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An endochitinase A from *Vibrio carchariae*: cloning, expression, mass and sequence analyses, and chitin hydrolysis

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Abstract

We provide evidence that chitinase A from *Vibrio carchariae* acts as an endochitinase. The *chitinase A* gene isolated from *V. carchariae* genome encodes 850 amino acids expressing a 95-kDa precursor. Peptide masses of the native enzyme identified from MALDI-TOF or nanoESIMS were identical with the putative amino acid sequence translated from the corresponding nucleotide sequence. The enzyme has a highly conserved catalytic TIM-barrel region as previously described for *Serratia marcescens* ChiA. The M_r of the native chitinase A was determined to be 62,698, suggesting that the C-terminal proteolytic cleavage site was located between R^{597} and K^{598}. The DNA fragment that encodes the processed enzyme was subsequently cloned and expressed in *Escherichia coli*. The expressed protein exhibited chitinase activity on gel activity assay. Analysis of chitin hydrolysis using HPLC/ESI-MS confirmed the endo characteristics of the enzyme.

Keywords: Endochitinase; Gene isolation; Cloning; Gene sequence; TIM barrel; Gel activity assay; Chitin hydrolysis; HPLC-ESI MS

Chitin is a polymer of β-1,4 linked *N*-acetylglucosamine and is widely distributed in nature as a structural component of fungi, protozoa, insects, and crustaceans. Chitin is degraded by the sequential action of two hydrolytic enzymes: chitinases (EC 3.2.1.14), followed by *N*-acetylglucosaminidase (EC 3.2.1.30) [1]. Chitinases are a diverse family of enzymes found in a wide variety of organisms [2,3] and classified into glycosidase families 18 and 19, depending on their amino acid sequences [4,5]. Structural analyses of mutant chitinase A (SmChiA) from *Serratia marcescens* complexed with chitooligosaccharides [6] and of the fungal chitinase CiX1 from *Coccidioides immitis* complexed with a known chitinase inhibitor, allosamidin, [7] suggested a substrate-assisted catalytic mechanism for family 18 chitinases. The mechanism involves protonation of the leaving group by the catalytic residue (E^{315} in SmChiA or E^{171} in CiX1), followed by substrate distortion into a 'boat' conformation at subsite −1 and the stabilization of an oxazolinium intermediate by the sugar acetamido moiety. The experimental data that showed glycosidic bond cleavage by family 18 chitinases, yielding retention of β-anomeric configuration in the products, supported the mechanism obtained from the structural information [8–10]. The X-ray structures of the hevamine chitinase/lysozyme complexed with allosamidin [9] and of the *S. marcescens* ChiA mutants complexed with octa- and hexasaccharide substrates [6] indicated that the catalytic sites of these two enzymes contained six substrate binding subsites, designated subsites −4, −3, −2, −1, +1, and +2. The scissile glycosidic bond is located between subsites −1 and +1.

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Two types of chitinases, exo- and endochitinases, have been distinguished and occur in both families. Exochitinase activity is defined as a progressive action that starts at the non-reducing ends of chitin chains and successively releases diacetylchitobiose units, while endochitinase activity involves random cleavage at internal points in chitin chains [11]. Based on the published 3D-structure information [12,13], the active site of family 18 endochitinases has a long, deep substrate-binding cleft with an opening on both sides. On the other hand, the active site of exochitinases has a tunnel-like morphology with a closure of the roof at the end of the tunnel [14,15].

The abundance of chitin has resulted in considerable interest in the possibilities of developing efficient bioconversion processes for recycling waste chitin based on chitinases. Marine bacteria are excellent sources of chitinases [16–19] and are potentially suitable sources of enzymes, especially for the recycling of waste crustacean chitin from the seafood industry. The highly insoluble polymer chitin is utilized rapidly as a sole source of carbon and nitrogen by marine bacteria, such as *Vibrios* [20]. The marine bacterium *Vibrio carchariae* is a particularly suitable source of chitinase A because it has been shown to express high levels of the enzyme within only 24 h of induction in the presence of chitin [21]. The enzyme is active as a monomer of M_r 63,000 as judged on SDS–PAGE. We report here the isolation of the gene encoding *V. carchariae* chitinase A and the determination of its sequence. The C-terminal proteolytic cleavage site of chitinase A was determined, permitting the gene that encodes chitinase A without the C-terminal proteolytic fragment to be cloned and functionally expressed in an *Escherichia coli* system.

Materials and methods

Bacterial strains and growth

A type strain of *V. carchariae* (LMG7890^T) was obtained from the type culture collection at Laboratorium voor Microbiologie Gent, Rijksuniversiteit, Gent, Belgium, and was a gift from Professor B. Austin, Heriot-Watt University, Edinburgh, UK. It is relevant to mention that *V. carchariae* and *V. harveyi* are very similar taxonomically, and it has recently been proposed to combine them into a single species as *V. harveyi* [22]. However, we retain the designation *V. carchariae* because the two relevant type strains displayed different properties with regard to the expression of chitinase activity [21]. The bacterium was grown and kept in marine medium, pH 7.6 [23]. Swollen chitin [24] prepared from chitin flakes (Sigma Practical Grade from crab shells) was included in the culture media for induction of chitinase expression as indicated.

Immunological

Antisera against *V. carchariae* chitinase A were obtained from a female New Zealand rabbit. Purified chitinase (MonoQ fraction, 50 μl containing 150 μg chitinase) [21] was emulsified with 50 μl TiterMax Gold adjuvant (CytRx, Norcross, Georgia) according to the manufacturer's instructions and injected intramuscularly into two sites. Antisera were collected at the second, third, and fourth weeks after immunization, and no boosting injections were required. Western blotting was done with detection by enhanced chemiluminescence (ECL, Amersham).

Viscosity reduction assay

The viscosity reduction assay was carried out according to Khasin et al. [25]. The reaction mixture (0.5 ml) contained 2% (w/v) glycol–chitin solution [26], *V. carchariae* chitinase A (5 μg) purified according to Suginta et al. [21] or *S. marcescens* chitinase (Sigma) (5 μg), and 250 mM MES[1] buffer, pH 6.0. The reaction was incubated at room temperature in a viscometer No. 42 (0–0.16 ml), and the flow time of the reaction mixture was measured at time intervals: 0 min, 15 min, 30 min, 1 h, 2 h, 12 h, 24 h, and 48 h. Decrease in viscosity of glycol–chitin versus reaction times (h) was plotted by means of the average data values obtained from three separate experiments.

Construction of a V. carchariae expression library, cDNA cloning, and sequencing

DNA fragments of 4–7 kb were isolated from a partial *Sau3* AI digest of *V. carchariae* genomic DNA prepared using the protocol of Ausubel et al. [27], ligated into the *Bam*HI site of pBluescript II KS(−), and transformed into *E. coli* type strain XL1 blue (Stratagene) by standard techniques. The library of 2100 transformants was screened for the expression of chitinase antigen using anti-chitinase A polyclonal antibodies. Positive clones were analyzed by restriction mapping, chitin plate assay, and Western blotting. Partial DNA sequencing was done manually by the dideoxy method according to the Sequenase PCR sequencing kit (USB) using T7 forward primer and SP6 reverse primer. Automated double-stranded DNA sequencing was carried out commercially by Oswel, Southampton, UK. The putative amino acid

[1] *Abbreviations used:* MES, 2-(4-morpholino)-ethanesulfonic acid; $(GlcNAc)_2$, *N,N'*-diacetylchitobioside; $(GlcNAc)_3$, *N,N',N''*-triacetylchitotrioside; $(GlcNAc)_4$, *N,N',N'', N'''*-tetraacetylchitotetraoside; IPTG, isopropylthiogalactoside; PMSF, phenylmethylsulfonyl fluoride; DMAB, *p*-dimethylaminobenzaldehyde; MALDI-TOF, matrix-assisted laser desorption ionizsation/time-of-flight; HPLC/ESI-MS, high performance liquid chromatography and electrospray mass spectrometry; SIM, single ion monitoring.

sequence of chitinase A was obtained from the back translation software in the DNA Star package. The signal peptide was predicted using SignalP V.1.1 program (http://www.cbs.dtu.dk/services/SignalP/). The nucleotide sequence of chitinase A has been deposited in the GenBank database under GenBank Accession No. Q9AMP1.

Amino acid sequence comparisons

The amino acid sequence alignment was made using "CLUSTALW" algorithm in a GCG package [28] and displayed using the Genedoc program (http://www.psc.edu/biomed/genedoc/). The putative *V. carchariae* chitinase A sequence was compared with three highly similar bacterial ChiA amino acid sequences available in the Swiss-Prot or TrEMBL database (http://us.expasy.org/). These sequences included *Altermonas* sp. strain O-7 (Accession No. P32823), *Pantoea* (*Enterobacter*) *agglomerans* (accession number P97034), and *S. marcescens* (accession number Q54275). Consensus motifs were analyzed based on the secondary structure of *S. marcescens* ChiA [13].

Mass analysis of the native chitinase A and its tryptic peptides

Chitinase A (2 μg) was applied in parallel onto a 12% SDS–PAGE gel using a Laemmli buffer system [29]. Following electrophoresis, protein was stained with Coomassie blue. After destaining, protein bands were excised from the gel and in-gel digested with trypsin (sequencing grade, Roche Diagnostics, Mannheim) using a standard protocol [30]. After overnight digestion at 37 °C, the peptides were extracted and dried in a SpeedVac vacuum centrifuge. A small fraction of these tryptic peptides was analyzed by high resolution MALDI-TOF MS (Voyager-DE Pro in reflective mode) in an α-cyano-4-hydroxycinnamic acid matrix for the peptide "mass fingerprinting". The majority was analyzed by nanoESI/MS (Thermo Finnigan LCQ Deca) using the proprietary "triple play" mode for obtaining MS/MS sequence information for the relevant peptides. Data bank searching was performed with "MS-Fit" (http://prospector.ucsf.edu/) for MALDI mass fingerprint data and with "Sequest search" (http://fields.scripps.edu/sequest/index.html). Mass spectra of intact protein were obtained with linear MALDI-TOF (Voyager-DE Pro) using sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) as the matrix.

Cloning of the DNA encoding C-terminally processed chitinase A

Two flanking primers were designed according to the nucleotide sequence of the *chitinase A* gene and compatible with cloning sites of the pQE60 expression vector (Qiagen). The forward primer included a *Nco*I cloning site, following the oligonucleotides that encode the chitinase A signal peptide. The reverse primer included a *Bgl*II cloning site, following the oligonucleotides encoding the C-terminal region starting from R^{597}. The primer sequences are:

*Nco*I
N-terminal: 5′-TATGCCATGGTAATTCGATTTACCTATG-3′
C-terminal: 5′-GAAGATCTACGGTTTGGTGGGGTAACGAC-3′
*Bgl*II

Thirty cycles of PCR were carried out using *Taq* DNA polymerase (Promega) with the following temperature profile: denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min except for the final cycle where extension proceeded for 7 min. A 1.7-kb amplified PCR product was purified using a DNA gel extraction kit (Qiagen) following the manufacturer's instructions. The purified fragment (5 μg) was then ligated to the pDrive cloning vector (Qiagen) and transformed into *E. coli* DH5α.The recombinant plasmid was prepared using the plasmid miniprep kit according to Qiagen's standard protocol. The plasmid was digested with *Nco*I and *Bgl*II, and then inserted into the *Nco*I/*Bgl*II sites of the pQE60 vector. The chitinase A lacking the C-terminal proteolytic cleavage fragment was expressed under the T5 promoter/*lac* operator element in *E. coli* type strain M15. Using such a vector system, the protein contained six histidine residues tagged at the C-terminal region.

Purification of chitinase A

Chitinase A secreted by *V. carchariae* culture was purified according to Suginta et al. [21]. Briefly, *V. carchariae* was grown at 30 °C in a marine medium. Swollen chitin (2.5% (w/v)) was added to induce chitinase expression. After about 40 h, the growth medium was collected by centrifugation. Swollen chitin was also used in the first step of the enzyme purification using chitin affinity chromatography. Binding of the secreted chitinase to chitin was carried out batchwise by adding 25 g of swollen chitin to a 2-L growth medium of *V. carchariae* and stirred for 5 min at 4 °C, and then the chitinase-bound chitin was collected by centrifugation. After washing with sodium carbonate buffer (pH 8.5) followed by sodium acetate buffer (pH 5.5), the enzyme bound to chitin was eluted with 2 M guanidine HCl and dialyzed immediately against 20 mM sodium phosphate buffer, pH 7.0, to remove guanidine HCl. The protein precipitated by 35–80% saturated ammonium sulfate was dissolved in 2 ml of the dialysis buffer containing 100 mM NaCl and then applied to a Sephacryl S300 HR column. Fractions containing chitinase activity were pooled, concentrated, and then reapplied to the same Sephacryl S300 HR column to further remove minor

contaminants. Chitinase-containing fractions were combined, concentrated using a vacuum centrifuge concentrator, and then stored at −30 °C. Chitinase activity was determined colorimetrically using DMAB method [31]. Protein concentrations were determined by Bradford's method [32]. Unless otherwise stated, experiments were carried out at 4 °C throughout the purification steps. For purification of chitinase A expressed in *E. coli* M15, the bacterial cells carrying the recombinant pQE60 plasmid were grown at 37 °C in LB medium containing 100 μg/ml ampicillin to an OD_{600} of about 0.6, and then IPTG was added to a final concentration of 0.5 mM. Incubation was continued at 25 °C for 5 h with shaking before harvesting the cells by centrifugation at 2500*g* for 20 min. The cells were resuspended in 15 ml of 20 mM Tris–HCl buffer, pH 8.0, containing 150 mM NaCl, 1 mM PMSF, and 1 mg/ml lysozyme. The suspended cells were kept on ice and broke open using an ultrasonicator (30 s, 6–8 times). Unbroken cells and cell debris were removed by centrifugation. The supernatant containing soluble chitinase A was purified using Ni–NTA agarose chromatography according to Qiagen's protocol. Fractions eluted with 250 mM imidazole, which contained soluble chitinase A, were pooled and concentrated using Vivaspin membrane concentrators (MW cut-off 10,000). Further purification was performed using an ÄKTA purifier system (Amersham Biosciences) on a Superdex S-200 HR 10/30 column (1.0 × 30 cm) using 20 mM Tris–HCl buffer, pH 8.0, containing 150 mM NaCl as running buffer. A flow rate of 250 μl/min was applied and fractions of 500 μl were collected. Chitinase-containing fractions were combined and stored at −30 °C until use.

Chitinase activity assay following SDS–PAGE

Chitinase A expressed in *E. coli* (2 μg) was treated with gel loading buffer without β-mercaptoethanol and electrophoresed in 12% polyacrylamide gel containing 0.1% glycol–chitin. After electrophoresis, the gel was washed at 37 °C for 1 h with 250 ml of 150 mM sodium acetate, pH 5.0, containing 1% Triton X-100 and 1% skimmed milk, followed by the same buffer without 1% skimmed milk for another hour to remove SDS and to allow the proteins to refold. The gel was stained with Calcoflour white M2R (0.01%) (Sigma) in 500 mM Tris–HCl, pH 8.5, and visualized under UV [33].

HPLC/ESI-MS analysis of chitin hydrolysis of chitinase A expressed in E. coli

Chitin hydrolysis was carried out in 50 mM ammonium acetate buffer, pH 7.1, at 20 °C with shaking. The concentrations of chitinase A and colloidal chitin suspension [34] were 750 ng/μl and 100 μg/ml, respectively. The reactions were quenched with 10% (v/v) acetic acid. Following a centrifugation at 5 °C, the supernatant containing chitooligosaccharide products formed after 5 min was immediately injected into a 150 × 2.1 mm (5 μm) Hypercarb HPLC (ThermoQuest, USA). The column was connected to an Agilent Technologies 1100 series HPLC system under the control of a Thermo Finnigan LCQ DECA electrospray mass spectrometer. The HPLC was operated at particularly low temperature (10 °C) and detected by ESI-MS. ESI-MS was conducted in positive single ion mode (SIM mode). Mass-to-charge ratios (*m/z*) of expected oligosaccharides were selected as follows: GlcNAc (221.9), $(GlcNAc)_2$ (425.5), $(GlcNAc)_3$ (627.6), $(GlcNAc)_4$ (830.8), $(GlcNAc)_5$ (1034.0), $(GlcNAc)_6$ (1237.2), and $(GlcNAc)_7$ (1440.0). Identification of β- and α-anomers was assessed from previous experiments with equivalent reverse-phase HPLC system and ^1H NMR [8].

Results and discussion

V. carchariae chitinase A is an endochitinase

The rate of glycol–chitin hydrolysis by *V. carchariae* chitinase A was determined using the viscosity reduction assay (Fig. 1) [25]. Prior to hydrolysis, the viscosity of the reaction mixture was high due to the presence of very long chains of the substrate polymer. In the first hour after adding chitinase, the viscosity dropped rapidly due to digestion of chitin by the added chitinase enzyme, leading to rapid breakdown of the polymer chains. A subsequent more gradual decrease in the vis-

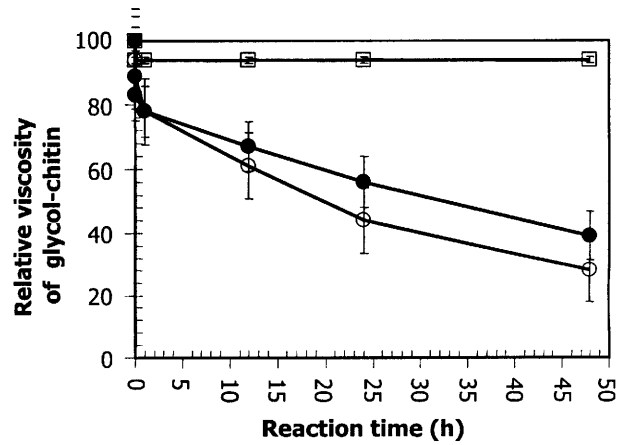

Fig. 1. Enzymatic viscosity reduction assay using glycol–chitin. Purified chitinase A (5 μg) was incubated with 2% (w/v) glycol–chitin solution at room temperature. The viscosity of glycol–chitin was measured at different time intervals from 0 min to 48 h. Reactions with *S. marcescens* chitinase (○), reactions with *V. carchariae* chitinase (●), and reactions without enzyme (□). Data are shown as means ± SEM ($n = 3$ for each protein).

cosity of the glycol–chitin solution was observed after 1 h of the hydrolytic reaction, at which time chitooligomers generated during initial hydrolysis were presumably further degraded into end products. However, the degradation of short intermediates appeared to have a less dramatic effect on the solution viscosity, compared to the initial viscosity change due to the hydrolysis of the long-chain substrate. After 48 h, the reaction became very slow and still did not reach complete hydrolysis. This might result from inhibition of the enzyme activity by high concentrations of the accumulated products.

A rapid reduction in viscosity at the very early stage of reaction indicated that the *V. carchariae* chitinase A has a characteristic endochitinase activity. The enzyme appears to be able to randomly cleave internal β-glycosidic bonds of the substrate polymer, leading to a rapid decrease in the chain length of the dissolved polymer and a rapid decrease in solution viscosity. Exochitinase activity, in contrast, would break down the polymer chains sequentially dimer by dimer from the non-reducing end, at a relatively constant rate over time [11]. Thus, a slow and steady reduction in viscosity would be expected upon addition of enzyme. Endochitinase activity was supported by results from paper chromatography (not shown) in which minor products: GlcNAc, $(GlcNAc)_3$, and $(GlcNAc)_4$, were detected along with the major product $(GlcNAc)_2$. These findings were later confirmed by analysis of the products of chitin hydrolysis by HPLC-MS, showing the production of chitosaccharide products ranging from GlcNAc to $(GlcNAc)_7$. The characteristic endochitinase activity was also observed for *S. marcescens* chitinase A (Fig. 1), which contrasts with a previous suggestion that *S. marcescens* chitinase A is an exochitinase [35].

Isolation of clones carrying the chitinase A gene

The genomic library consisting of 2100 colonies was screened for the expression of chitinase antigen, and six single colonies gave positive signals, and could be isolated. The library was estimated to correspond to 1.3 genome equivalents by assuming that the size of the *V. carchariae* genome is the same as that of *E. coli* (4.2×10^6 bp) [36]. The positive clones were cultured individually in the presence or absence of swollen chitin, and samples of cell extracts and culture supernatants were examined by Western blotting (Fig. 2). It can be seen that all six clones expressed chitinase antigen in the cell extracts, with highest expression in the presence of the swollen chitin inducer (Fig. 2B and D). Clone P3C1 gave the highest expression, but apparently only in the presence of swollen chitin.

The M_r observed for the chitinase antigen expressed in *E. coli* was approx. 95,000, whereas that for chitinase purified from *V. carchariae* was 63,000 [21]. The expression pattern in the culture supernatants showed a much greater bias in favor of induced expression than the cell extracts, as only very faint signals were found in the absence of swollen chitin (Fig. 2A and C). Moreover, only four of the six clones (clones P2C1, P2C2, P2C3, and P3C1) appeared to give secretion into the culture supernatant, and only these four clones exhibited chitinase activity on a chitin agar plate (not shown). Immunoblotting of both fractions from untransformed *E. coli* cells showed no expression of chitinases upon induction with chitin (not shown). Expression of the 95-kDa chitinase in response to chitin implies that all the clones carried a DNA insert that contains the *chitinase A* gene with its own control element (a *ChiA* promoter) (Fig. 3).

Nucleotide sequence analysis

Restriction mapping with *Eco*RI showed that all the clones typically carried apparently identical 5.5-kb inserts, with the exception of clone P3C1 that had a 4.0-kb insert. Because P3C1 contained the smallest DNA fragment, it was therefore chosen for sequence analysis. DNA sequence determination of 100 bp at the 5′ end of the insert in clone P3C1 showed 76% sequence identity to the corresponding region of *ChiA* from *S. marcescens* [37]. The P3C1 clone was later fully analyzed to give the complete sequence (Fig. 3). The *chitinase A* gene contains a putative open reading frame of 2550 bases

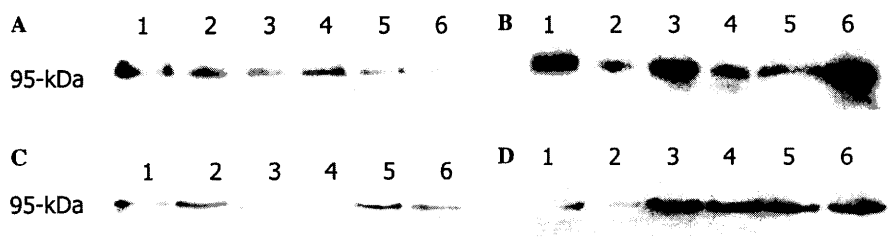

Fig. 2. Western blot analysis of clones expressing chitinase antigen. Single colonies were grown in the presence or absence of 1% (w/v) swollen chitin, and cell extracts and culture supernatants were prepared. The proteins present in these samples were analyzed by Western blotting. (A) Cell extracts, absence of swollen chitin; (B) cell extracts, presence of swollen chitin; (C) culture supernatants, absence of swollen chitin; and (D) culture supernatants, presence of swollen chitin. The tracks had the following clones: 1, P1C1; 2, P1C2; 3, P2C1; 4, P2C2; 5, P2C3; and 6, P3C1 (nomenclature: P refers to the number of the culture plate and C to the colony number).

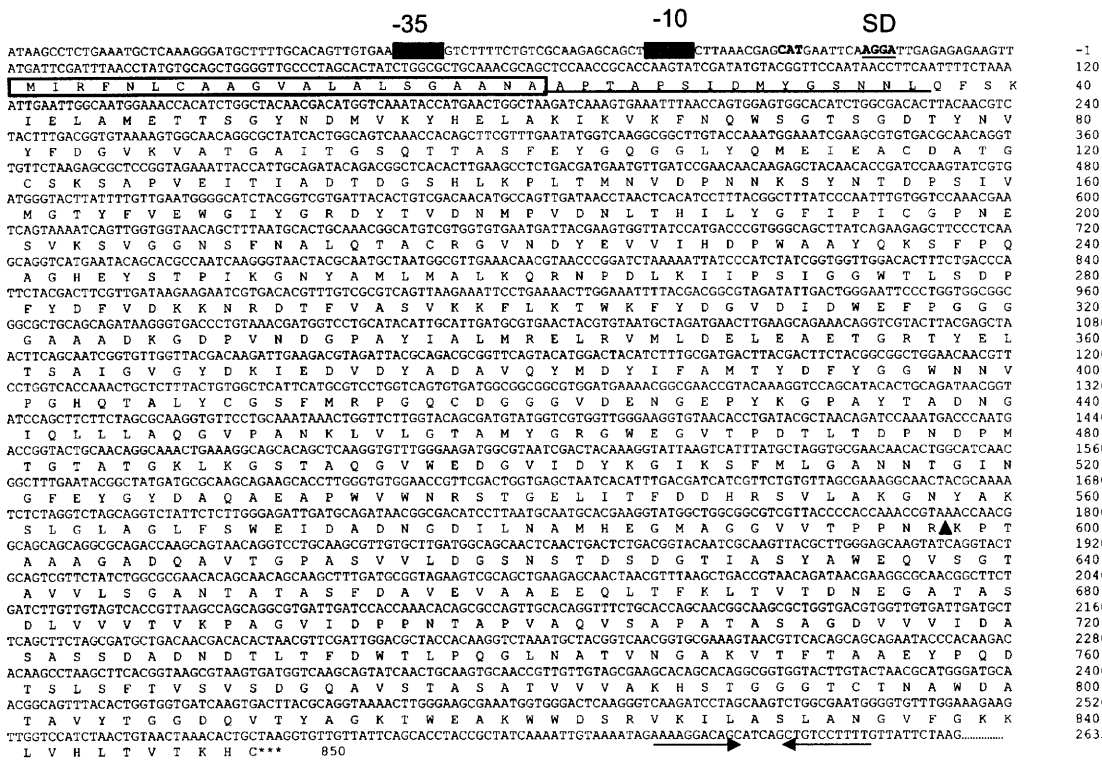

Fig. 3. Determination of the DNA sequence of the *V. carchariae* chitinase gene. Essential elements of the *chitinase A* gene comprise: (i) the −10 and −35 regions of a possible promoter sequence (highlighted); (ii) transcription start site (bold); (iii) the putative ribosome-binding site (AGGA) (bold and underline); (iv) the signal peptide containing 21 amino acids (boxed); (v) the structural *chitinase A* gene; (vi) the termination codon TAA (asterisks); and (vii) the putative inverted-repeat sequence downstream of the termination codon (horizontal arrows). The N-terminal sequence of secreted chitinase A determined by microsequencing [21] is underlined. The predicted C-terminal proteolytic cleavage site is indicated by a triangle.

starting at the ATG codon, ending with the stop codon TAA, and encodes 850 amino acid residues. The ATG initiation codon is preceded by a Shine–Dalgarno sequence (AGGA) that is identical to that of the ribosome binding site in the chitinase gene of *Enterobacter agglomerans* [38]. Typical promoter sequences [39] were found at the −35 region (TTGATT) and the −10 region (TATGTT), although the distance between these two sites is longer than customary (24 bp instead of 17 bp). The sequence at the −10 region is identical to that in the *E. coli lac* operon promoter. An inverted repeat sequence (AAAAGGACAGC----GCTGTCCTTTT) is located 44 nucleotides downstream from the termination codon.

The chitinase precursor has a typical N-terminal secretion signal peptide of 21 residues with a positively charged arginine near the N-terminus followed by a hydrophobic region. It is especially rich in alanine (36%) and leucine (14%) [40]. A signal-sequence cleavage site was predicted by the Signal P V1.1 program as described in Materials and methods to be located between A^{21} and A^{22}, and corresponded precisely with the N-terminal sequence of the mature chitinase secreted by *V. carchariae* as determined by microsequencing [21]. Alanine has also been found to be on the N-terminal side of the signal peptide cleavage site in other bacterial chitinases [38,41,42].

Amino acid sequence analysis

The deduced amino acid sequence of chitinase A from *V. carchariae* was compared with other bacterial ChiA sequences. The putative mature chitinase A showed highest identity with ChiA from *Vibrio parahaemolyticus* (94%), followed by ChiA from *Serratia liquefaciens* (48%), ChiA from *Alteromonas* sp. (47%), and ChiA from *Enterobacter* sp. (47%), ChiA from *S. marcescens* (47%), and ChiA from *Pantoea agglomerans* (44%). *V. carchariae* chitinase A aligned with ChiA from *Bacillus circulans* with low identity (18%). Fig. 4 shows the amino acid sequence comparison of *V. carchariae* chitinase A with *Alteromonas* sp ChiA, *P. agglomerans* ChiA, and *S. marcescens* ChiA. The secondary structure of the *S. marcescens* ChiA structure is also shown to locate the positions of an N-terminal chitin binding domain connected with a small hinge region, a typical $(\alpha/\beta)_8$ TIM barrel catalytic domain, and an extra $\alpha + \beta$ domain. As expected, bacterial chitinase A appears to be highly conserved in the catalytic region, with two completely conserved motifs SxGG (located in the β3

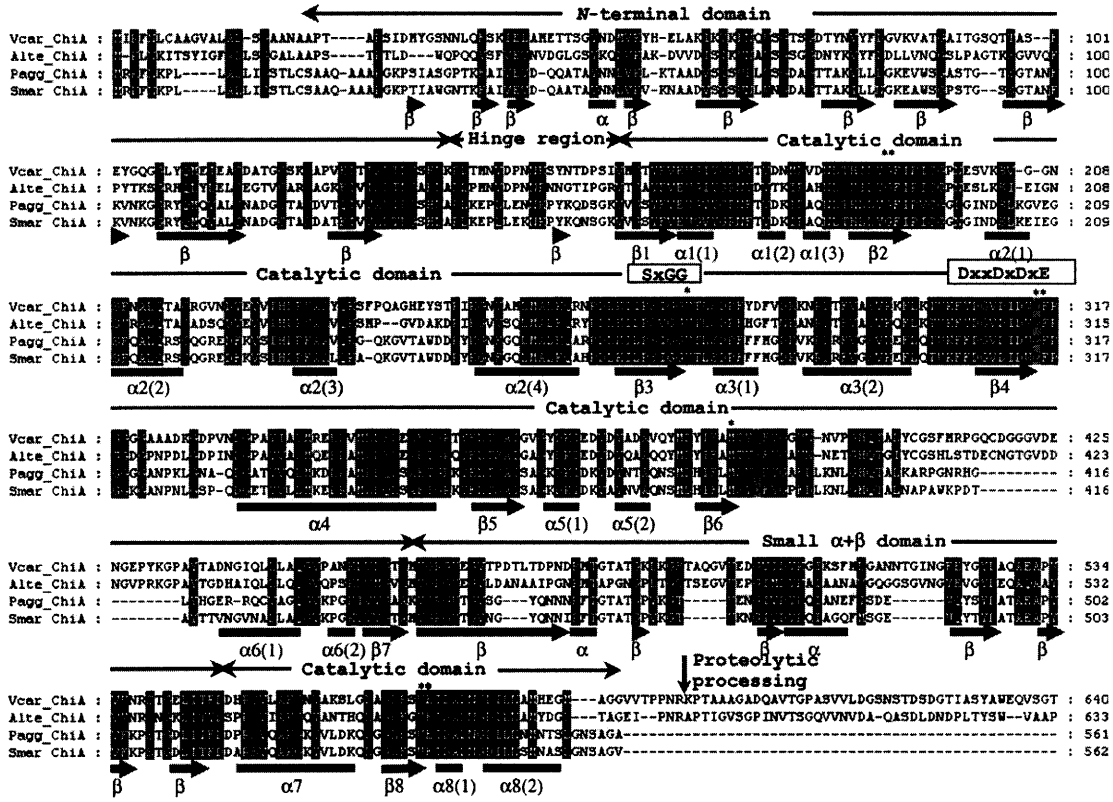

Fig. 4. Alignment of *V. carchariae* chitinase A with other bacterial chitinase A sequences. Bacterial chitinase A sequences were retrieved from the Swiss-Prot/TreEMBL protein databases, aligned using "CLUSTALW" and displayed in Gendoc. The secondary structure elements are those observed in the crystal structure of *S. marcescens* ChiA [13]. Completely conserved regions are shaded in dark blue and the catalytic residues in magenta. The amino acid residues that are suggested to form three cis peptide bonds or to provide hydrophobic environments in the active site of *S. marcescens* ChiA are indicated (asterisks). Key: Vcar_ChiA: chitinase A from *V. carchariae* (*harveyi*) (Q9AMP1); Alte_ChiA: ChiA from *Alteromonas* sp. strain O-7 (P32823); Pagg_ChiA: ChiA from *Pantoea* (*Enterobacter*) *agglomerans* (P97034); and Smar_ChiA: ChiA from *S. marcescens* (P07254, Q54275) ▬▶: β-strand ▬▬: α-helix.

strand) and DxxDxDxE (located in the β4 strand) within the TIM barrel catalytic domain. These motifs have been found in all family 18 chitinases [14].

Amino acid residues that were suggested to form three *cis* peptide bonds (equivalent to residues G^{190}–F^{191}, E^{315}–F^{316}, and W^{539}–E^{540}) or to provide hydrophobic environments (equivalent to residues F^{191}, W^{275}, F^{316}, and M^{388}) in the active site of *S. marcescens* ChiA are completely conserved in all the aligned sequences (Fig. 4).

C-terminal proteolytic cleavage site of V. carchariae chitinase A

The ensemble of peptides, which were eluted from in-gel digestion of chitinase A, was analyzed by means of MALDI-TOF or nanoESI mass spectrometry and subjected to a data-bank search. This process, commonly denoted as mass fingerprinting, resulted in an unambiguous match of this protein to the chitinase A gene (Table 1). Moreover, the molecular mass of peptide T1 (2040.7) agreed well with the theoretical mass (2040.0) of the N-terminal peptide identified previously by microsequencing [21]. In addition, peptide T39, identified as being nearest to the C-terminus, had a mass of 551.0, which matched the mass of the tryptic peptide sequence GNYAK.

The chitinase A precursor had a calculated M_r of 90,249, which was slightly less than indicated by SDS–PAGE (95,000). Because the chitinase precursor was inactive and its molecular mass was approx. 23 kDa larger than the native enzyme, the precursor must be cleaved by a proteinase in *V. carchariae* to form the active 63-kDa enzyme. MALDI-TOF measurement yielded a peak of M_r 62,698 (Fig. 5), which corresponded to the mass of chitinase A predicted to end at R^{597} (calculated M_r 62,718.12). C-terminal processing has also been detected in other chitinases, for example, in two *Serratia* chitinases: a 52-kDa chitinase cleaved between F^{324} and K^{325} to produce an active proteolytic 35-kDa product, and a 54-kDa chitinase cleaved between I^{482} and T^{483} to produce an active proteolytic 22-kDa product [43,44]. The 59-kDa chitinase isolated from *Streptomyces olivaceoviridis* was also found to be proteolytically processed to a 47-kDa truncated chitinase lacking the chitin binding domain [45]. However, chitinases from *Aeromonas hydrophila* [46] and

Table 1
Mass identification of tryptic peptides of *V. carchariae* chitinase A by MALDI-TOF or nanoESI mass spectrometry

Position in the sequence[a]	Tryptic peptide	Expected mass	Observed mass	Peptide AA sequence
22–40	T1	2040.0	2040.7	APTAPSIDMYGSNNLQFSK
41–56	T2	1800.8	1800.5	IELAMETTSGYNDMVK
57–62	T3	759.4	759.3	YHELAK
67–86	T6	2284.0	2283.8	FNQWSGTSGDTYNVYFDGVK
124–151	T8	2976.5	2976.6	SAPVEITIADTDGSHLKPLTMNVDPNNK
152–173	T9	2554.2	2553.6	SYNTDPSIVMGTYFVEWGIYGR
204–218	T11	1523.7	1523.6	SVGGNSFNALQTAC
219–236	T12	2103.0	2102.8	GVNDYEVVIHDPWAAYQK
237–250	T13	1560.8	1560.7	SFPQAGHEYSTPIK
251–262	T14 + T15	1394.7	1394.5	GNYAMLMALKQR
268–288	T17 + T18	2397.2	2397.0	IIPSIGGWTLSDPFYDFVDKK
289–298	T19 + T20	1135.6	1135.6	NRDTFVASVK
303–326	T23 + T24	2601.2	2601.2	TWKFYDGVDIDWEFPGGGGAAADK
327–341	T25	1587.8	1587.6	GDPVNDGPAYIALMR
345–356	T27	1361.7	1361.7	VMLDELEAETGR
454–463	T31	1079.6	1079.6	LVLGTAMYGR
464–487	T32	2460.1	2460.1	GWEGVTPDTLTDPNDPMTGTATGK
488–505	T33 + T34	1965.0	1964.8	LKGSTAQGVWEDGVIDYK
509–538	T36	3377.5	3378.5	SFMLGANNTGINGFEYGYDAQAEAPWVWNR
539–550	T37	1389.7	1389.6	STGELITFDDHR
551–555	T38	516.3	516.5	SVLAK
556–560	T39	551.3	551.3	GNYAK

[a] Unidentified peptides are not included.

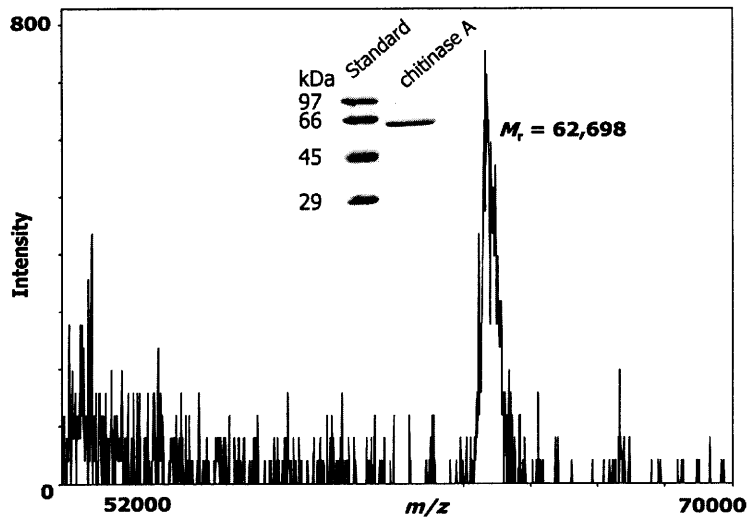

Fig. 5. MALDI-TOF spectrogram of chitinase A purified from *V. carchariae*. The purified *V. carchariae* chitinase A (2 μg) was mixed in serial dilutions (1/10) with sinapinic acid, 1 mg/ml in 0.3% TFA/ACN (1:1) and deposited on a stainless steel sample stage. After rapid drying, samples in crystalline form were submitted for Voyager Elite MALDI-TOF (linear mode).

Alteromonas sp. [41] are expressed as large proteins (866 and 820 residues, respectively), and apparently are not subjected to C-terminal processing.

Expression of chitinase A in E. coli

Chitinase A expressed in *E. coli* as the unprocessed precursor was much less active than the enzyme purified from *V. carchariae*. Taking advantage of the M_r of the native enzyme obtained from MALDI-TOF measurement, two oligonucleotides were designed to generate the mature protein without the 23-kDa C-terminal proteolytic peptide (see Materials and methods). A 1.7-kb DNA fragment encoding the C-terminally processed chitinase A was cloned into pDrive cloning vector and later transferred to the pQE60 expression vector. The protein was expressed under the T5 promoter in *E. coli* M15 under optimized conditions with high yield (~10 mg/100 ml culture).

Using such a system, the expressed chitinase was a hybrid protein with six histidines tagged at the C-terminus. The protein was purified using Ni–NTA agarose

affinity chromatography, followed by Superdex-S200 HR FPLC (Fig. 6, lane 1). Electrospray MS confirmed the M_r of the expressed protein to be 63,823 (±15), corresponding to the calculated M_r of the mature chitinase (62,718.12 Da) plus two additional amino acids: arginine and serine (260.27 Da). These amino acids were encoded by the six nucleotides (AGATCT) corresponding to the *Bgl*II cloning site following the codon of

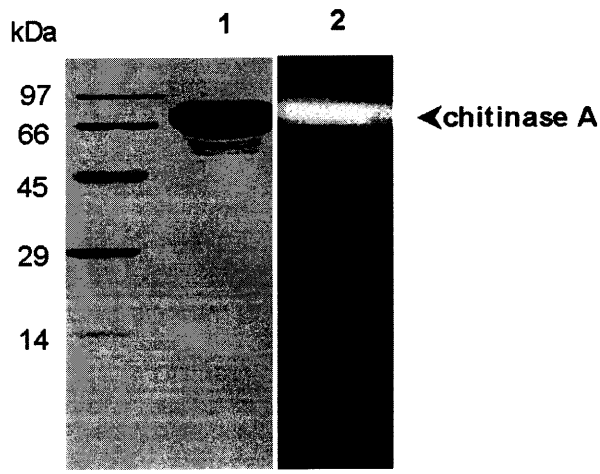

Fig. 6. Gel activity assay of the chitinase A expressed in *E. coli*. The purified chitinase A (3 μg) was electrophoresed in 12% SDS–PAGE. After electrophoresis, protein bands were stained with Coomassie blue (lane 1). In the case of the gel activity assay (lane 2), the protein was prepared in the absence of β-mercaptoethanol and was not heated prior to subjecting to SDS–PAGE gel in the presence of glycol-chitin. The gel was stained for chitinase activity using fluorescent Calcoflour white M2R.

R^{597} of the mature chitinase A (see Fig. 3) and formed a link to the C-terminal histidine tag residues (839.85 Da). The total calculated mass of the expressed protein is therefore 63,818.24 Da. The expressed protein exhibited chitinase activity using the gel activity assay with glycol–chitin substrate (Fig. 6, lane 2).

As analyzed by HPLC/ESI-MS, chitinase A expressed in *E. coli* was able to hydrolyze colloidal chitin. Fig. 7 shows a HPLC-MS chromatogram of chitooligosaccharide products acquired after 5 min of reaction time. The enzyme degraded chitin polymer releasing chitooligosaccharide products ranging from GlcNAc to $[GlcNAc]_7$ with $[GlcNAc]_2$ as the major product (>80% of the total products). Although $[GlcNAc]_5$ and $[GlcNAc]_7$ were not clearly seen in the HPLC-MS chromatogram, their molecular masses were certainly observed in the MS spectrum (Fig. 7 inset). The release of chitooligosaccharide products with various sizes confirmed the endo characteristic of *V. carchariae* chitinase A.

With the HPLC system used, the β- and α-anomers were also separated. The cleavage pattern was assessed from a separation profile of chitooligosaccharides as previously been published using reverse-phase HPLC and ^1H NMR [8]. The earlier appearing peak represented the β-anomer and the later peak corresponded to the α-anomer of the oligomeric products. Production of higher level of the β- over α-anomers at initial stage of reaction indicated that the hydrolysis of chitin polymer by chitinase A is employing a mechanism that is in an agreement with the substrate-assisted catalysis as suggested earlier [6,7].

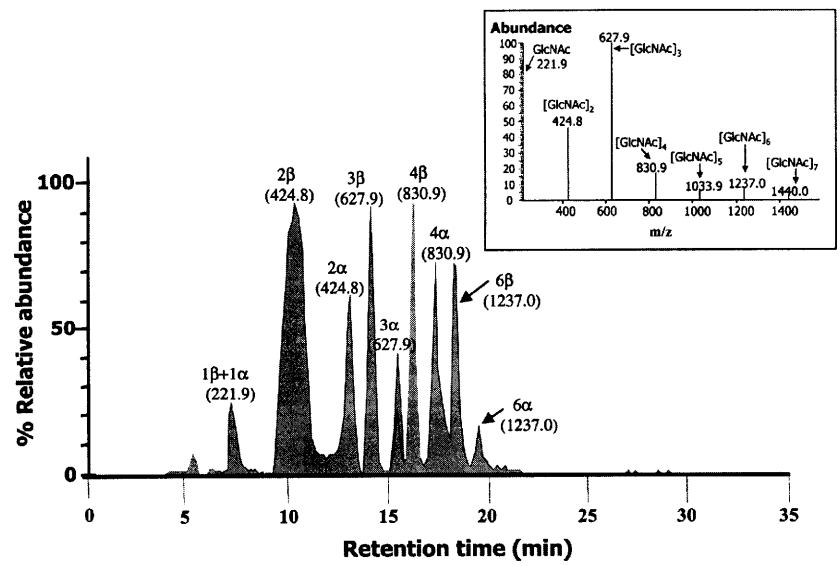

Fig. 7. A HPLC-MS chromatogram of hydrolytic products of chitinase A expressed in *E. coli*. Chitinase A (75 ng) was added to 100 μg/ml colloidal chitin and incubated at 20 °C for 5 min. After centrifugation, a 10-ml supernatant was immediately subjected to a hypercarb HPLC and eluted at 250 μl/min with a linear gradient from 5 to 40% acetronitrile into a LCQ ESI-MS. The relative abundance of the product peaks is plotted as a function of the elution time. Numbers indicate the amount of GlcNAc units in an oligomer, α, and β indicate their isoforms. The inset represents the MS signal recorded in the SIM mode set for *m/z* 222, 425, 628, 831, 1034, and 1237.

In conclusion, we provided evidence that *V. carchariae* chitinase A is an endochitinase. We also report gene isolation and sequence comparison based on the closely related *S. marcescens* ChiA. Determination of the C-terminal proteolytic site of the enzyme allowed the mature chitinase A to be generated in vitro, permitting successful expression of the functional protein in *E. coli*. This will lead to structural investigation and elucidation of the mode of enzyme action of *V. carchariae* chitinase A in great detail using both X-ray crystallographic and genetic engineering techniques, thus paving the way for the biotechnological application in bioconversion of chitin based on chitinase A.

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References

[1] C. Jeuniaux, Methods Enzymol. 8 (1966) 644–650.
[2] R.A.J. Warren, Annu. Rev. Microbiol. 50 (1996) 183–212.
[3] R.S. Patil, V. Ghormade, M.V. Deshpande, Enzyme Microb. Technol. 26 (2000) 473–483.
[4] B. Henrissat, Biochem. J. 280 (1991) 309–316.
[5] B. Henrissat, A. Bairoch, Biochem. J. 316 (1993) 695–696.
[6] Y. Papanikolau, G. Prag, G. Tavlas, C.E. Vorgias, A.B. Oppenheim, K. Petratos, Biochemistry 40 (2001) 11338–11343.
[7] K. Bortone, A.F. Monzingo, S. Ernst, J.D. Robertus, J. Mol. Biol. 320 (2002) 293–302.
[8] S. Armand, H. Tomita, A. Heyraud, C. Gey, T. Watanabe, B. Henrissat, FEBS Lett. 343 (1994) 177–180.
[9] A.C. Terwisscha van Scheltinga, S. Armand, K.H. Kalk, A. Isogai, B. Henrissat, B.W. Dijkstra, Biochemistry 34 (1995) 15619–15623.
[10] C. Sasaki, A. Yokoyama, Y. Itoh, M. Hashimoto, T. Watanabe, T. Fukamizo, J. Biochem. (Tokyo) 131 (2002) 557–564.
[11] P.W. Robbins, C. Albright, B. Benfield, J. Biol. Chem. 263 (1998) 443–447.
[12] A.C. Terwisscha van Scheltinga, K.H. Kalk, J.J. Beintema, B.W. Dijkstra, Structure 2 (1994) 1181–1189.
[13] A. Perrakis, I. Tews, Z. Dauter, A.B. Oppenheim, I. Chet, K.S. Wilson, C.E. Vorgias, Structure 2 (1994) 1169–1180.
[14] D.M.F. van Aalten, B. Synstad, M.B. Brurberg, E. Hough, B.W. Riise, V.G.H. Eijsink, R.K. Wierenga, Proc. Natl. Acad. Sci. USA 97 (2000) 5842–5847.
[15] D.M.F. van Aalten, D. Komander, B. Synstad, S. Gaseidnes, M.G. Peter, V.G.H. Eijsink, Proc. Natl. Acad. Sci. USA 98 (2001) 8979–8984.
[16] B.L. Bassler, C. Yu, Y.C. Lee, S. Roseman, J. Biol. Chem. 266 (1991) 2476–2486.
[17] I. Hirono, M. Yamashita, T. Aoki, J. Appl. Microbiol. 84 (1998) 1175–1178.
[18] H. Tsujibo, Y. Yoshida, K. Miyamoto, Can. J. Microbiol. 38 (1992) 891–897.
[19] T. Lonhienne, K. Mavromatis, C.E. Vorgias, L. Buchon, C. Gerday, V. Bouriotis, J. Bacteriol. 183 (2001) 1773–1779.
[20] B.L. Bassler, P.J. Gibbons, C. Yu, S. Roseman, J. Biol. Chem. 266 (1991) 2468–2475.
[21] W. Suginta, P.A.W. Robertson, B. Austin, S.C. Fry, L.A. Fothergill-Gilmore, J. Appl. Microbiol. 89 (2000) 76–84.
[22] K. Pedesen, L. Verdonck, B. Austin, D.A. Austin, A.R. Blanch, P.A.D. Grimont, J. Jofre, S. Koblavi, J.L. Larsen, T. Tiainen, M. Vigneulle, J. Swings, Int. J. Syst. Bacteriol. 48 (1998) 749–758.
[23] R.T. Dando, E.P.S. Young, In the NCIMB Catalogue of Strains, Aberdeen University Press, Aberdeen, 1990.
[24] J. Monreal, E.T. Reese, Can. J. Microbiol. 15 (1968) 689–696.
[25] A. Khasin, I. Alchanati, Y. Shoham, Appl. Environ. Microbiol. 59 (1993) 1725–1730.
[26] E. Cabib, Methods Enzymol. 161 (1988) 425–435.
[27] F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.A. Smith, K. Strukh, Short Protocols in Molecular Biology, fourth ed., Wiley, New York, 1999.
[28] J.D. Thompson, D.G. Higgins, T.J. Gibson, Nucleic Acids Res. 22 (1994) 4673–4680.
[29] U.K. Laemmli, Nature 227 (1970) 680–685.
[30] A. Shevchenko, M. Wilm, Vorm, O.M. Mann, Anal. Chem. 68 (1996) 850–858.
[31] J.L. Reissig, J.L. Strominger, L.F. Leioir, J. Biol. Chem. 217 (1955) 959–966.
[32] M.M. Bradford, Anal. Biochem. 72 (1976) 248–254.
[33] J. Trudel, A. Asselin, Anal. Biochem. 178 (1989) 362–366.
[34] W.K. Roberts, C.P.J. Selitrennikoff, Gen. Microbiol. 134 (1988) 169–176.
[35] R.L. Roberts, E. Cabib, Anal. Biochem. 127 (1982) 402–412.
[36] K. Kaiser, N.E. Murray, P.A. Whittaker, in: D.M. Glover, B.D. Hames (Eds.), DNA Cloning 1: A Practical Approach, second ed., Oxford University Press, New York, 1996, pp. 37–84.
[37] J.D.G. Jones, K.L. Grady, T.V. Suslow, J.R. Bedbrook, EMBO J. 5 (1986) 467–473.
[38] L.S. Chernin, L. de la Fuente, V. Sobolev, S. Haran, C. Vorgias, A.B. Oppenheim, I. Chet, Appl. Environ. Microbiol. 63 (1997) 834–839.
[39] W.R. McClure, Annu. Rev. Biochem. 54 (1985) 171–204.
[40] G. von Heijne, Eur. J. Biochem. 133 (1983) 17–21.
[41] H. Tsujibo, H. Orikoshi, H. Tanno, K. Fujimoto, K. Miyamoto, C. Imada, Y. Okami, Y. Inamori, J. Bacteriol. 175 (1993) 176–181.
[42] H. Tsujibo, H. Orikoshi, K. Shiotani, M. Hayashi, J. Umeda, K. Miyamoto, C. Imada, Y. Okami, Y. Inamori, Appl. Environ. Microbiol. 64 (1998) 472–478.
[43] S.W. Gal, J.Y. Choi, C.Y. Kim, Y.H. Cheong, Y.J. Choi, J.D. Bahk, S.Y. Lee, M.J. Cho, FEMS Microbiol. Lett. 151 (1997) 197–204.
[44] S.W. Gal, J.Y. Choi, C.H. Kim, Y.H. Cheong, Y.J. Choi, S.Y. Lee, J.D. Bahk, M.J. Cho, FEMS Microbiol. Lett. 160 (1998) 151–158.
[45] H. Blaak, H. Schrempf, Eur. J. Biochem. 229 (1995) 132–139.
[46] J.P. Chen, F. Nagayama, M.C. Chang, Appl. Environ. Microbiol. 57 (1991) 2426–2428.