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ORIGINAL RESEARCH PAPER

A new approach for discovering cold-active enzymes in a cell mixture of pure-cultured bacteria

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Abstract To overcome the intrinsic problems of conventional approaches, such as the unavailability of source microorganisms in metagenomic libraries and the production of inactive aggregates, a new method was tested for discovering new enzymes (e.g. cold-active chitinase). A metagenome-like library was constructed using genomes extracted from a cell mixture of pure-cultured chitinolytic bacteria, followed by activity-based screening for *Escherichia coli* clones that exhibit chitinase activity on selective medium. Within one positive chitinolytic clone, one chitinase gene (*chi22718_III*) was detected and assigned to the arctic marine bacterium, *Pseudoalteromonas issachenkonii* PAMC 22718, by colony-PCR with *chi22718_III*-specific primers. When expressed in *E. coli*, recombinant R-Chi22718_III lost 85 % of its enzyme activity when pre-incubated at 40 °C for 1 h, whereas its mesophilic counterpart R-ChiK only lost 10 % of its activity under the same conditions indicating that R-Chi22718_III is thermolabile, a characteristic of cold-active enzymes.

Keywords Activity-based enzyme screen · Chitinase · Cold-active · Culture mixture · Functional expression · Metagenomic library · *Pseudoalteromonas issachenkonii*

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Introduction

Historically, two conventional methods have been widely used to isolate new enzymes from environmental samples. The first technique uses enrichment culturing followed by screening, or direct plating of environmental samples for screening, of bacteria using selective media. The second method involves the construction of metagenomic libraries and activity- or sequence-based screening. In addition, a combination of these methods (enrichment culturing, metagenomic library construction, and functional screening) has been tested for the ability to detect novel genes encoding alcohol oxidoreductases in environmental samples (Knietsch et al. 2003). For research and commercial applications, which require considerable amounts of active recombinant protein, the enzymes of interest were produced using a heterologous overexpression system using *Escherichia coli* as a host. This host was because it has numerous advantages, including inexpensive culture conditions, rapid growth, and easy manipulation (Rosano and Ceccarelli 2009).

However, the conventional approaches to discovering new enzymes, and the later steps of heterologous expression for downstream applications, have several intrinsic problems. First, the source bacterium for enzymes is often unavailable in the metagenomic library. Second, the detection rate is extremely low due to the difficulty of functionally expressing the enzyme genes in heterologous *E. coli*. Finally, the recombinant proteins, although overexpressed, frequently form inactive insoluble aggregates (Heath et al. 2009).

To overcome the difficulties of the aforementioned conventional approaches, we developed and tested a new approach to discover new enzymes (in the present study, a cold-active chitinase was used as a model), which should be produced as a soluble and functional protein from *E. coli*. Specifically, we mixed purely cultured chitinolytic bacteria obtained from the Polar and Alpine Microbial Collection (PAMC) in the Korea Polar Research Institute (KOPRI) to generate a homogeneous microbial consortium, and then constructed a library of their genomes. We subsequently conducted an activity-based screening for an *E. coli* clone producing a halo on chitinase selective medium, indicating the production of chitinases.

Chitin, β-(1, 4)-linked homopolymer of *N*-acetyl-D-glucosamine (GlcNAc), is one of the most abundant natural biopolymers and it serves as a nutrient source for microbes. The first step in chitin microbial degradation is the hydrolysis of the β-(1, 4)-glycosidic bonds between GlcNAc subunits catalyzed by chitinases. Cold-active chitinase is a useful biocatalyst for the production of pure GlcNAc monomers or oligomers through cold-condition processing in industrial biotechnology. However, until now, only a few cold-active bacterial chitinases from cold natural environments have been characterized (Bendt et al. 2001; Lonhienne et al. 2001; Mavromatis et al. 2003).

Materials and methods

Screening of chitinase-producing cold-adapted bacteria

Korea Polar Research Institute (KOPRI) has been operating a culture collection (PAMC, http://pamc.kopri.re.kr) of cold-adapted bacterial strains as a platform to develop potential industrial enzymes. The bacteria were originally isolated for their hydrolyzing protease, chitinase, or lipase activities from terrestrial and marine samples in polar and alpine regions.

Among approx. 6,500 strains in PAMC, 169 strains have been classified as chitinolytic in 20 % (v/v) glycerol at −80 °C. To select excellent strains producing cold-active chitinases, the 169 strains were revived from glycerol stocks by inoculation on ZoBell plates (5 g peptone, 1 g yeast extract, 0.01 g $FePO_4$, 15 g agar, 750 ml sea water, 250 ml distilled water per liter) and cultivation at 25 °C for several days. Following suspension in ZoBell medium, the bacterial cells were dispensed into 96-well microplates, inoculated on ZoBell containing 0.4 % colloidal chitin with a 96-pin replicator, and incubated at 25 °C for 7 days. Chitinase activity was scored according to the difference between the diameter of the clear zone and the colony diameter.

Genomic library construction and screening of chitinolytic fosmid clones

Ten chitinolytic bacteria were cultured by inoculating a small amount of glycerol stock on Difco Marine Broth 2216 (MB) plates, and one single colony was transferred to 50 ml MB broth and incubated at 25 °C for 1 day. Cell growth was determined from the OD_{600} value; cells of each bacterium were mixed together at 2 ml per OD_{600} value of 1. From the cell mixture, total genomic DNAs were extracted using two different common methods for genomic DNA that were optimized for Gram-negative and Gram-positive bacteria, respectively. Each extracted DNA (300 ng/μl) was mixed at the same ratio. A metagenome-like library of the mixed genomic DNAs was constructed using a pCC1FOS vector and the EPI300-$T1^R$ *E. coli* plating strain (CopyControl Fosmid Library Production Kit, Epicentre), as described by the manufacturer. The resulting fosmid clones were screened on LB plates containing chloramphenicol (12.5 μg/ml), colloidal chitin (0.05 %), and Epicentre CopyControl solution. After incubation at 25 °C for 4 days, chitinolytic clones producing a halo around the colonies were selected as those containing chitinase gene(s).

Small-insert DNA library construction and the screening of chitinolytic plasmid clones

A recombinant fosmid was isolated from one strongly positive chitinolytic fosmid clone (EPI-C78) and

digested with three different restriction enzyme sets (A set: *Sph*I–*Pst*I–*Sal*I–*Eco*RI; B set: *Kpn*I–*Sac*I; C set: *Xba*I–*Sma*I), each of which recognizes multiple cloning sites on the high-copy vector pUC19. The DNA fragments from each restriction reaction were mixed, purified, and ligated into linear pUC19, which was treated with the same restriction enzyme sets and dephosphorylated. The ligation reaction was transformed into *E. coli* Rosetta competent cells. The plasmid clones were screened for chitinase activity on LB plates supplemented with carbenicillin (100 μg/ml) and colloidal chitin (0.05 %). After a 3 day incubation at 25 °C, plasmid clones producing a clear halo were selected for their chitinolytic activity.

Sequencing and analysis of insert DNA in chitinolytic plasmid clone

The *Sal*I-*Eco*R1 DNA fragment in recombinant plasmid from one strongly positive chitinolytic plasmid clone (Rosetta-C78-4) was completely sequenced by primer walking from both ends of the pUC19 cloning site, and the nucleotide sequences were assembled using DNAstar Lasergene software (DNAstar, USA). Similarity analysis of the sequences was performed using the BLAST program by searching against GenBank database, and protein domains were predicted using the Conserved Domain Database in NCBI.

Chitinase gene cloning and homologous expression

A chitinase gene (*chi22718_III*) was PCR-amplified from the recombinant plasmid in chitinolytic Rosetta-C78-4 and cloned into pEXP5-CT/TOPO, generating the recombinant plasmid pDOC166, which was transferred into *E. coli* BL21 star (DE3) for the heterologous production of recombinant R-Chi22718_III. Recombinant *E. coli* culture (5 ml) grown in LB medium was inoculated into 500 ml LB/carbenicillin (100 μg/ml) and cultured to $OD_{600} = 0.6$–0.8 (approx. 2 h) at 37 °C. Following cooling to 15 °C for 1 h, the culture was induced by adding 0.25 mM IPTG and incubated further for 48 h at 15 °C. Subsequently, the culture was harvested, washed in 50 mM sodium phosphate buffer (pH 7.6, standard buffer), and disrupted by sonication in 10 ml of the same buffer. Unbroken cells and cell debris were removed by centrifugation (8,000×*g*, 15 min, 4 °C). The resulting supernatant was used as crude enzyme solution for the chitinase assay and Western blotting.

Determination of chitinase activity and thermostability at various temperatures

Crude R-Chi22718_III (1.0 mg) was added to 1 ml of standard buffer containing 0.1 mM *p*NP-GlcNAc. After incubating the reaction mixture at 30 °C for 1 h, absorbance was determined at 400 nm, and one unit was defined as the enzyme activity sufficient to produce 1 nmol *p*-nitrophenol (extinction coefficient of 17,000 M^{-1} cm^{-1}) per h per mg protein at 30 °C.

To determine the optimal temperature, R-Chi22718_III and *p*NP-GlcNAc were added to standard buffer that was pretreated from 10 to 60 °C. After 1 h incubation, the relative activity of R-Chi22718_III was measured. To test the thermostability of the enzyme, R-Chi22718_III in standard buffer was incubated at 10–60 °C for 1 h. Subsequently, *p*NP-GlcNAc was added to each of the pretreated enzyme-buffer mixtures, and then the residual activity of R-Chi22718_III was measured after another 1 h-incubation.

Results and discussion

Screening of chitinase-producing cold-adapted bacteria

After measuring the relative chitinase activity of 169 bacteria on colloidal chitin plates, 10 strains were selected due to their strong activity. For chitinase typing, the culture supernatants of these strains were assayed with synthetic chromogenic substrates, *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide [*p*NP-GlcNAc] and *p*-nitrophenyl-β-D-*N*, *N*'-diacetylchitobiose [*p*NP-$(GlcNAc)_2$] (Table 1). Each of the ten chitinolytic bacteria was purely cultured, after which the bacteria were mixed together and used to construct a metagenome-like library using CopyControl fosmid pCC1FOS and *E. coli* EPI300.

Screening for chitinolytic fosmid and plasmid clones

The metagenome-like library, which consisted of approx. 2.9×10^4 fosmid clones, was screened for

Table 1 A list of the chitinolytic cold-adapted bacteria used for metagenome-like library construction

PAMC number	Isolation site	Identification	Activity at colloidal chitin plate (%)[a] at 25 °C	Classification[b]
21693	Antarctic soil	*Pseudoalteromonas* sp.	53	Exochitinase
22644	Arctic sea	*Paenibacillus* sp.	40	Endochitinase
22655	Arctic sea	*Paenibacillus* sp.	50	Endochitinase
22688	Arctic sea	*Paenibacillus* sp.	36	Exochitinase
22701	Arctic sea	*Paenibacillus* sp.	45	Exochitinase
22718	Arctic sea	*Pseudoalteromonas issachenkonii*	38	Exochitinase
22723	Arctic sea	*Bacillus thuringiensis*	31	Exochitinase
24697	Arctic sea	*Bacillus* sp.	42	Endochitinase
24716	Arctic sea	*Paenibacillus tundrae*	32	Endochitinase
24812	Arctic sea	*Actinotalea fermentans*	59	Exochitinase

[a] Activity (%) = (halo diameter − colony diameter)/halo diameter on colloidal chitin plates × 100

[b] Chitinases were putatively classified according to their preference to synthetic substrates: exochitinase for *p*NP-GlcNAc and endochitinase for *p*NP-$(GlcNAc)_2$

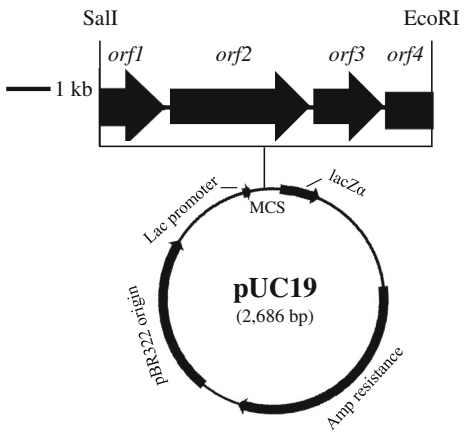

Fig. 1 Map of one strongly positive chitinolytic plasmid clone, Rosetta-C78-4. The Sal1–EcoR1 fragment (7464 bp) from Arctic marine bacterium *Pseudoalteromonas issachenkonii* PAMC 22718 was cloned into high-copy plasmid pUC19 and produced an active recombinant chitinase in *E. coli* Rosetta

the production of a clear halo around the colony on LB plates supplemented with colloidal chitin. As a result, 74 chitinolytic fosmid clones were selected for their chitinolytic activities, and the most active clone (EPI-C78) among them was used to construct a small-insert DNA library using high-copy plasmid pUC19 and *E. coli* Rosetta that was further screened for the clear halo production on the same plates. Subsequently, one positive clone (Rosetta-C78-4) was finally selected owing to its highest activity among six chitinolytic plasmid clones.

Sequence analysis of insert DNA in chitinolytic plasmid clone

The 7464-bp insert DNA in Rosetta-C78-4 was completely sequenced and analyzed, and one partial gene (collagenolytic protease *C*-terminal region; *orf1*), two chitinase genes (*orf2* and *orf3*), and one partial gene (chitinase *N*-terminal region; *orf4*) were detected (Fig. 1; Table 2). When submitted to the NCBI protein–protein BLAST program, their deduced amino acid sequences displayed overall high identities with those of protease and chitinases that were obtained and functionally characterized from different marine *Pseudoalteromonas* sp. strains. Conserved domain searching available on the NCBI website revealed that the protein sequence of intact *orf2* possessed a catalytic domain for chitin hydrolysis that was previously identified in GH18 (glycosyl hydrolases, family 18) type II chitinases, whereas intact *orf3* had only a chitin-binding domain. Considering that the Rosetta-C78-4 clone produced a clear halo on colloidal chitin, it appears likely that a recombinant chitinase from intact *orf2* would display chitinolytic activity.

A specific primer set for the full sequence of *orf2* in Rosetta-C78-4 was applied for chitinase amplification from the genomic DNA extracted from each of the 10 chitinolytic bacteria that were used to construct the metagenome-like library. Consequently, the target PCR product of 3171 bp was amplified from only one strain, psychrotolerant *Pseudoalteromonas issachenkonii*

Table 2 ORFs identified in a 7464-bp insert DNA in the chitinolytic plasmid clone Rosetta-C78-4

ORF	Size (aa)	Representative homologue[a] (identity[b])	Microorganism	Description
orf1 (partial)	1,396 (464)	Deseasin MCP-01 464/464 (100 %)	*Pseudoalteromonas* sp. SM9913	Collagenolytic protease
orf2	3,171 (1,056)	Chitinase A 743/1060 (70 %)	*Pseudoalteromonas* sp. S9	Chitinase with a catalytic domain
orf3	1,596 (531)	Chitinase B 305/529 (58 %)	*Pseudoalteromonas* sp. S9	Putative chitinase with a chitin-binding domain
orf4 (partial)	933 (311)	Chitinase 305/311 (98 %)	*Pseudoalteromonas* sp. PAMC 21693	Chitinase with catalytic and chitin-binding domains
Total	7,464			

[a] Homolog searches were based on a comparison with functionally characterized proteins

[b] Percentage of identity was obtained by aligning the deduced amino acid sequences using Blastp

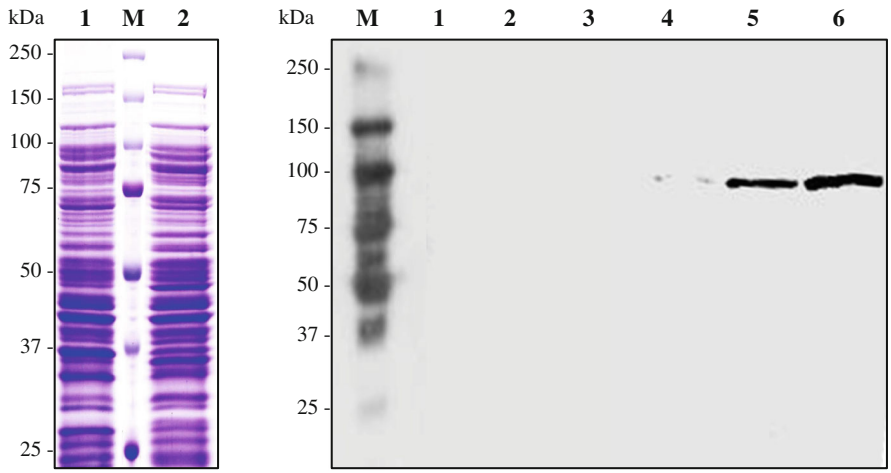

Fig. 2 SDS-PAGE (**a**) and Western blot (**b**) analyses of proteins present in the crude cell lysates. **a** The soluble fractions of *E. coli* cells containing pDOC166 (pEXP5-CT/TOPO plus *chi22718_III*) or pEXP5-CT/TOPO were boiled for 10 min at 95 °C and separated on a 10 % acrylamide gel using a Tris–glycine buffer system. The separated proteins were stained using Coomassie brilliant blue solution. *Lane M* protein size marker; *lane 1 E. coli*/pEXP5-CT/TOPO (25 μg); *lane 2 E. coli*/pDOC166 (25 μg). **b** The separated proteins were transferred onto an Immobilon-P Transfer Membrane (Millipore) at 100 V for 1 h. Following a 12-h incubation at 4 °C with 5 % (v/v) skim milk in PBS/Tween 20 (0.1 %, PBST) as a blocking reagent, the membrane was probed for 2 h at room temperature with anti-His (1:20,000), washed three times for 10 min each in PBST, and incubated for 1 h with goat anti-mouse IgG secondary antibodies (1:5,000) in the blocking solution. After three washes, blots were revealed with a chemiluminescence assay kit and detected on an LAS-3000 imager (Fujifilm). *Lane M*, protein size marker; *lanes 1–3 E. coli*/pEXP5-CT/TOPO (each 5, 10, and 25 μg); *lanes 4–6 E. coli*/pDOC166 (each 5, 10, and 25 μg)

PAMC 22718, which was isolated for its high chitinase and protease activities from cold Arctic seawater. Previously, the genome sequence of PAMC 22718 was deposited by Park et al. (2000) in NCBI Genome Database (BioProject Accession No. PRJNA159457); thus, *orf2* was designated as *chi22718_III* (GenBank Accession No. KF574005). *chi22718_III* consists of 3171 bp and encodes a 1,056-amino acid protein with a calculated molecular weight of approx. 114 kDa.

Cloning, expression, and functional analysis of Chi22718_III

chi22718_III was PCR-amplified and cloned into the expression vector pEXP5-CT/TOPO to generate the recombinant plasmid pDOC166, which was transferred into *E. coli* BL21 star (DE3). Following heterologous expression, the soluble cell lysate containing recombinant R-Chi22718_III produced a high

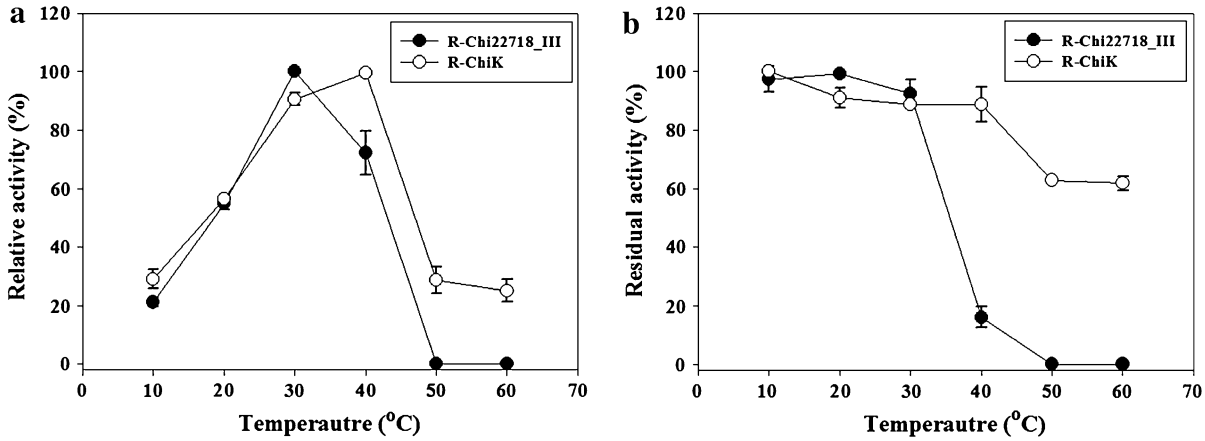

Fig. 3 Determination of the optimal activity (**a**) and thermostability (**b**) of R-Chi22718_III at various temperatures. The relative activity of R-Chi22718_III (100 %, 20.5 ± 0.4 U/mg) was measured in 50 mM sodium phosphate buffer (pH 7.6) containing 0.1 mM *p*NP-GlcNAc. As a mesophilic counterpart, endochitinase R-ChiK was produced from *E. coli* Top10/pBAD-TOPO harboring *chiK* (chitinase K), which was cloned from *Vibrio* sp. 98CJ11027 (Park et al. 2000). This strain was isolated from the coastal area of Cheju Island in Korea. R-chiK was homogeneously purified using a Ni–NTA column (unpublished data). One unit of R-ChiK was defined as the enzyme activity sufficient to produce 1 μmol of *p*-nitrophenol from 0.1 mM *p*NP-$(GlcNAc)_2$ per hour per mg of protein at 40 °C. The relative activity of R-ChiK (100 %, 15.2 ± 0.2 U/mg) was measured in 50 mM sodium phosphate buffer (pH 7.6)

absorbance value at 400 nm for *p*NP-GlcNAc (specific activity, 20.5 ± 0.4 U/mg at 30 °C), indicating the release of the chromogenic residue (*p*-nitrophenol) from the substrate due to the cleavage of glycosidic bonds, which is catalyzed by β-*N*-acetylglucosaminidase. Conversely, a control cell lysate of *E. coli* BL21 star (DE3) cells containing only pEXP5- CT/TOPO did not display any chitinase activity for *p*NP-GlcNAc. To determine the size and expression level of R-Chi22718_III, SDS-PAGE and western blot analyses were performed on a 10 % acrylamide gel using a Tris/glycine buffer system and on an immunoblot membrane with anti-His and goat anti-mouse IgG secondary antibodies, respectively (Fig. 2a, b). SDS-PAGE analysis did not reveal the overexpression of R-Chi22718_III in *E. coli*/pDOC166 compared to its expression in control *E. coli*/pEXP5- CT/TOPO, but western blotting clearly revealed a protein blot with a size of ~100 kDa only in *E. coli*/pDOC166, and its size was similar to the predicted size (~114 kDa) of R-Chi22718_III.

Effects of temperature on R-Chi22718-III activity and thermostability

R-Chi22718_III was active from 10 to 40 °C, with maximal activity at 30 °C (Fig. 3a). Preincubation of R-Chi22718_III at different temperatures had a dramatically different effect on the activity of the enzyme (Fig. 3b). For example, preincubation at 40 °C for 1 h resulted in an approx. 85 % loss of enzyme activity. When a mesophilic counterpart R-ChiK, which displayed maximal activity at 40 °C, was analyzed under the same conditions, its thermostability profile was substantially different from that of R-Chi22718_III, resulting in an approx. 10 % loss of activity. These experimental data demonstrate that R-Chi22718_III possesses one characteristic property of cold-active enzymes opposite to the mesophilic ones: thermolability to be rapidly inactivated at moderate temperatures exceeding 30 °C.

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