish, such as shrimp, crab, and krill, consists of nonedible material, i.e., “heads” and exoskeletons rich in chitin but also protein, which form, on the one hand, major environmental pollutants as a result of uncontrolled dumping (12). On the other hand, however, due to their chemical composition (20 to 30% chitin, 20 to 40% protein, 30 to 60% minerals, and 0 to 14% lipids) and their actual availability from seafood industries, shrimp waste also constitutes the major source for chitin and chitosan production (21). Currently applied methods to purify and modify chitin from such material and to transform it to useful carbohydrate products involve harsh chemical treatments accompanied by uncontrollable hydrolysis and chemical modifications that eventually result in the formation of undesired by-products such as irregularly deacetylated polymers (33).

Exploitation limits are mainly set by the purification costs, which mainly arise from removal of proteins and calcium carbonate by alternating acid and alkali treatment, ultimately resulting in large amounts of aqueous waste. Bioconversion is, thus, of considerable interest. Since the application of enzymes, although effectively used in laboratory scale (6), causes uneconomical production costs, the use of living microbes facilitating efficient chitin purification is desirable. A panoply of microorganisms secrete proteolytic enzymes that routinely have wide substrate specificities (13). Thus, shrimp waste deproteinization with different pro- and eukaryotic microorganisms, such as *Pseudomonas aeruginosa*, *Enterococcus faecium*, *Bacillus subtilis*, and *Candida parapsilosis*, was attempted (3, 31, 33). However, either the degree of deproteinization was insufficient or the process was too time-consuming and/or costly sterilization processes had to be included.

In the present study, the isolation and phenotypic, as well as molecular genetic, characterization of new chitinase-deficient *Bacillus licheniformis* strains able to efficiently deproteinize shrimp shell waste, eventually resulting in chitin of superior quality, is described.

MATERIALS AND METHODS

Isolation of strains. Shrimp shell waste (10 g [dry weight]) was suspended in 100 ml of sterile saline solution (0.5% KH_2PO_4 , 0.5% $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.5% NaCl [pH 7.0]) and incubated for 48 h at 42, 50, or 55°C. Pasteurized (15 min 80°C) dilutions (in 0.9% NaCl) of the culture were spread onto shrimp protein agar plates and incubated again as described above. Shrimp protein medium consisted of 5 g of yeast extract, 5 g of KH_2PO_4 , 5 g of NaCl, 1 g of $\text{Mg}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$, and 100 g of freshly ground, dried shrimp shell waste, which was washed and separated from insoluble material by filtration and filled up to a total volume of 1 liter; the pH was adjusted to 7.0. Material from single colonies was transferred

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TABLE 1. Oligonucleotides used as primers in this study

Oligonucleotide	Sequence (5'-3')
amyB2	AGCGATCCGGTCGAGAAAC
amyB10	CGGAATCCGTCTGCTGACATG
aprE18	CAAAGCACCGAACGATTG
aprE_2888	TATCGCTGGTGCGTACGCTTC
celA1	AACTGCAGGGATATGGTGCGCGTTG
celA4	TTTCGGCGTCATCATGCCTC
chiB1	TTCTGCAGAGAAAGGTGACAAATCGTCTG
chiA6	GTCGATCATTCGGATACTC
sacU1	GGCCAAATGAACCCCTTGTGTCC
yviA2	TTTTATACTGTATACAGCGCATACCC
mpr2	CCATTCCGCCATTCAATAAAG
mpr4	CCCGAACGAAACGCTGCAGTAAAG
pga3	GATAATTTGGCGCATCCG
pga8b	AACGGCGTCGCTATTTC
16S-27f	AGAGTTTGATCMTGGCTCAG
16S-1492r	TACGGYTACCTTGTTACGACTT

to calcium-caseinate agar plates (Merck, Darmstadt, Germany) and incubated for 24 h to determine the proteolytic activity. Colonies forming a clearing halo were subjected to further analysis.

Batch fermentation and shrimp shell analysis. Precultures were grown overnight in 100 ml of shrimp protein broth (see above). Wet frozen shrimp waste from Palembang (Sumatra, Indonesia) was ground in a mill (pore size, 0.5 cm) and washed repeatedly with tap water. Standard medium consisted of 200 g of this shell waste, which was mixed with 1 liter of fermentation broth containing 5 g of KH_2PO_4 , 5 g of NaCl, and 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, whereas improved medium was also supplemented with 5 g of yeast extract and 1 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. Fermentation media were inoculated with 100 ml of preculture. Fermentation was carried out in a Labfors Master fermentor (Infors, Bottmingen, Switzerland) at 42, 50, or 55°C at 500 rpm and 2 volumes of air per volume of liquid per minute for 48 h. After 48 h, the fermented shrimp shells were harvested and washed. Finally, the fermentation product was demineralized with 0.9% lactic acid for 3 h and then oven dried.

Total nitrogen was measured by the Kjeldahl method as described by the supplier (Büchi, Flawil, Switzerland). Chitin nitrogen was measured and calculated as described previously (1). The protein content of the dried material was measured after hydrolyzing the shrimp shell protein with 1 M NaOH at 55°C for 24 h (15). To determine the ash content, samples were treated at 800°C for 3 h in a muffle furnace (Kendro, Langensfeld, Germany).

Viscometry. Insoluble chitin particles were removed by treatment with *N*-methyl-2-pyrrolidone-LiCl (19:1), agitation for 48 h (at room temperature), and centrifugation ($10,000 \times g$, 1 h, room temperature). The obtained pellet was dried, and the chitin concentration was determined gravimetrically. The soluble fraction was diluted in *N*-methyl-2-pyrrolidone-LiCl to a final concentration of 0.1%, and the viscosity was measured in a Viskotester 7L Plus (Haake, Karlsruhe, Germany) at a shear rate of 50 m/s; the results were analyzed by using the software RheoWin 3.12 (Haake).

Strain classification. For morphological classification, bacteria were cultivated on blood agar plates (Merck, Darmstadt, Germany). Microscopic characterizations were done applying the BX51 system equipped with differential interference contrast optics using the analySIS^B software package (Olympus, Hamburg, Germany). For DAPI (4',6'-diamidino-2-phenylindole) staining the cells were grown in Luria-Bertani broth at 37°C, harvested at mid-log phase, fixed in ethanol, and stained as described previously (11). Gram staining, Bactident oxidase strips (Merck), and 3% H_2O_2 solution were used for physiological tests. Carbon source utilization was performed with the API 50 CHB system (bio-Mérieux, Nürtingen, Germany) and analyzed by applying APILAB PLUS 3.3.3 software.

For checking protease, amylase, chitinase, and cellulase activities we used minimal medium agar plates (19) containing 2% (wt/vol) skim milk, 1% (wt/vol) soluble starch, 1% (wt/vol) swollen chitin (8), or 0.02% (wt/vol) lichenin, respectively. After incubation at 37°C for 24 to 48 h, amylase plates were overlaid with Lugol solution and cellulose plates were overlaid with 0.1% (wt/vol) Congo red. Protease and chitinase plates were incubated at 37°C for 1 to 11 days, and clearing halos were determined.

Molecular techniques. Molecular procedures were essentially carried out as described previously (23). Genomic DNA from *B. licheniformis* was isolated (7), and PCRs were performed as previously described (19). PCR primers (Table 1) were designed on the basis of the *B. licheniformis* DSM13 (isogenic to ATCC 14580) genome sequence (22, 30) or *B. licheniformis* MD1 sequences (accession no. AJ786636 [*amyB*], AJ616005 [*celA*], AJ786637 [*chiB*, *chiA*, and *mpr*] and AJ616006 [*degS-degU* operon]). All fragments obtained were sequenced on both strands using fluorescence-labeled dideoxynucleotides of the BigDye Terminator v3.1 sequencing kit (Applied Biosystems, California) and an ABI Prism capillary sequencer (model 3730) and then submitted to the EMBL database.

Amylase and protease activity. Amylase activities were measured by using the Phadebas test (Pharmacia Diagnostics, Freiburg, Germany) as recommended by the supplier. Proteolytic activities were assayed by using azocasein (Sigma, St. Louis, MO). Samples were prepared as described previously (18) and incubated at 55°C for 10 min. Nondigested azocasein was precipitated by centrifugation ($10,000 \times g$, 10 min), and the supernatant (750 μl) was transferred into a cuvette containing 250 μl of 0.5 M NaOH and measured at 440 nm. One protease unit equals the amount of enzyme releasing 1 μmol of azocasein per min under reaction conditions.

FIG. 1. Microscopic and macroscopic examination of the *B. licheniformis* F5 and F11 strains. F5 and F11 cells observed by differential interference contrast (DIC) and fluorescence microscopy (DAPI). The colony morphology (CM) of isolates F5 and F11 on blood agar is also shown.

TABLE 2. Physiological and biochemical characteristics of isolates F5 and F11 based on the ABI 50 CHB system and additional tests described in Materials and Methods

Substrate	Presence (+) or absence (–) in isolate:		Substrate	Presence (+) or absence (–) in isolate:	
	F5	F11		F5	F11
Glycerin	+	+	Melezitose	–	–
Erythritol	–	–	D-Raffinose	–	+
D-Arabinose	–	–	Amidon/starch	+	+
L-Arabinose	+	+	Glycogen	+	+
Ribose	+	+	Xylitol	–	–
D-Xylose	+	+	β-Gentiobiose	–	–
L-Xylose	–	–	D-Turanose	+	+
Adonitol	–	–	D-Lyxose	–	–
β-Methylxyloside	–	–	D-Tagatose	+	+
Galactose	+	+	D-Fucose	–	–
D-Glucose	+	+	L-Fucose	–	–
D-Fructose	+	+	D-Arabitol	–	–
D-Mannose	+	+	L-Arabitol	–	–
L-Sorbose	+	+	Gluconate	–	–
Rhamnose	–	+	2-Ketogluconate	–	–
Dulcitol	–	–	5-Ketogluconate	–	–
Inositol	+	+	ONPG ^a	+	+
Mannitol	+	+	Arginine	+	+
Sorbitol	+	+	Lysine	–	–
α-Methyl-D-mannoside	–	–	Tryptophan	–	–
α-Methyl-D-glucoside	+	+	Ornithine	–	–
N-Acetylglucosamine	+	–	Citrate	+	+
Amygdalin	+	+	H ₂ S	–	–
Arbutin	+	+	Urease	–	–
Esculin	+	+	Indol	–	–
Salicin	+	+	Gelatin	–	+
Cellobiose	+	+	Casein	+	+
Maltose	+	+	Collagen	+	+
Lactose	–	–	Carboxymethyl cellulose	+	+
Melibiose	–	+	Lichenan	+	+
Sucrose	+	+	Voges-Proskauer	+	+
Trehalose	+	+	Oxidase	–	–
Inulin	–	–	Catalase	+	+

^a ONPG, *o*-nitrophenyl-β-D-galactopyranoside.

RESULTS

Isolation and identification of proteolytic strains from shrimp shell waste. Following the procedure outlined in Materials and Methods, a total of 109 proteolytic microbial colonies from shrimp shell waste were isolated. When these isolates were also tested for their lack of chitinase activity, only one sample originating from Indonesian material (Palembang, Sumatra) displayed no discernible chitinolytic activity (not shown). Since the microorganisms from that sample appeared to be promising candidates for the deproteinization of shrimp shell waste concomitantly leaving the chitin unaffected, they were analyzed in more detail.

Initial observation suggested a mixed culture; thus, cells were separated by streaking them onto blood agar plates, and finally bacteria displaying two different colony morphologies were obtained (Fig. 1). These two isolates were designated F5 and F11. F5 had smooth, convex colonies, presumably due to excessive slime formation, whereas the F11 displayed a rough colony morphology. Microscopic investigation revealed no major differences between F5 and F11 cells; both form motile rods of equal sizes (2.9 by 0.75 μm) as depicted in Fig. 1 with terminal endospores (data not shown).

Further physiological and biochemical characterization applying the API 50 CHB system, the oxidase, catalase, and Voges-Proskauer test, disclosed rather faint differences (Table 2). According to the physiological and microscopic tests, both F5 and F11 are suggested to be representatives of the *B. licheniformis* species. These findings agree with the fact that no extrachromosomal elements (approximately 1 to 20 kb in size) could be detected within cells of F5 and F11 (data not shown) since small plasmids are rarely found in *B. licheniformis* strains (6 to 25%) (17, 34). However, definite evidence for the affiliation of the isolates with the species *B. licheniformis* was obtained from sequencing the 16S rRNA gene, including the hypervariable regions V1 to V3; 100% identity to *B. licheniformis* DSM13/ATCC 14580, which was totally sequenced only recently, was found (Fig. 2).

Deproteinization of shrimp shell waste. Parallel to the species identification of the isolate comprising F5 and F11, deproteinization experiments were performed, since removing proteins from shrimp shell waste constitutes a crucial step in the extraction procedure for obtaining chitin.

First of all, the optimal temperature for efficient deproteinization was checked. At the lowest temperature tested

FIG. 2. Phylogenetic tree constructed with 16S rRNA gene sequences comprising hypervariable regions V1 to V3 of *Bacillus* strains using neighbor-joining method of the RDP II software package and database (5). The bar indicates the 0.1% difference in nucleotide sequence.

(42°C [Fig. 3]) the resident spoilage flora strongly retarded the growth of the inoculum (not shown), and also the protein content stayed at levels significantly higher than in experiments performed at 50 and 55°C (Fig. 3). To minimize the growth of the accompanying natural (chitinolytic) spoilage flora and to produce conditions that are not ideal for pathogenic organisms, further experiments were carried out at 55°C, since this also approximately meets the temperature requirements of alkaline proteases from *B. licheniformis* (24). In addition, fastest digestion of proteins occurred at 55°C (Fig. 3). Since the protein content (ca. 7%) was still relatively high, the fermentation broth was adjusted as outlined in Materials and Methods. Upon applying this improved medium, the protein content finally dropped below 1% (Fig. 4). As shown in Fig. 4, such a decline in protein content came along with a significantly enhanced proteolytic activity in the culture broth. After demineralization with 0.9% lactic acid the purity of the obtained product was determined (Table 3). The total N content (%) was compared to that of commercially available chitin. A slightly lower N content (6.44% versus 6.68%) was obtained for our sample, presumably due to N-free impurities, such as salts and pigments. Significant impurities caused by proteins, however, were seen in the sample that was treated in stan-

FIG. 3. Protein content of shrimp shell waste (%) during a 142-h fermentation at various temperatures in standard medium. Values are the means of duplicates, each displaying a standard deviation of less than 5%. The protein content was determined as described previously (15).

FIG. 4. Proteolytic activity of the culture supernatant and protein content of shrimp shell residues during fermentation at 55°C in standard and improved medium. All values are means of triplicates; the standard deviation was less than 10% in each instance.

dard medium, which has an N content of 7.14%. As shown in Table 3, the protein and ash content of the obtained product meet the standard of an average-quality chitin.

Besides purity, the molecular mass or chain length is of utmost importance. In general, high-molecular-mass chitin is desirable since it facilitates the production of a wide range of different products. Since the molecular mass of a chitin preparation correlates with the viscosity of the product, chitin, obtained from shrimp waste in improved medium, was compared to the commercially available chitin, which has an average molecular mass of 400,000 Da. Since the viscosity is significantly higher (80 versus 10 mPa; see Table 3), the chitin that was deproteinized with the bacterial isolates F5 and F11 is apparently superior to the commercially available material.

Characterization of genes involved in extracellular enzyme activity, secretion, and slime formation. When both F5 and F11 were checked for extracellular enzyme activities, it turned out from plate assays that the size of clearing halos on media containing various polymers differed significantly for the two strains (Fig. 5A); the results for assays for chitinases are not given because they were negative in both instances. On all media tested (starch, skim milk, and carboxymethyl cellulose) F11 displayed greater degrading activities than did F5. To

TABLE 3. Chemical composition related to dry matter and viscosity of shrimp shell waste^a

Chemical composition or viscosity	Raw material	Pretreated	Standard medium		Improved medium		Commercial chitin
			DP	DM	DP	DM	
Protein (%)	32	11	3	6.3	0.8	2.1	ND
Chitin (%)	22	32	32	81	38	87	ND
Ash (%)	31.5	42	48	2.9	52	1.2	ND
N (%)	ND	ND	ND	7.14	ND	6.44	6.68
Viscosity (mPas)	ND	ND	ND	ND	ND	80	10

^a Determinations for before processing (raw material), after pretreatment and fermentational deproteinization (DP), and after demineralization with lactic acid (DM) were performed in standard and in improved medium (for details, see Materials and Methods). ND, not determined.

FIG. 5. Analysis of extracellular enzyme activities of F5 and F11 and determination of specific amylase activities. (A) *Bacillus* sp. strain F5 and F11 cultures containing equal numbers of cells were spotted onto agar plates containing starch (st), skim milk (sm), and carboxymethyl cellulose (cmc). Clearing halos around the colonies depict enzyme activities. (B) Extracellular amylase activity after 72 h of batch fermentation; mean values were calculated from triplicates of two independent experiments; the standard deviation was less than 10% in either case.

substantiate the findings from the plate assays, enzyme activities were exemplified for the amylase by determining specific activities in the culture supernatant. The results obtained clearly match the findings obtained in the plate assays (Fig. 5B). As anticipated, the amylase activity of F11 explicitly exceeded F5.

To check whether differences in enzyme activity or colony morphology are due to nucleotide or amino acid exchanges, a number of loci encoding extracellular degradative enzymes,

including adjacent regions, and the *degS-degU* operon instrumental in the regulation of such enzymes, as well as the *pga* locus, which encodes the polyglutamic acid synthetase, were amplified and sequenced. No differences could be seen between F5 and F11 on the nucleotide level for any of the loci investigated (altogether 24,310 and 24,359 bp, respectively). When a Blastp search was performed with the deduced polypeptides, 99 to 100% identity to known *B. licheniformis* loci became evident, with only one exception, i.e., *chiA* (see Table 4 for details). The predicted ChiA protein displays only 86% identity to the corresponding protein of another *B. licheniformis* strain in a relatively short overlap. It is noteworthy that chitinases encoded by *chiA* of *B. licheniformis* MD1 and DSM13/ATCC 14580 each comprise 693 amino acids (22, 30); however, the overlap identified in Blastp analysis spans only 160 amino acids. By aligning the corresponding predicted proteins and DNA sequences (Fig. 6), it became evident that there is a frameshift mutation in *chiA* in both F5 and F11 due to the deletion of an A at position 412, which eventually results in the formation of a truncated nonfunctional protein comprising 160 amino acids only. However, there is another intact gene (*chiB*) encoding a nonhomologous chitin-degrading enzyme, and it remains to be noticed that chitinase activities could not be obtained in both F5 and F11.

DISCUSSION

Since members of the gram-positive bacterial genus *Bacillus* are well known for their potential to secrete a number of degradative enzymes (25), it was not surprising that the iso-

TABLE 4. Amino acid identities of the predicted gene products of *B. licheniformis* F5 and F11^a

Predicted protein	Primer pair	Identity			Accession no.	
		%	aa overlap	Protein, <i>B. licheniformis</i> strain (accession no.)	F5	F11
'YjeA	amyB2/amyB10	100	278/278	YjeA, DSM13 (AAU39593)	AM183787	AM183788
AmyB	amyB2/amyB10	99	510/512	AmyB, MD1 (CAH10338)	AM183787	AM183788
YvdE'	amyB2/amyB10	100	248/248	YvdE, MD1 (CAH10339)	AM183787	AM183788
FenH	celA1/celA4	100	113/113	YngL, ATCC 14580 (AAU23612)	AM183789	AM183790
CelA	celA1/celA4	100	517/517	CelA (BglC), MD1 (CAE82178)	AM183789	AM183790
YnfE	celA1/celA4	98	84/85	YnfE, DSM13 (AAU40978)	AM183789	AM183790
ChiB	chiB1/chiA6	99	594/595	YvbX, ATCC 14580 (AAU21943)	AM183793	AM183794
ChiA^b	chiB1/chiA6	86	139/160	ChiA, MD1 (CAH10341)	AM183793	AM183794
Mpr	mpr2/mpr4	99	315/316	Mpr, ATCC 14580 (AAU21945)	AM183793	AM183794
YcdF	mpr2/mpr4	99	256/257	YcdF, ATCC 14580 (AAU21946)	AM183793	AM183794
YdeD'	aprE18/aprE.2888	100	166/166	YdeD, DSM13 (AAU40018)	AM183795	AM183796
AprE	aprE18/aprE.2888	99	378/379	KerA, RG2 (AAB34259)	AM183795	AM183796
YhfN	aprE18/aprE.2888	99	240/241	YhfN, ATCC 14580 (AAU22668)	AM183795	AM183796
YvhJ'	SacU1/yviA2	100	75/75	YvhJ, MD1 (CAE82202)	AM183797	AM183798
YvyE	SacU1/yviA2	99	211/212	YvyE, MD1 (CAE82203)	AM183797	AM183798
DegS	SacU1/yviA2	100	385/385	DegS, MD1 (CAE82204)	AM183797	AM183798
DegU	SacU1/yviA2	100	229/229	DegU, MD1 (CAE82205)	AM183797	AM183798
YviA'	SacU1/yviA2	100	230/230	YviA (DegV), MD1 (CAE82205)	AM183797	AM183798
RbsR'	pga3/pga8b	100	150/150	RbsR, DSM13 (AAU42656)	AM231531	AM231532
YwsC	pga3/pga8b	100	393/393	YwsC, DSM13 (AAU42655)	AM231531	AM231532
YwtA	pga3/pga8b	100	149/149	YwtA, DSM13 (AAU42654)	AM231531	AM231532
YwtB	pga3/pga8b	100	377/377	YwtB, DSM13 (AAU42653)	AM231531	AM231532
YwtC	pga3/pga8b	100	55/55	YwtC, DSM13 (AAU42652)	AM231531	AM231532
YwtD	pga3/pga8b	99	413/414	YwtD, DSM13 (AAU42651)	AM231531	AM231532

^a The loci coding for selected extracellular enzymes (indicated in boldface) including their flanking region(s) were isolated using the quoted oligonucleotides (see also Table 1). The *degS-degU* and *pga* operons (consisting of the *ywsC*, *ywtA*, *ywtB*, and *ywtC* genes) are also given. The corresponding accession numbers of homologous proteins (synonyms in parentheses) and of the respective strain are indicated in parentheses. aa, amino acids.

^b Truncated *chiA* product. The predicted transcription length is estimated to be 2,082 bp (693 amino acids) in *B. licheniformis* DSM13.

FIG. 6. Alignment of predicted chitinase A from strains F5 and F11 with predicted chitinases of *B. licheniformis* DSM13 and MD1. (A) N termini with putative signal peptides in boldface italics. Chitinases from DSM13 and MD1 span 693 amino acids (aa), whereas the truncated chitinase A of F5 and F11 comprise 160 amino acids only. (B) The frameshift mutation caused by deletion of an A and the eventually resulting translational stop are highlighted. BacliMD1, chitinase A of *B. licheniformis* MD1 (accession no. CAH10341); BacliDSM13, chitinase A of *B. licheniformis* DSM13 (accession no. AAU39297); BacliF5, chitinase A of *B. licheniformis* F5; BacliF11, chitinase A of *B. licheniformis* F11.

lated proteolytic strains turned out to be members of this taxon. *B. licheniformis* is a ubiquitously occurring bacterium that can routinely be isolated from a number of different sources, such as soil (17) and wasted organic material (the present study). Physiological tests (API) (14) and 16S rRNA gene comparison (9), as well as microscopic and macroscopic investigations, revealed only faint differences between the bacterial isolates F5 and F11. It was suggested that 16S rRNA gene sequence comparisons should be performed only in conjunction with a number of phenotypic and phylogenetic properties because two distinct species may exist with identical 16S rRNA gene sequences (2, 20, 27).

To inspect whether the different extracellular enzymatic activities are due to nucleotide or amino acid exchanges, a number of loci encoding degradative extracellular enzymes, as well as an operon influencing secretion (*degS-degU*), were analyzed in addition to the *pga* locus. In each case the genetic organization was found to correspond to that published previously (22, 30). In addition, intergenic sequences, as well as coding regions, are almost identical between all *B. licheniformis* strains (data not shown). In general, amino acid alignments revealed routinely 99 to 100% identity to known *B. licheniformis* loci, with only one exception, i.e., *ChiA*, in which a frameshift mutation was detected in both F5 and F11.

Thus, it can be concluded that the observed lack of chitinase activity in F5 and F11 is due to the deletion of an A within the coding region of *chiA*. Additional support for such argumentation comes from the proven chitinase activity of *B. licheniformis* DSM13 (4), which exhibits essentially the same genetic organization but has an intact *chiA* gene (22, 30). The efficient secretion of amylase, protease, and cellulase by F5 and espe-

cially F11 for which intact genes were proven also bears out such reasoning. Hence, the polypeptide encoded by the second chitinase encoding gene, i.e., *chiB*, either is not expressed or on its own does not have the ability to degrade chitin efficiently, although the respective catalytic domain and substrate-binding domain, as well as a fibronectin type III-like domain, are present (29, 32). Synergistic effects of different chitin-degrading enzymes (28) may be an additional reason for the observed lack of activity (at least beyond detection level). Nevertheless, the *chiA* gene product is evidently crucial for efficient chitin degradation in *B. licheniformis*.

Although F5 and F11 are almost identical, there are differences in their abilities to secrete extracellular degradative enzymes. Compared to F5 and other *B. licheniformis* strains, such as DSM13, F11 displays a hypersecreting phenotype. In *B. subtilis*, such hypermutants are characterized by enhanced secretion capabilities due to mutations in the two-component regulatory system encoded by *degS-degU* (16). We were therefore eager to find out whether such alterations occur in *degS-degU* of F11 as well. Since both F5 and F11 have identical operons, which correspond to the DSM13 locus, hypermutations can clearly be excluded. Hence, the reason for the hypersecretion phenotype of F11 remains to be elucidated.

Similarly, although the capsule formation (polyglutamic acid) is clearly different in both strains, the underlying genetic basis (*pga* operon) is identical. Taking the determined identical mutations in *chiA* and the identity of all other sequenced loci into account, F5 and F11 presumably constitute variants of the same *B. licheniformis* strain.

Applying the optimal temperature for deproteinization (55°C) reduces the risk of contamination by the naturally oc-

curing spoilage flora and also avoids costly sterilization processes. Since the residual protein and ash content met the average of a standard-quality, commercially available chitin, the process developed here is a competitive procedure for industrial chitin production. The demineralization performed with pure lactic acid in the present study can generally be substituted by environment-friendly fermentation with lactic acid bacteria (10).

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