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Proteomic characterization of transient expression and secretion of a stress-related metalloprotease in high cell density culture of *Bacillus megaterium*

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Abstract

Intracellular and extracellular proteome analysis was carried out by combined two-dimensional gel electrophoresis and mass spectrometric analysis (2DE/MS) for high cell density fed-batch culture of recombinant *Bacillus megaterium* strains. In the early feeding phase with a constant growth rate of $0.12\,h^{-1}$ under glucose limitation, high expression and secretion of a metalloprotease (referred as Bmg1465) was detected. The transient appearance of this metalloprotease was found both as cell-associated and as secreted into the culture medium. Searching homologous proteins for functional assignment led to an unambiguous identification of Bmg1465 as a zinc-binding metalloprotease of the type immune inhibitor A (InhA). Metalloproteases of this type are currently considered as typical virulence factors associated with pathogenic *Bacillus* species. The result raises questions concerning the intrinsic function(s) of Bmg1465 in *B. megaterium*, which has the GRAS status, with respect to its stress response in high cell density culture.
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Keywords: *Bacillus megaterium*; Proteome analysis; High cell density; Metalloprotease; Immune inhibitor A; Stress response

1. Introduction

The Gram-positive bacterium *Bacillus megaterium* is a promising host for the production of diverse heterologous enzymes and vitamins, etc. due to its intrinsic favorable properties such as low protease activity and high secretion capability (Vary, 1994; Malten et al., 2005), and, not the least, due to its GRAS (generally recognized as safe) status. The availability of a strain-specific protein database, which was developed based on the genome sequence of *B. megaterium* DSM319 (Sun et al., 2006), has enhanced our proteome analysis based on two-dimensional gel electrophoresis/mass spectrometric analysis (2DE/MS). We have recently

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performed intracellular proteome analysis to characterize protein expressions of *B. megaterium* in batch cultures for the production of a heterologous dextransucrase (Wang et al., 2005).

To understand the physiological responses of *B. megaterium* to a high cell density culture (HCDC) process that was developed for recombinant protein productions with *B. megaterium* strains (Hollmann and Deckwer, 2004; Malten et al., 2005), we have now carried out more extensive intracellular and extracellular proteome analysis. In general, protein expression patterns are different to that observed in batch cultures (Wang et al., 2005), and there are also significant discrepancies between the two *B. megaterium* strains under investigation. One of the most striking results of our proteomic study with *B. megaterium* in HCDC was, however, the transient expression and secretion of a metalloprotease. In this paper we report on this surprising phenomenon. Mass spectrometric analysis and database search confirmed that the metalloprotease is a homologous protein of a potential virulence factor, namely the zinc-binding metalloprotease commonly known as immune inhibitor A. This potential virulence factor can be normally only found in the pathogenic *Bacillus* strains *Bacillus anthracis*, *Bacillus cereus* and *Bacillus thuringiensis*. *B. anthracis* is the aetiological agent of the acute fatal disease anthrax and a potential biological weapon due to its high toxicity. *B. cereus* is an ubiquitous non-specific opportunistic pathogen that is the common cause of food poisoning as well as a variety of other diseases. *B. thuringiensis* produces intracellular protein crystals toxic to a wide number of insect larvae and is the most commonly used biological pesticide worldwide. Therefore, it would be of great interest to further elucidate the function of this protein in *B. megaterium*.

2. Materials and methods

2.1. Bacterial strains and cultivation conditions

Two mutant strains of *B. megaterium* DSM 319, namely the defined mutant MS941 by gene replacement of a major extracellular protease ΔnprM (Wittchen and Meinhardt, 1995) and the chemical mutant *B. megaterium* WH320 (Rygus and Hillen, 1991), were transformed with the plasmid pMM1520*dsrS* which carries the gene of a dextransucrase from *Leuconostoc mesenteroides* (Malten et al., 2005). These two strains were compared for recombinant protein production of a heterologous dextransucrase in fed-batch cultures. The cultivation conditions and the analytical procedures for the determination of cell dry weight, concentrations of glucose, xylose and extracellular metabolites were the same as described in details before for the high cell density culture of strain WH320 (Malten et al., 2005), except that the amount of trace elements added during the batch phase of the cultivation for strain MS941 was twice as much as that for strain WH320, because MS941 has demonstrated a higher demand on trace elements than WH320 to achieve a comparable cell growth (unpublished experimental results). In addition to the feeding solution, 1 ml of trace element solution and 0.3 g of $MgSO_4{\cdot}H_2O$ were added at 7-h intervals during the feeding phase.

2.2. Preparation of protein samples and two-dimensional gel electrophoresis

Immediately after harvesting, cell samples were chilled on ice, centrifuged at 6500 rpm (Sorvall RT 6000B, DuPont) for 30 min at 4 °C to separate the supernatants from the cell pellets. The supernatants were stored at −20 °C and used for the 2DE analysis of the extracellular proteome (secretome), whereas the cell pellets were washed twice with a buffer containing 0.1 M Tris–HCl (pH 7.0), 10 mM DTT, 20 mM KCl, 5 mM $MgCl_2$ and 1 mM EDTA, and stored at −80 °C for the 2DE analysis of the intracellular proteome.

For intracellular proteome analysis cell pellets were resuspended with a lysis buffer containing 7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 1% (w/v) DTT, 0.8% (w/v) Pharmalyte™ pH 3–10, and 5 mM Pefabloc and disrupted by ultrasonication (Bandelin Sonopuls HD207 with power set at 60%) in an ice bath for 15 × 30 s with 60 s interval between each cycle. Cell debris was removed by centrifugation at 13,000 × *g* for 30 min at 4 °C. The raw protein extracts were treated further by a phenol precipitation with subsequent acetone extraction according to the method described before (Wang et al., 2005). Purified protein pellets were stored at −80 °C until use.

For extracellular proteome (secretome) analysis proteins in the supernatants were precipitated overnight

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using 10% (w/v) trichloracetic acid (TCA), then centrifuged at 4 °C for 45 min at 33,000 × *g*. The pellets obtained were further washed three times with 10 ml of cold 96% ethanol (4 °C), dried and stored at −80 °C until use.

2.3. Two-dimensional gel electrophoresis

Purified protein pellets (either intracellular or extracellular) were reconstituted in a rehydration buffer containing 7 M urea, 2 M thiourea, 0.5% (w/v) CHAPS, 1% (w/v) tritonX-100, 1% (w/v) ASB-14, 5 mM TCEP, 0.5% IPG buffer pH 4–7 and trace amount of bromphenol blue. The total protein concentration in the supernatant was determined using the PlusOne 2D-Quant kit (GE Healthcare) according to the manufacturer's instruction. 2DE was carried out as described previously (Wang et al., 2005). The first dimension was performed on immobilized pH gradient (IPG) strips (24 cm in length, pH gradient 4–7, GE Healthcare). Each IPG strip was loaded with 250 μg of proteins for intracellular proteome analysis and 70–240 μg of proteins for extracellular proteome analysis. The second dimension was run on self-cast 12.5% polyacrylamide gels. Duplicate or triplicate gels were analyzed for each protein sample under same experimental conditions.

2.4. Gel staining, scanning and image analysis

Gels were stained according to the improved fluorescent staining method described by Lamanda et al. (2004) using ruthenium II tris-bathophenanthroline disulfonate (RuBPS), which was prepared according to the method described by Rabilloud et al. (2001). Fluorescent images were obtained by scanning gels with the CCD-based Fujifilm LAS-1000 image analyzer using the software Image Reader LAS-1000 Pro Version 2.5. The scanning parameters are set as follows: excitation wavelength 470 nm (Blue-SQW-LED), lens filter Y515-Di for fluorescence detection, exposure time 60 s, 14 bits images saved as .tif files for later image analysis. Computer image analysis for protein spot detection, matching and quantification were performed with the Phoretix 2D Advanced Software Version 2003.02 (Phoretix, Newcastle upon Tyne, UK) as described by Wang et al. (2005).

2.5. Mass spectrometric protein identification

2DE gels were further stained using Brilliant Blue G-Colloidal Concentrate (Sigma, St. Louis, MO, USA) according to the manufacturer's instruction. Protein spots excised from 2DE gels were subjected to tryptic digestion according to a method described previously (Wang et al., 2003, 2005) with minor modifications. Briefly, protein spots were washed twice with Milli-Q water, dehydrated with acetonitrile, digested overnight with trypsin (sequencing grade modified, Promega Corp.) at 37 °C, and desalted using the Montage ZipPlateC18 (Millipore Corp.) for parallel desalting of 96 digested protein samples. Subsequently, the tryptic peptides were analyzed by MALDI-TOF MS with a Bruker Ultraflex time-of-flight mass spectrometer (Bruker Daltonics GmbH, Germany) and, when necessary, by ESI-QqTOF MS/MS with a Q-TOF 2 mass spectrometer (Micromass, Manchester, England) as described before (Wang et al., 2003). Peptide masses obtained from MALDI-TOF MS analysis were used for protein identification by peptide mass fingerprinting (PMF) by searching a strain-specific protein database "bmegMECI" with the MASCOT program licensed in-house. This protein database was developed from a low-coverage, unfinished genomic sequence of *B. megaterium* wild strain DSM319 (Sun et al., 2006). If necessary, ESI-QqTOF MS/MS analysis were performed and partial amino acid sequences obtained by automatic or manual sequencing with the PepSeq program of the software package Masslynx Version 3.5 (Micromass) were used for the verification of MALDI-TOF MS results.

3. Results

3.1. Fed-batch cultures with two recombinant B. megaterium strains

To achieve high cell densities of the two *B. megaterium* strains MS941 and WH320, a fed-batch strategy was chosen by controlling the substrate glucose at very low concentration to avoid oxygen limitation and, as the consequence, the formation of fermentation byproducts. As depicted in Fig. 1 the time courses of the cell growth of the two *B. megaterium* strains are comparable to each other, as well as the changes of the

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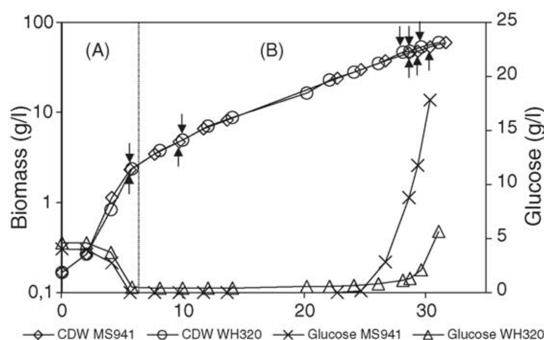

Fig. 1. High cell density fed-batch cultivation of two recombinant *B. megaterium* strains, MS941 and WH320, for the production of a heterologous dextransucrase. (A) Batch phase; (B) exponential feeding phase. Arrows indicate time points of sampling for the 2DE analysis of intracellular and extracelluar protein expressions. The first sample (S1) was taken at the end of the batch phase, when the glucose concentration became limited. The second sample (S2) was from the early exponential feeding phase about 4 h after the start of feeding. The last three samples (S3, S4 and S5) were taken at the late stage of the exponential feeding phase shortly before the induction of the recombinant protein production and 0.5 and 1.5 h after the induction, respectively.

glucose concentrations—at least during the batch phase and the first 20 h of the fed-batch phase. The specific growth rate was well controlled at 0.12 h^{-1} for both cultures during the first 20 h of the exponential feeding phase. However, it decreased at the late stage to about 0.08–0.09 h^{-1}. The reason for this decrease is still not clear. But during this period of time the computer-aided regulation of feeding still kept at the pace for a growth rate of 0.12 h^{-1}. Consequently, the glucose concentration increased, especially noticeable in the culture with strain MS941.

3.2. Identification of a metalloprotease and its degradation products

2DE experiments were carried out to characterize physiological responses of the two *B. megaterium* strains to the fed-batch cultivation process, as well as to the induction of recombinant protein production. Arrows in Fig. 1 indicate the time points where cell samples were taken for 2DE analysis.

The expression patterns of intracellular (cell-associated) proteins at different cultivation times were compared quantitatively. Unusual high expressions of several protein spots, signed as spots 1–5 in Fig. 2,

Table 1
Protein spots detected on 2D gels of samples taken at the early exponential feeding phase (S2) and identified as a zinc-binding metalloprotease, immune inhibitor A (Bmg1465)

Spot number	p*I*[a]	M_r (kDa)[a]	Top score[b]
1	5.21	86.1	102
2	5.27	84.6	155
3	5.33	85.9	167
4	5.40	86.1	136
5	5.48	86.8	125
6	4.99	80.1	60
7	5.02	80.2	164
8	5.34	61.1	62
9	5.50	48.4	73
10	5.65	49.8	87
11	5.81	50.2	118
12	5.03	38.0	67
13	5.43	26.3	50
14	6.07	30.0	38

[a] p*I* and M_r indicate the apparent isoelectric point and molecular weight determined on 2D gels.

[b] Top score indicates the score of the top candidate on the MASCOT hit list. It is the probability-based Mowse score used in the MASCOT search program. Using the database bmgEMCI scores >50 are significant (probability of a random match $p < 0.05$).

were found in the samples taken in the early exponential feeding phase (S2) for both *B. megaterium* strains. They appeared as a chain of spots on the 2D gels with apparent molecular weights (M_r) ranged from 85 to 87 kDa and isoelectrical points (p*I*) between 5.21 and 5.48. By MALDI-TOF MS analysis and peptide mass fingerprinting using the strain-specific protein database “bmegMECI” (Sun et al., 2006), all of these spots were clearly identified with significant high matching scores (Table 1) as a zinc-binding metalloprotease, the immune inhibitor A (Bmg1465). This protein is predicted to have 783 amino acid residues, with a calculated isoelectrical point (p*I*) of 6.08 and a calculated molecular weight (M_r) of 85684 Da. The protein sequence of Bmg1465 and its corresponding DNA sequence have been submitted to GenBank (http://www.ncbi.nlm.nih.gov/Genbank/index.html) and are now available under the Accession Number DQ372704.

Protein domain structure of Bmg1465 was analyzed by querying its protein sequence against InterPro database, a database of protein families, domains and functional sites (Mulder et al., 2005, http://www.ebi.ac.uk/interpro). The domain

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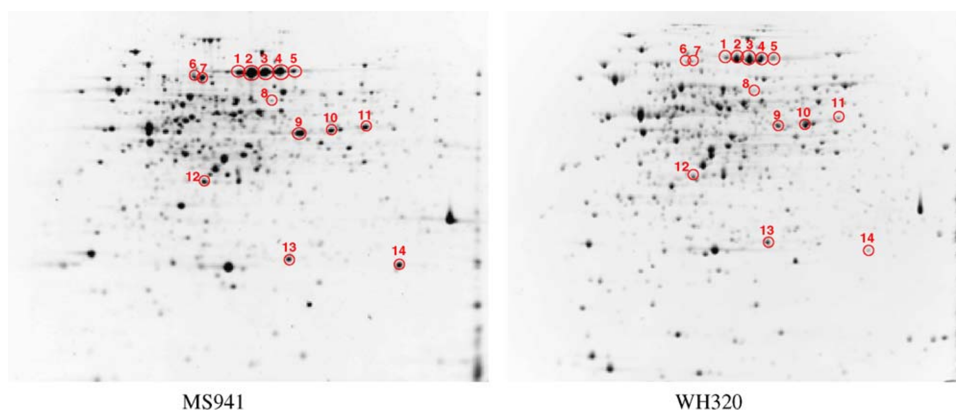

Fig. 2. Two-dimensional gel electrophoretic analysis of intracellular protein extracts of the two *B. megaterium* strains, MS941 and WH320, in the pH range of 4–7. Both samples were taken at the early exponential feeding phase (S2). Protein spots identified as a zinc-binding metalloprotease, immune inhibitor A (Bmg1465) are highlighted as spots 1–14.

structure contains the characteristic zinc-binding motif (HEXXH) of the zinc-metalloprotease family (Jongeneel et al., 1989). A potential signal peptide cleavage site is predicted between position 34 and 35 by the signal peptide prediction program SignalP 3.0 (Bendtsen et al., 2004, http://www.cbs.dtu.dk/services/SignalP/) using both neural networks and hidden Markov models trained for Gram-positive bacteria. The domain structure prediction clearly demonstrates that Bmg1465 belongs to the subfamily peptidase M6 (immune inhibitor A) of the superfamily zinc-dependent metallopeptidases (metzincins).

Interestingly, in addition to spots 1–5 Bmg1465 was also found to be the first candidate on the MASCOT hit lists for additional nine protein spots, as marked as spots 6–14 in Fig. 2. However, as shown in Table 1, most of these spots have apparent molecular weights according to their locations on the 2D gels clearly lower than the theoretical molecular weight calculated from the intact amino acid sequence of Bmg1465. Besides, the matching scores for spots 13 and 14 were not significant enough for an unambiguous identification. In order to confirm the MALDI-TOF MS results, ESI-QqTOF MS/MS analysis were performed. MS spectra of peptide precursors obtained for all the 14 protein spots demonstrated great similarity to each other. Altogether seven double charged peptide precursors were found. Through further fragmentation to acquire the corresponding MS/MS spectra, five of these precursors could be sequenced to give the partial amino acid sequences as given in Table 2. Using each of the partial amino acid sequences obtained to search the protein database "bmegMECI" led to unambiguous identification of them as amino acid fragments derived from Bmg1465, not from any other proteins of *B. megaterium* which might be homologous or functionally related to Bmg1465. MS spectra of all 14 protein spots possesses at least two of the sequenced double charged peptide precursors. This means that all the 14 spots were, undoubtedly, either the intact protein or the degradation products of Bmg1465. As an example shown in Fig. 3 are the MS spectra for spot 13 and spot 14 in comparison with spot 3 which showed the highest matching score by MALDI-TOF MS analysis (Table 1). Arrows indicate double charged peptide precursors with known sequences. While the MS spectrum of spot 3 possessed four double charged peptide precursors at m/z 625.7, 646.8, 867.8 and 922.4, only two double-charged peptide precursors at m/z 646.8 and 867.8 were found in the MS spectra of spot 13 and spot

Table 2
Double charged peptide precursor in MS spectra and their corresponding amino acid sequences derived from MS/MS spectra by ESI-QqTOF MS/MS analysis

m/z ($z=2$)	Corresponding sequences
625.70	412 TVFTDDAEGAPK 423
646.80	250 SGSVSVVDQSVTK 262
688.30	518 YQIADAAFSFDK 529
867.30	52 EYGADNPAGGNDNAPGSK 69
922.40	501 VLYFNVNGISTTANSSR 517

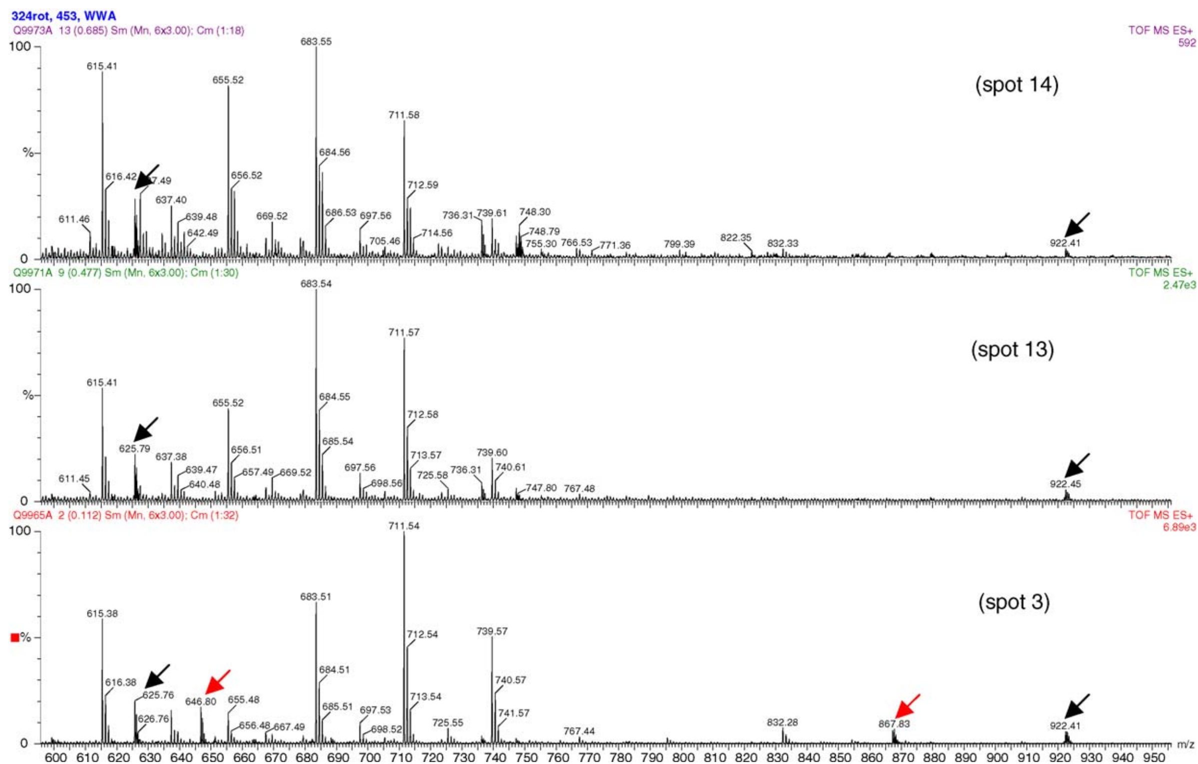

Fig. 3. MS spectra of peptide precursors in ESI-QqTOF MS/MS analysis. Arrows indicate double charged peptide precursors of *m/z* 625.7, 646.8, 867.8 and 922.4, which were further fragmented to obtain the corresponding MS/MS sequence spectra. Partial amino acid sequences derived were used for the verification of protein identification results of MALDI-TOF MS analysis.

14. Therefore, spots 6–14 were degradation products of Bmg1465 resulted from proteolysis that happened under the cultivation conditions applied. Possibility of fragmentation due to instability during protein sample preparation can be excluded, as no degradation products were found in samples taken at the end of the batch phase (S1), and all protein samples were treated exactly the same way. It is worth to mention that both MALDI-TOF MS and ESI-QqTOF MS/MS results indicated that spots 8–11 were more likely degradation products with C-terminal amino acid sequence of the intact protein being cleaved, whereas spots 12–14 lacked the N-terminal amino acid sequence of the intact protein.

3.3. Expression and degradation of the metalloprotease

As shown in Fig. 4, the expression of Bmg1465 was detected already in the samples taken at the end of the batch phase (S1) for both *B. megaterium* strains. At this stage Bmg1465 appeared mainly as a chain of protein spots (spots 1–5) having apparent molecular weights that approximate to the M_r of the mature protein with intact amino acid sequence. The expression of these spots were all strongly up-regulated in the samples taken 4 h later in the early exponential feeding phase (S2), especially in the fed-batch culture with MS941. In the sample S2, almost all the spots of the degradation products of Bmg1465 (spots 6–14) were also found on the 2D gels both for MS941 and WH320. The results indicates that although the production and accumulation of Bmg1465 were notably high between the sampling times S1 and S2, the degradation of Bmg1465, possibly resulted from intracellular protease activities, has also become obvious. In contrast to samples of S1 and S2, none of the spots related to Bmg1465 were visualized anymore on the 2D gels of the last three samples taken at the late stage of the

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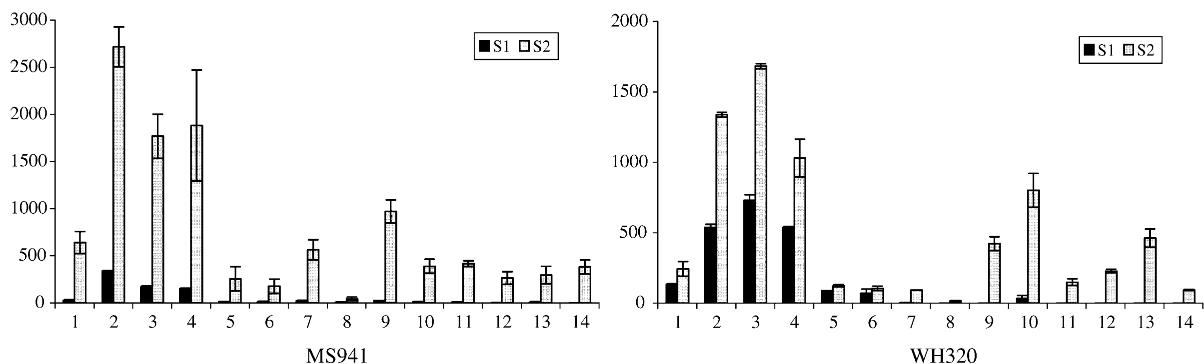

Fig. 4. Intracellular (cell-associated) accumulation and degradation of the zinc-binding metalloprotease, immune inhibitor A (Bmg1465) of the two *B. megaterium* strains, MS941 and WH320.

cultivations (S3, S4 and S5) for both *B. megaterium* strains. It is likely that at the late stage the production of Bmg1465 ceased or, at least, the production rate became much lower than the degradation rate. In addition, all Bmg1465 accumulated before, whether in non-degraded or degraded forms, were all subjected to further degradations, leading possibly to the formation of degradation products that might be too small to be detected on the 2D gels by the 2DE technique applied in this study or they might be consumed by the cells as a kind of amino acid source. We can see a correlation between the expression change of Bmg1465 and the change of the glucose concentration. It was clear that the accumulation and secretion of Bmg1465 were only detected in 2D samples, when glucose in the culture medium was limited (S1 and S2). Once the glucose concentration increased (S3, S4 and S5) at the late stage of the feeding phase (Fig. 1), the production of Bmg1465 was shut down or strongly down-regulated. It is conceivable that the expression of Bmg1465 might be controlled by a regulation factor which acts in response to limitation of a nutrient not necessarily restricted to glucose.

3.4. Secretion of the metalloprotease

Since Bmg1465 has a signal peptide for secretion, the extracellular proteome of the samples S2 and S3 were also analyzed by 2DE. The secretion of Bmg1465 was verified by MALDI-TOF MS identification of spots which appeared as a chain on the 2D gels of sample S2 in the form of its mature protein for both *B. megaterium* strains (Fig. 5), but was not found on the gels of sample S3. This means that the secretion of Bmg1465 was in line with its intracellular accumulation and dissipation. No degradation products were

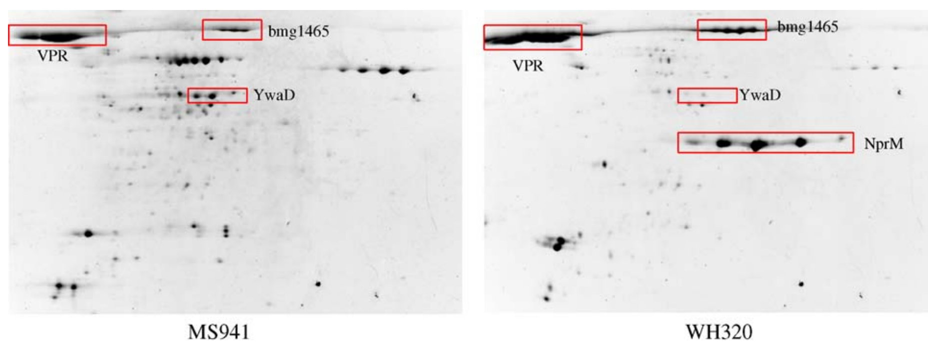

Fig. 5. Two-dimensional gel electrophoretic analysis of extracellular protein extracts of the two *B. megaterium* strains, MS941 and WH320, in the pH range of 4–7. Both samples were taken at the early exponential feeding phase (S2). Highlighted in boxes are, in addition to the zinc-binding metalloprotease, immune inhibitor A (Bmg1465), an extracellular serine protease (VPR), an aminopeptidase (YwaD), and a neutral metalloprotease bacillolysin (NprM).

found among the protein spots identified. As shown in Fig. 5, the secretome of both strains were highly heterogeneous. Beside the metalloprotease Bmg1465, additional proteases and/or peptidases were also identified as large, dense protein spots/bands. For instances, an extracellular serine protease VPR belonged to the most abundant secreted proteins of both strains. While the secretion of an aminopeptidase YwaD was much higher in MS941 than in WH320, a high amount of an neutral metalloprotease bacillolysin (NprM) was detected for strain WH320 but not for MS941 as expected, because the nprM gene coding for the extracellular protease NprM was already deleted in the strain MS941 (Wittchen and Meinhardt, 1995). Interestingly, both NprM and Bmg1465 are functionally categorized as zinc metalloproteases and are predicted as extracellular proteins. The expression and secretion pattern of NprM was, however, different to those of Bmg1465. Bmg1465 was inclined to accumulate a great amount within the cells (cell associated) and was susceptible to proteolytic degradations (Figs. 2 and 4), whereas no intracellular accumulation of NprM was detected, and the amount of NprM secreted remained nearly constant from the early stage (S2) to the late stage (S3) of the feeding phase (data not shown). It is noticeable that proteases made up of the biggest part of the secretome under the experimental conditions, especially in strain WH320. This seems not to support the view of a natural low protease activity in *B. megaterium* made before (Vary, 1994; Malten et al., 2005). In order to produce proteins in secreted form extracellular degradation of the desired proteins should be avoided. Thus, it might be of importance to knock out the genes coding for these extracellular proteases, or to choose cultivation conditions that can suppress the expression of these proteases.

4. Discussions

4.1. Homologous proteins of Bmg1465 and their functions

To understand the function of Bmg1465, its amino acid sequence was used to search for homologous proteins with the BLAST search program in a protein database constituted with protein sequences of the 13 *Bacilli* whose genomes have already been completely sequenced and are publicly available (Sun et al., 2006). Eighteen proteins were revealed to have amino acid sequences highly similar to the sequence of Bmg1465, as given in Table 3. Sequence identities vary from 55 to 59%. It should be noted that Bmg1465 is not as conserved as its homologs in other *Bacilli* strains, as shown by the multiple sequence alignment in the supplementary material. Nearly all the homologous proteins have been assigned the function of the metalloprotease immune inhibitor A (InhA), and all contain the zinc-binding motifs (HEXXH), as highlighted (boxed section) in the multiple sequence alignment. Interestingly, no proteins homologous to Bmg1465 can be found in *Bacillus subtilis*, *Bacillus clausii*, *Bacillus haludurans* and *Bacillus lichenniformisis*, a group of *Bacillus* species that, like *B. megaterium*, are known for their GRAS status and are widely used as hosts for industrial productions of diverse enzymes and biochemicals like vitamins. In contrast, all the 18 homologs of Bmg1465 are from different subspecies of three well known pathogenic *Bacillus* species, namely *B. anthracis*, *B. cereus* and *B. thuringiensis*, members of the *B. cereus* group. Genetic analysis has provided evidences for a close similarity of the genomes of these three *Bacillus* species, suggesting that they should be considered as members of the same species (Helgason et al., 2000).

Most of the secreted zinc metalloproteases of the InhA type have been shown to be associated with stress phenomena. They contribute to protect the bacteria against the attacks of the host immune system. Back to 1976, Edlund et al. (1976) already suggested that the production of two immune inhibitors, inhibitor A and inhibitor B, which selectively block the humoral defense system developed by insects, contribute to the insecticidal nature of *B. thuringiensis*. InhA1 of *B. thuringiensis* has been found to selectively hydrolyzes cecropins and attacins, two antibacterial peptides produced by insects in response to bacterial invasion (Dalhammar and Steiner, 1984; Lövgren et al., 1990), suggesting that it may contribute to the overall virulence of *B. thuringiensis*. InhA2 of *B. thuringiensis*, a paralog of InhA1 sharing 66% sequence identity, has been demonstrated recently to play a vital role in virulence when the host is infected via the oral route (Fedhila et al., 2002). It is involved in the synergistic effect of spores on the insecticidal activity of the crystal toxins in *B. thuringiensis* and,

Table 3
Homologous proteins of the zinc-binding metalloprotease, immune inhibitor A (Bmg1465) found in 13 *Bacillus* strains with known genome sequences

Strain name	Abbreviation	Protein name		
B. anthracis Ames	ban	BA1295	BA0672	
B. anthracis A2012	baa	BA_1822	BA_1259	
B. anthracis Ames 0581	bar	GBAA1295	GBAA0672	
B. anthracis Sterne	bat	BAS1197	BAS0638	
B. cereus ATCC 14579	bce	BC2984	BC1284	BC0666
B. cereus ATCC 10987	bca	BCE3037	BCE1396	BCE0740
B. cereus ZK	bcz	BTZK1177	BTZK0581	
B. thuringiensis	btk	BT9727_1175	BT9727_0582	
B. licheniformis ATCC 14580	bli			
B. licheniformis DSM13	bld			
B. clausii	bcl			
B. halodurans	bha			
B. subtilis	bsu			
B. megaterium	bmg	Bmg1465		

The protein names are according to the genomic database of KEGG (http://www.genome.jp/kegg), except for the strain *B. megaterium*.

consequently, it was considered to be a virulence factor.

InhA metalloprotease was found to be not only secreted during cell growth cycles but was also identified as an exosporium component of *B. cereus*. Exosporium is a loose-fitting balloon-like layer enclosing the spore, possibly involved in adhesion and pathogenicity (Charlton et al., 1999). As the major component of the exosporium, InhA1 has been found recently to be essential for spores of the *B. cereus* group to survive and escape macrophages and induce phagocytic cell death through involvement in processes that altered the macrophage membranes (Ramarao and Lereclus, 2005). It has been reported that many zinc-containing proteases in pathogenic organisms cause necrotic and hemorrhagic tissue damage in the host by digesting structural components such as collagen (Miyoshi and Shinoda, 2000).

All three pathogenic strains possess multiple copies of the *inhA* gene (Table 3). A recent 2DE comparative study of their extracellular proteomes has also identified three metalloproteases, named as InhA1, InhA2 and InhA3, in the culture supernatants of the *B. cereus* strain ATCC14579 and the *B. thuringiensis* strain 407 Cry$^-$ harvested 2 h after the onset of the stationary phase, respectively (Gohar et al., 2005). In addition, a fragment of the mature InhA2 protein was also found in the secretome of *B. cereus* strain ATCC14579. *B. megaterium*, the wild strain DSM319 and its derivatives MS941 and WH320, seems to have only one copy of the inhA gene. Through "reversed" searching in the database "bmegEMCI" with the amino acid sequences of any of the 18 homologs described above, no additional homologous protein other than Bmg1465 can be found. Thus, all spots highlighted in Fig. 2 were related to one and the same protein Bmg1465.

4.2. Expression and transcriptional regulation of the homologous proteins of Bmg1465

Many potential virulence factors, including haemolysins, enterotoxins and degradative enzymes such as metalloproteases of the InhA type, are secreted at the onset of the stationary phase by the pathogenic *Bacillus* strains. InhA1 and InhA2 of *B. thuringiensis* were found to be produced and secreted at the beginning of the stationary phase (Dalhammar and Steiner, 1984; Lereclus et al., 1996; Grandvalet et al., 2001; Fedhila et al., 2002). This was also demonstrated, in addition to *B. thuringiensis*, for *B. anthracis* and *B. cereus* by 2DE analysis of their culture supernatants harvested 2 h after the onset of the stationary phase (Gohar et al., 2002, 2005). Our 2DE results revealed that the expression time course of Bmg1465 was in good agreement with the expression of its homologous proteins. Sample S1 was taken at the end of the batch phase that was about 1.5 h after the beginning of the declining growth phase or early stationary phase (defined as the break point of the exponential growth phase). Bmg1465 then accumulated and secreted in

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the following fed-batch phase for additional 4 h or even longer, during which the glucose concentration was controlled as the growth-limiting factor.

Many of the potential virulence factors have been reported to be transcriptionally regulated, directly or indirectly, by the pleiotropic transcriptional activator, PlcR, which was first identified in *B. thuringiensis*. PlcR expression starts at the end of the vegetative phase and is maximal 2 h after the onset of the stationary phase. It is both auto-regulated and under the control of the transition state regulator Spo0A (Lereclus et al., 1996, 2000; Agaisse et al., 1999; Gohar et al., 2002). 2DE analysis of the extracellular proteome of *B. cereus* revealed that InhA2 was among the most abundant proteins, accounted for 7.6% of the total extrocellular proteins. Its secretion was strongly decreased (by 13-folds) in a ΔplcR mutant (Gohar et al., 2002). Later, Fedhila et al. (2003) found out that in *B. thuringiensis*, *inhA2*, the gene encoding InhA2, is a PlcR-regulated gene. PlcR activates *inhA2* transcription directly by binding to a DNA sequence in the *inhA2* promoter region showing a one-residue mismatch with the previously reported regulatory sequence known as the PlcR box. However, they also pointed out that *inhA2* alone is not sufficient for pathogenicity when the other members of the PlcR regulon are lacking, suggesting that InhA2 may act in concert with other PlcR-regulated gene products to provide virulence.

The *inhA* gene of *B. thuringiensis* is confirmed to be also repressed by AbrB during the exponential growth phase and is expressed only during the stationary phase, when the transcription of AbrB is repressed (Grandvalet et al., 2001). AbrB protein is one of the most pleiotropic transition-state regulators that acts as repressor during vegetative growth to prevent expressions of stationary-phase-specific genes. The transcription of itself is repressed by phosphorylated Spo0A, namely Spo0A ∼ P, at the onset of the transition phase, when Spo0A is subjected to phosphorylation by a regulatory phosphorelay cascade that is activated in response to nutrient deprivation (Strauch and Hoch, 1993).

In the protein database "bmegMECI" of *B. megaterium* an ortholog of the AbrB protein of *B. thuringiensis* (BT9727_1828) exists as the protein Bmg1537 with 53% sequence identity. A homologous protein (Bmg0831) can be found in *B. megaterium* for the PlcR protein (BT9727_5033) of *B. thuringiensis* with 27% sequence identity. Bmg0831 is, however, more similar to another transcriptional regulator of *B. thuringiensis*, namely BT9727_1061 with a sequence identity of 33%. Neither AbrB nor PlcR was among the proteins that have been so far identified on the 2D gels.

5. Conclusion remarks

B. megaterium does not only possess a gene that is highly similar to genes encoding metalloproteases of InhA type, which are suggested to be virulence factors of many pathogenic *Bacillus* strains, the transcription of this gene was also confirmed through the identification of its translation product Bmg1465 by proteome analysis of cells in a HCDC process for recombinant protein production. Indeed, Bmg1465 was among the most abundant proteins accumulated and secreted when the substrate glucose was limiting for cell growth, a strategy chosen for the achievement of high cell density cultures of *B. megaterium*. The results presented in this work arise several important questions regarding the intrinsic functions of the protein Bmg1465 and the GRAS status of *B. megaterium* as an industrial host strain for the production of vitamins and recombinant proteins. More work needs to be done to answer these questions. It should be, however, mentioned that proteins that are reported to be associated with pathogenicity in some organisms cannot be considered as virulence factors *per se*. In a recent comparative study of six pathogenic and non-pathogenic *Escherichia coli* strains including the probiotic *E. coli* strain Nissle 1917, Sun et al. (2005) showed that the latter possesses 130 "common *E. coli* virulence factors". Surprisingly, *E. coli* strain Nissle 1917 and the well-known pathogenic *E. coli* CFT073 showed very little difference in this regard. It is understood that many of the so-called "virulence factors" may actually function for general fitness of the cells or for adaptation purpose under stress conditions.

For a more detailed characterization of the formation and secretion of Bmg1465 in *B. megaterium* in association with high cell density cultivation it would be of interest to know why a big amount of Bmg1465 was not readily secreted into the growth medium after its production but remained cell-associated; where the cell-associated part exactly locates, still in the cytosol or already transported outside of the

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cytoplasmic membrane but retained by the cell wall; and why Bmg1465 was subjected to further processing (proteolytic degradation).

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jbiotec.2006.05.005.

References

Agaisse, H., Gominet, M., Økstad, O.A., Kolstø, A.-B., Lereclus, D., 1999. PlcR is a pleiotropic regulator of extracellular virulence factor gene expression in *Bacillus thuringiensis*. 32 (5), 1043–1053.

Bendtsen, J.D., Nielsen, H., von Heijne, G., Brunak, S., 2004. Improved prediction of signal peptides: SignalP 3.0. J. Mol. Biol. 340 (4), 783–795.

Charlton, S., Moir, A.J., Bailie, L., Moir, A., 1999. Characterization of the exosporium of *Bacillus cereus*. J. Appl. Microbiol. 87 (2), 241–245.

Dalhammar, G., Steiner, H., 1984. Characterization of inhibitor A, a protease from *Bacillus thuringiensis* which degrades attacins and cecropins, two classes of antibacterial proteins in insects. Eur. J. Biochem. 139 (2), 247–252.

Edlund, T., Siden, I., Boman, H.G., 1976. Evidence for two immune inhibitors from *Bacillus thuringiensis* interfering with the humoral defense system of saturniid pupae. Infect Immun. 14 (4), 934–941.

Fedhila, S., Nel, P., Lereclus, D., 2002. The InhA2 metalloprotease of *Bacillus thuringiensis* strain 407 is required for pathogenicity in insects infected via the oral route. J. Bacteriol. 184 (12), 3296–3304.

Fedhila, S., Gohar, M., Slamti, L., Nel, P., Lereclus, D., 2003. The *Bacillus thuringiensis* PlcR-regulated gene inhA2 is necessary, but not sufficient, for virulence. J. Bacteriol. 185 (9), 2820–2825.

Gohar, M., Okstad, O.A., Gilois, N., Sanchis, V., Kolsto, A.B., Lereclus, D., 2002. Two-dimensional electrophoresis analysis of the extracellular proteome of *Bacillus cereus* reveals the importance of the PlcR regulon. Proteomics 2 (6), 784–791.

Gohar, M., Gilois, N., Graveline, R., Garreau, C., Sanchis, V., Lereclus, D., 2005. A comparative study of *Bacillus cereus*, *Bacillus thuringiensis* and *Bacillus anthracis* extracellular proteomes. Proteomics 5 (14), 3696–3711.

Grandvalet, C., Gominet, M., Lereclus, D., 2001. Identification of genes involved in the activation of the *Bacillus thuringiensis* inhA metalloprotease gene at the onset of sporulation. Microbiology 147 (Pt 7), 1805–1813.

Helgason, E., Okstad, O.A., Caugant, D.A., Johansen, H.A., Fouet, A., Mock, M., Hegna, I., Kolsto, A.-B., 2000. *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*—one species on the basis of genetic evidence. Appl. Environ. Microbiol. 66 (6), 2627–2630.

Hollmann, R., Deckwer, W.-D., 2004. Pyruvate formation and suppression in recombinant *Bacillus megaterium* cultivation. J. Biotechnol. 111, 89–96.

Jongeneel, C.V., Bouvier, J., Bairoch, A., 1989. A unique signature identifies a family of zinc-dependent metallopeptidases. FEBS Lett. 242 (2), 211–214.

Lamanda, A., Zahn, A., Roder, D., Langen, H., 2004. Improved ruthenium II tris (bathophenantroline disulfonate) staining and destaining protocol for a better signal-to-background ratio and improved baseline resolution. Proteomics 4 (3), 599–608.

Lereclus, D., Agaisse, H., Gominet, M., Salamitou, S., Sanchis, V., 1996. Identification of a *Bacillus thuringiensis* gene that positively regulates transcription of the phosphatidylinositol-specific phospholipase C gene at the onset of the stationary phase. J. Bacteriol. 178 (10), 2749–2756.

Lereclus, D., Agaisse, H., Grandvalet, C., Salamitou, S., Gominet, M., 2000. Regulation of toxin and virulence gene transcription in *Bacillus thuringiensis*. Int. J. Med. Microbiol. 290 (4/5), 295–299.

Lövgren, A., Zhang, M., Engstrom, A., Dalhammar, G., Landen, R., 1990. Molecular characterization of immune inhibitor A, a secreted virulence protease from *Bacillus thuringiensis*. Mol. Microbiol. 4 (12), 2137–2146.

Malten, M., Hollmann, R., Deckwer, W.D., Jahn, D., 2005. Production and secretion of recombinant *Leuconostoc mesenteroides* dextransucrase DsrS in *Bacillus megaterium*. Biotechnol. Bioeng. 89 (2), 206–218.

Miyoshi, S., Shinoda, S., 2000. Microbial metalloproteases and pathogenesis. Microbes Infect. 2 (1), 91–98.

Mulder, N.J., Apweiler, R., Attwood, T.K., Bairoch, A., Bateman, A., Binns, D., Bradley, P., Bork, P., Bucher, P., Cerutti, L., Copley, R., Courcelle, E., Das, U., Durbin, R., Fleischmann, W., Gough, J., Haft, D., Harte, N., Hulo, N., Kahn, D., Kanapin, A., Krestyaninova, M., Lonsdale, D., Lopez, R., Letunic, I., Madera, M., Maslen, J., McDowall, J., Mitchell, A., Nikolskaya, A.N., Orchard, S., Pagni, M., Ponting, C.P., Quevillon, E., Selengut, J., Sigrist, C.J., Silventoinen, V., Studholme, D.J., Vaughan, R., Wu, C.H., 2005. InterPro, progress and status in 2005. Nucleic Acids Res. 33 (Database Issue), D201–D205.

Rabilloud, T., Strub, J.M., Luche, S., van Dorsselaer, A., Lunardi, J., 2001. A comparison between Sypro Ruby and ruthenium II tris (bathophenanthroline disulfonate) as fluorescent stains for protein detection in gels. Proteomics 1 (5), 699–704.

Ramarao, N., Lereclus, D., 2005. The InhA1 metalloprotease allows spores of the *B. cereus* group to escape macrophages. Cell Microbiol. 7 (9), 1357–1364.

Rygus, T., Hillen, W., 1991. Inducible high-level expression of heterologous genes in *Bacillus megaterium* using the regulatory elements of the xylose-utilization operon. Appl. Microbiol. Biotechnol. 35 (5), 594–599.

Strauch, M.A., Hoch, J.A., 1993. Transition-state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. Mol. Microbiol. 7 (3), 337–342.

Sun, J., Gunzer, F., Westendorf, A.M., Buer, J., Scharfe, M., Jarek, M., Gossling, F., Blocker, H., Zeng, A.-P., 2005. Genomic peculiarity of coding sequences and metabolic potential of probiotic *Escherichia coli* strain Nissle 1917 inferred from raw genome data. J. Biotechnol. 117 (2), 147–161.

Sun, J., Wang, W., Hundertmark, C., Zeng, A.-P., Jahn, D., Deckwer, W.-D., 2006. A protein database constructed from low-coverage genomic sequences of *B. megaterium* and its use for accelerated proteomic analysis. J. Biotechnol. March 24 [Epub ahead of print].

Vary, P.S., 1994. Prime time for *Bacillus megaterium*. Microbiology 140 (5), 1001–1013.

Wang, W., Sun, J., Nimtz, M., Deckwer, W.-D., Zeng, A.-P., 2003. Protein identification from two-dimensional gel electrophoresis analysis of *Klebsiella pneumoniae* by combined use of mass spectrometry data and raw genome sequences. Proteome Sci. 1 (1), 6.

Wang, W., Hollmann, R., Furch, T., Nimtz, M., Malten, M., Jahn, D., Deckwer, W.-D., 2005. Proteome analysis of a recombinant *Bacillus megaterium* strain during heterologous production of a glucosyltransferase. Proteome Sci. 3, 4.

Wittchen, K.D., Meinhardt, F., 1995. Inactivation of the major extracellular protease from *Bacillus megaterium* DSM319 by gene replacement. Appl. Microbiol. Biotechnol. 42 (6), 871–877.