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Characterization of genes for chitin catabolism in *Haloferax mediterranei*

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Abstract Chitin is the second most abundant natural polysaccharide after cellulose. But degradation of chitin has never been reported in haloarchaea. In this study, we revealed that *Haloferax mediterranei*, a metabolically versatile haloarchaeon, could utilize colloidal or powdered chitin for growth and poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) accumulation, and the gene cluster (HFX_5025-5039) for the chitin catabolism pathway was experimentally identified. First, reverse transcription polymerase chain reaction results showed that the expression of the genes encoding the four putative chitinases ($ChiA_{Hme}$, $ChiB_{Hme}$, $ChiC_{Hme}$, and $ChiD_{Hme}$, HFX_5036-5039), the LmbE-like deacetylase (Dac_{Hme}, HFX_5027), and the glycosidase ($GlyA_{Hme}$, HFX_5029) was induced by colloidal or powdered chitin, and $chiA_{Hme}$, $chiB_{Hme}$, and $chiC_{Hme}$ were cotranscribed. Knockout of $chiABC_{Hme}$ or $chiD_{Hme}$ had a significant effect on cell growth and PHBV production when chitin was used as the sole carbon source, and the $chiABCD_{Hme}$ knockout mutant lost the capability to utilize chitin. Knockout of dac_{Hme} or $glyA_{Hme}$ also decreased PHBV accumulation on chitin. These results suggested that $ChiABCD_{Hme}$, Dac_{Hme}, and $GlyA_{Hme}$ were indeed involved in chitin degradation in *H. mediterranei*. Additionally, the chitinase assay showed that each chitinase possessed hydrolytic activity toward colloidal or powdered chitin, and the major product of colloidal chitin hydrolysis by $ChiABCD_{Hme}$ was diacetylchitobiose, which was likely further degraded to monosaccharides by Dac_{Hme}, $GlyA_{Hme}$, and other related enzymes for both cell growth and PHBV biosynthesis. Taken together, this study revealed the genes and enzymes involved in chitin catabolism in haloarchaea for the first time and indicated the potential of *H. mediterranei* as a whole-cell biocatalyst in chitin bioconversion.

Keywords Chitin · Catabolism · Haloarchaea · Bioplastic · Bioconversion

Introduction

With the urgent need of transition to a more environmentally sustainable bioeconomy, the biocatalysts that can effectively convert the recalcitrant organic carbon polymers into useful compounds have attracted special interest (Himmel et al. 2007; Vaaje-Kolstad et al. 2010). Chitin is a highly insoluble polymer composed of linear chains of β-1,4-linked *N*-acetyl-D-glucosamine (GlcNAc) residues, which are tightly held by a large number of intra- and inter-chain hydrogen bonds. Chitin is widespread in nature as a structural component of insect exoskeletons, crustacean shells, and cell walls of most fungi and some algae. Chitin is the second most abundant natural polymer after cellulose and has an estimated biological production of more than 10^{11} metric tons per year in the aquatic ecosystems alone (Lutz et al. 1994). A large amount of chitin waste is generated from the seafood industry, which has caused severe

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environmental problems. Thus, bioconversion of chitin waste to value-added products is environmentally and economically beneficial (Songsiriritthigul et al. 2010).

Chitinases catalyze the first step of chitin degradation by cleaving the β-1,4-glycosidic bonds of the chitin chain. According to the amino acid sequence similarity, chitinases fall into either glycoside hydrolase (GH) family 18 or 19 (Tsuji et al. 2010). GH family 18 chitinases are distributed in various organisms, including microbes, plants, and animals, and have a relatively high sequence divergence. Thus, these chitinases are further divided into three subfamilies of A, B, and C (Karlsson and Stenlid 2009). Conversely, GH family 19 chitinases are almost exclusively from plants and possess a high degree of sequence similarity (Tsuji et al. 2010). Additionally, chitinases are classified as endochitinases or exochitinases based on their mode of action. During the degradation of chitin, endochitinases first cleave the β-1,4-glycosidic bonds randomly at the internal sites of the chitin chain to provide a variety of chitooligosaccharides. The exochitinases then remove the $GlcNAc_2$ units from the non-reducing end of the chitooligosaccharides in a progressive manner (Cohen-Kupiec and Chet 1998). Chitinases from bacteria and eukarya have been well studied, but only a limited number of archaeal chitinases have been characterized. To our knowledge, only five archaeal strains, including *Thermococcus chitinophagus* (Andronopoulou and Vorgias 2004), *Thermococcus kodakaraensis* KOD1 (Tanaka et al. 2001), *Pyrococcus furiosus* (Gao et al. 2003; Kreuzer et al. 2013), *Sulfolobus tokodaii* (Staufenberger et al. 2012), and *Halobacterium salinarum* strain NRC-1 (Yoshinobu et al. 2006), have been reported to possess chitinases, which all belong to GH18A or GH18C. It is noteworthy that studies regarding these archaeal chitinases have focused primarily on biochemical analysis and structural determination (Nakamura et al. 2008; Oku and Ishikawa 2006; Tsuji et al. 2010).

The chitin catabolism pathway in bacteria and eukarya has also been well characterized; however, the archaeal chitin catabolism pathway has been established only in *T. kodakaraensis* KOD1 (Tanaka et al. 2004), in which the concerted action of three key enzymes of $ChiA_{Tk}$ (chitinase, TK1765), Dac_{Tk} (deacetylase, TK1764), and $GlmA_{Tk}$ (exo-β-glucosaminidase, TK1754) converts chitin into glucosamine (GlcN). All of the chitin catabolism-related genes cluster together in *T. kodakaraensis* KOD1. The chitin degradation pathway identified in *T. kodakaraensis* KOD1 is different from those of bacteria or eukarya. Whether there are alternative chitin degradation pathways in archaea remains to be determined.

Haloarchaea represent a distinct archaeal group that inhabits hypersaline environments. Knowledge regarding haloarchaeal chitin utilization is very limited; so far, only a chitinase homolog in *H. salinarum* strain NRC-1 has been heterogeneously expressed and characterized (Yoshinobu et al. 2006). *Haloferax mediterranei* is a good model for the study of haloarchaeal metabolism (Han et al. 2012), as it can utilize a variety of compounds as the sole carbon source, including carbohydrates, polyalcohols, organic acids, and amino acids (Rodriguez-Valera et al. 1983). In the presence of excessive carbon sources, *H. mediterranei* is capable of accumulating a large amount of poly(3-hydroxybutyrate-*co*-3-hydroxyvalerate) (PHBV) (Lu et al. 2008b), a desired bioplastic with a variety of applications such as packaging and medical materials. Interestingly, bioinformatic analysis of the *H. mediterranei* genome revealed a gene cluster (HFX_5025-5039) that might encode the chitin catabolism pathway. Whether *H. mediterranei* could use chitin for growth and convert it into PHBV remained to be determined.

This study revealed for the first time that *H. mediterranei* was able to use colloidal or powdered chitin for cell growth and PHBV production. Notably, four important chitinases involved in chitin degradation by *H. mediterranei* were identified through bioinformatic prediction, transcription analysis, genetic verification, chitinase assay, and detection of the chitinolytic products. In addition, two other key enzymes, the LmbE-like deacetylase and the glycosidase, were also verified to be involved in chitin utilization by *H. mediterranei*. Taken together, this study has enriched our understanding of the enzymes in haloarchaeal chitin catabolism and provided the first evidence for the potential of haloarchaea in chitin bioconversion, especially in hypersaline environments.

Materials and methods

Strains and culture conditions

The strains used in this study are listed in Table 1. Colloidal chitin was prepared as described by Tanaka et al. (1999), except that concentrated hydrochloric acid rather than 85 % phosphoric acid was used to dissolve the powdered chitin. *Escherichia coli* JM109 was cultivated in Luria–Bertani medium at 37 °C (Sambrook et al. 1989). When needed, 100 μg/ml ampicillin was added to the media. *H. mediterranei* was first cultured at 37 °C in AS-168 medium (Han et al. 2007) for 36 h as seed culture. Subsequently, 2.5 ml of the seed culture was harvested, washed with sterile NaCl solution, and inoculated into 50 ml basal medium (per liter, 200 g NaCl, 20 g $MgSO_4 \cdot 7H_2O$, 2 g KCl, 2 g NH_4Cl, 37.5 mg KH_2PO_4, 5 mg $FeSO_4 \cdot 7H_2O$, 0.036 mg $MnCl_2 \cdot 4H_2O$, 15 g PIPES, pH 7.2) with 5 g/l glucose or colloidal chitin or powdered chitin (Sangon Biotech, Shanghai, China) as the sole carbon

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source and reference
Strains		
H. mediterranei CGMCC 1.2087	Wild-type strain (ATCC 33500)	CGMCC
H. mediterranei DF50	*pyrF*-deleted mutant of *H. mediterranei*	Liu et al. (2011)
E. coli JM109	*recA1 supE44 endA1 hsdR17 gyrA96 relA1 thi*	Sambrook et al. (1989)
H. mediterranei Δ*chiABC*$_{Hme}$	*chiABC*$_{Hme}$-deleted mutant of *H. mediterranei* DF50	This study
H. mediterranei Δ*chiD*$_{Hme}$	*chiD*$_{Hme}$-deleted mutant of *H. mediterranei* DF50	This study
H. mediterranei Δ*chiABCD*$_{Hme}$	*chiABCD*$_{Hme}$-deleted mutant of *H. mediterranei* DF50	This study
H. mediterranei Δ*dac*$_{Hme}$	*dac*$_{Hme}$-deleted mutant of *H. mediterranei* DF50	This study
H. mediterranei Δ*glyA*$_{Hme}$	*glyA*$_{Hme}$-deleted mutant of *H. mediterranei* DF50	This study
Plasmids		
pHFX	4.0 kb; integration vector containing *pyrF* and its native promoter	Liu et al. (2011)
pHFXDABC	5.2 kb; pHFX-derived integration vector for knockout of *chiABC*$_{Hme}$	This study
pHFXDD	5.2 kb; pHFX-derived integration vector for knockout of *chiD*$_{Hme}$	This study
pHFXDABCD	5.2 kb; pHFX-derived integration vector for knockout of *chiABCD*$_{Hme}$	This study
pHFXDdac	5.3 kb; pHFX-derived integration vector for knockout of *dac*$_{Hme}$	This study
pHFXDglyA	5.3 kb; pHFX-derived integration vector for knockout of *glyA*$_{Hme}$	This study
pWL502	7.9 kb; expression vector containing *pyrF* and its native promoter	Cai et al. (2012)
pWLA	9.5 kb; pWL502-derived expression vector for expression of ChiA$_{Hme}$	This study
pWLB	9.5 kb; pWL502-derived expression vector for expression of ChiB$_{Hme}$	This study
pWLC	9.8 kb; pWL502-derived expression vector for expression of ChiC$_{Hme}$	This study
pWLD	9.7 kb; pWL502-derived expression vector for expression of ChiD$_{Hme}$	This study

source for additional cultivation of 24 h (for reverse transcription polymerase chain reaction, RT-PCR) or until stationary phase (for growth monitoring and PHBV accumulation analysis in *H. mediterranei* strains). In the experiment of comparing the PHBV production from glucose, colloidal chitin, and powdered chitin, the culture conditions were the same as described above, except that 1 g/l sodium glutamate was added to the basal medium (modified basal medium) for a short lag phase. For *H. mediterranei* DF50 (a uracil auxotroph of the wild-type strain) and the DF50-based knockout mutants, 50 μg/ml uracil was added into the media (Liu et al. 2011). Cell numbers (expressed as colony-forming units (CFU) per milliliter) were determined by plating an aliquot of the diluted cell culture onto the surface of AS-168 Petri plates, which were then incubated at 37 °C for about 72 h. The colonies were counted and the cell numbers in the original culture were determined.

RT-PCR

Total RNA of *H. mediterranei* was isolated with the TRIzol reagent (Invitrogen^{TM}-Life Technologies, USA). RT-PCR was performed as described previously (Han et al. 2007), except that the primers for the RT reaction were random hexamer primers (Thermo Scientific) and the specific primers used for the PCR reactions are listed in Table S1.

Knockout mutant construction and verification

Gene knockout was performed as previously described (Lu et al. 2008b). The primers and plasmids used for gene knockout are listed in Table S1 and Table 1, respectively. Briefly, the fragments (approximately 650 bp for each) located immediately upstream or downstream of the target gene were amplified, sequenced, and inserted into the plasmid pHFX (Liu et al. 2011). The resulting plasmids were then transformed into the host strain *H. mediterranei* DF50 to delete the target gene through homologous recombination. The knockout mutant strains were all verified by PCR using the primers shown in Table S1.

PHBV analysis

The PHBV content and its monomer composition in the cells were determined by gas chromatography as described previously (Han et al. 2007). Benzoic acid was used as the

internal standard for quantitative analysis of the PHBV content.

Protein preparation and chitinase assay

For chitinase assay, four plasmids (pWLA, pWLB, pWLC, and pWLD) listed in Table 1 were constructed for the expression of $ChiA_{Hme}$, $ChiB_{Hme}$, $ChiC_{Hme}$, and $ChiD_{Hme}$, respectively. The coding regions under the *hsp5* promoter (Lu et al. 2008a), without putative signal peptides, were amplified and inserted into the plasmid pWL502 (Cai et al. 2012) to generate the expression plasmids. These plasmids were then transformed individually into the $\Delta chiABCD_{Hme}$ mutant strain. The resulting strains were first cultivated in AS-168 medium without yeast extract and then inoculated as 5 % of the total volume into the basal medium (as described in the "Strains and culture conditions" section) with glucose as the sole carbon source for an additional 36 h of cultivation. These cells were harvested by centrifugation (10,000×*g* for 10 min at 4 °C) and then suspended with high salt buffer (HSB; 3.4 M KCl in 20 mM Tris–HCl (pH 7.5)) for ultrasonication. Intact cells and debris were removed by centrifugation (10,000×*g* for 10 min at 4 °C) to obtain crude extracts. The protein concentration of the crude extracts was determined by a bicinchoninic acid protein assay kit (Thermo Scientific). The standard chitinase assay mixture contained 100 μg of crude protein and 3 mg of colloidal or powdered chitin in the HSB in a total volume of 500 μl. After incubation at 37 °C for 15 min, the reaction was terminated by boiling for 3 min, and the reaction mixture was centrifuged to obtain the supernatant. The amount of liberated reducing sugar was measured with a modified Schales procedure (Imoto and Yagishita 1971). One unit of activity was defined as the amount of protein that produces 1 μmol of reducing sugars per minute. The specific activity was expressed as unit per gram crude protein. The chitinase assay with the crude extracts from *H. mediterranei* DF50 $\Delta chiABCD_{Hme}$ harboring the plasmid pWL502 was used as the negative control.

Analysis of the chitinolytic products by high-performance anion exchange chromatography

The chitinase assay was performed as described in the "protein preparation and chitinase assay" section, except that the crude extracts used were the mixture of the four crude extracts containing $ChiA_{Hme}$, $ChiB_{Hme}$, $ChiC_{Hme}$, and $ChiD_{Hme}$, respectively; the substrate was 3 mg of colloidal chitin, and the reaction time was extended to 1 h. The high-performance anion exchange chromatography (HPAEC) analysis of the hydrolysis products was performed as described previously (Lü et al. 2009). The reaction mixture containing the crude extracts from the $\Delta chiABCD_{Hme}$ strain with the plasmid pWL502 and 3 mg of colloidal chitin in HSB was used as the negative control. The standards of GlcNAc, $GlcNAc_2$, and $GlcNAc_3$ (Northstar BioProducts™-Associates of Cape Cod, USA) were used to calibrate the retention time.

Protein sequence analysis and phylogenetic tree construction

Signal peptides were predicted with the SignalP 4.0 server (http://www.cbs.dtu.dk/services/SignalP/). Domain annotation was performed by using the NCBI conserved domain search service (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi), InterProScan program (http://www.ebi.ac.uk/Tools/pfa/iprscan/), and referring to the CAZy database (http://www.cazy.org/). Protein modeling was performed on the SWISS-MODEL server using the automated mode (http://swissmodel.expasy.org/workspace/index.php?func=modelling_simple1). Sequence homology was analyzed using the BLAST service (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and the GeneDoc program (http://www.nrbsc.org/gfx/genedoc/). The phylogenetic tree was constructed in MEGA4 software using the neighbor-joining method. The topology of the phylogenetic tree was evaluated by bootstrap analysis on the basis of 1,000 replicates.

Results

Bioinformatic analysis of putative chitinases in *H. mediterranei*

Our recent genome sequencing of *H. mediterranei* (Han et al. 2012) revealed a gene cluster that might encode the enzymes involved in chitin catabolism. This gene cluster (HFX_5025-5039) includes the genes encoding four potential chitinases (HFX_5036-5039), a putative ABC transport system (HFX_5025, HFX_5030-5032), a glycosidase (HFX_5029), and an LmbE-like deacetylase (HFX_5027), which might be directly involved in chitin degradation. In addition, a putative galactonate dehydratase (HFX_5033) and a glucose-1-dehydrogenase (HFX_5035) were also found in this gene cluster (Fig. 1a and Table S2).

The four putative chitinases, which were designated as $ChiA_{Hme}$, $ChiB_{Hme}$, $ChiC_{Hme}$, and $ChiD_{Hme}$, respectively, may catalyze the first step of chitin degradation by *H. mediterranei*. Among them, $ChiA_{Hme}$ and $ChiB_{Hme}$ were more closely related (45 % identity), and $ChiC_{Hme}$ and $ChiD_{Hme}$ were more similar (41 % identity). The protein sequence analysis revealed that the four chitinases all harbored a signal sequence in the N terminus, followed by a chitin binding domain (ChtBD; accession number IPR003610), a polycystic

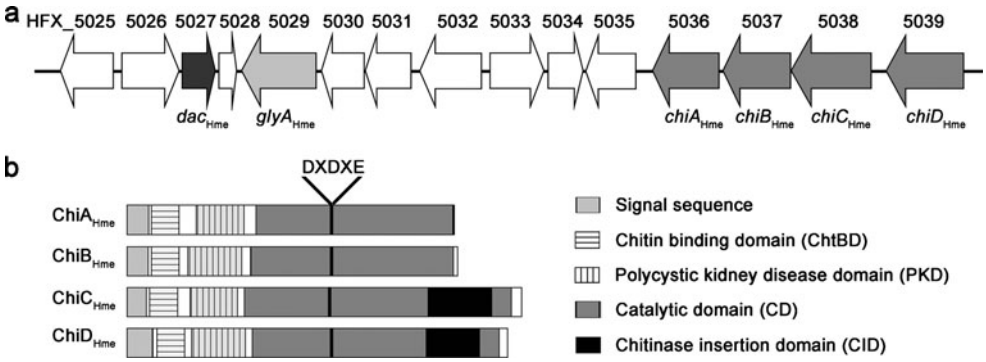

Fig. 1 Bioinformatic analysis of the four putative chitinases in *H. mediterranei*. **a** The putative chitin catabolism-related gene cluster in *H. mediterranei*. The locus tag of each gene is indicated. The corresponding gene names are listed in Table S2. **b** The schematic representation of the domain organization in the four chitinases. The *dark line* in the catalytic domain represents the conserved "DXDXE" motif in the GH family 18 chitinases

kidney disease domain (PKD; accession number IPR000601), and a C-terminal catalytic domain (CD; accession number IPR017853) (Fig. 1b). The N-terminal signal sequence may direct the chitinase to the outside of the cell. The ChtBD, a member of the carbohydrate-binding module family 5/12 (CBM5/12), may be responsible for concentrating the CD on the chitin surface and disrupting the hydrogen bonds of the chitin chains (Nakamura et al. 2008). PKD was reported to be involved in the degradation of powdered chitin in a marine bacterium (Orikoshi et al. 2005). The CDs of all the four chitinases adopted the triosephosphate isomerase $(\beta/\alpha)_8$-barrel fold (Fig. S1a) and had a conserved sequence motif of "DXDXE" (Fig. 1b and Fig. S1b), which are characteristics of GH family 18 chitinases (Tsuji et al. 2010). In addition to these domains, $ChiC_{Hme}$ and $ChiD_{Hme}$ contained an additional chitinase insertion domain (CID; accession number SSF54556) in the CD (Fig. 1b). The CID was found to occur in the bacterial exochitinases and may enhance the enzyme activity of exochitinases by increasing the depth of the substrate-binding cleft (Li and Greene 2010). Hence, $ChiC_{Hme}$ and $ChiD_{Hme}$ might be exochitinases, and $ChiA_{Hme}$ and $ChiB_{Hme}$ might be endochitinases. Phylogenetic tree analysis of the chitinases from archaea and the chitinases with definitive classifications revealed that the archaeal chitinases belonged to either GH18A or GH18C subfamily. $ChiC_{Hme}$ and $ChiD_{Hme}$ were members of GH18A subfamily, while $ChiA_{Hme}$ and $ChiB_{Hme}$ belonged to GH18C (Fig. 2 and Table S3). This result was consistent with a previous report that the CID seemed specific to GH18A (Li and Greene 2010).

Bioconversion of chitin to PHBV by *H. mediterranei*

To test the capability of chitin utilization by *H. mediterranei*, the commercially available powdered chitin or the hydrochloric acid-treated colloidal chitin was used as the carbon source. During the colloidal chitin preparation, some hydrogen bonds and glycosidic bonds are disrupted; thus, colloidal chitin has a lower degree of crystallinity and polymerization than powdered chitin (Kurita 2006). Such structural changes in the colloidal chitin make it more susceptible to degradation. *H. mediterranei* was found to be able to grow on colloidal or powdered chitin, and it could also produce PHBV from colloidal or powdered chitin. The final PHBV concentration from colloidal chitin was nearly as high as that from glucose in shake flask culture (1 g/l sodium glutamate added only in this experiment, see "Materials and methods"), although the accumulation rate was slower from colloidal chitin than that from glucose during the first 48 h (Fig. 3). This relatively slow accumulation rate is possibly due to the need of a degradation process, which converts the

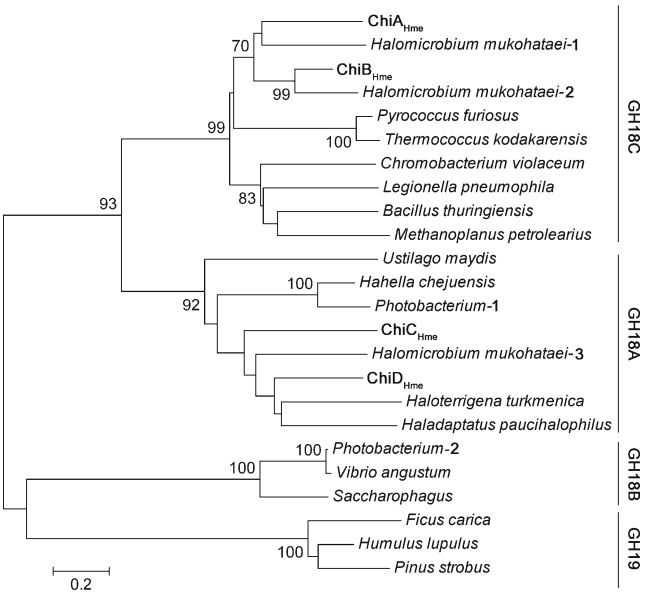

Fig. 2 The phylogenetic tree of the chitinases from archaea and the chitinases with certain subfamily classifications of GH18A, GH18B, GH18C, and GH19. *Numbers* at nodes represent the percentage bootstrap values based on 1,000 replicates. Only values above 70 % are considered significant and are shown here. The *scale bar* indicates a difference of 0.2 substitutions per site. The accession numbers of these proteins are shown in Table S3

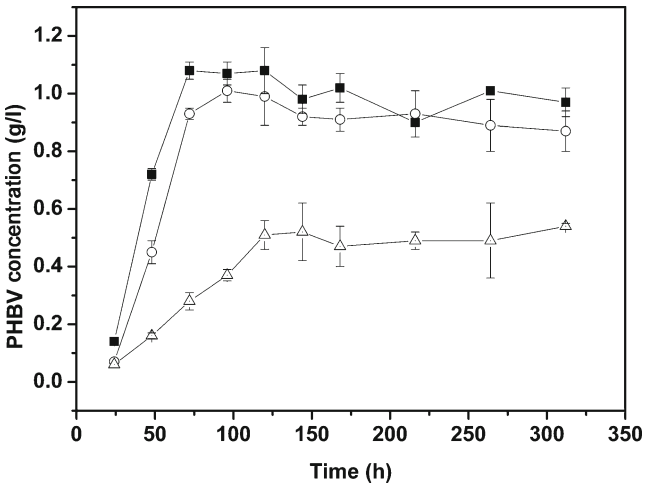

Fig. 3 The time course of PHBV accumulation from glucose (*filled square*), colloidal chitin (*empty circle*), or powdered chitin (*empty triangle*) in *H. mediterranei* wild-type strain. *H. mediterranei* cells were cultivated in modified basal medium with 5 g/l glucose, or colloidal chitin, or powdered chitin, respectively (see "Materials and methods")

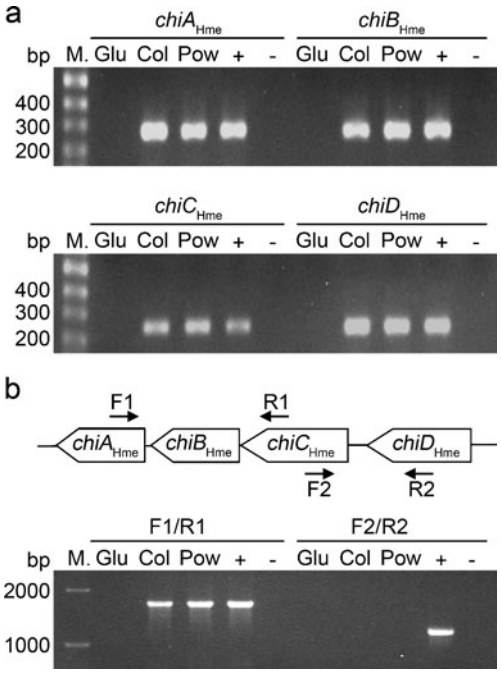

Fig. 4 RT-PCR analysis of the transcripts of the four chitinase genes. **a** Expression of the four chitinase genes under different culture conditions. **b** Transcriptional pattern of the four chitinase genes. The primer positions (F1/R1 and F2/R2) are indicated in the gene cluster of $chiABCD_{Hme}$. *Lanes M* marker; *lanes Glu*, *Col*, and *Pow* the reverse transcripts of total RNA from *H. mediterranei* grown with glucose (Glu), colloidal chitin (Col), and powdered chitin (Pow), respectively, as the template; *lanes* + genome as the template, positive control; *lanes* − negative control. The primers used here are listed in Table S1

polymeric chitin into usable monomers. A lower PHBV concentration was obtained from powdered chitin than from colloidal chitin (Fig. 3), possibly because the highly crystalline structure of powdered chitin is not readily accessible to the chitinases. These results show for the first time that *H. mediterranei* can efficiently utilize colloidal or powdered chitin as a carbon source for PHBV production.

Induced expression of the four chitinase genes

To investigate the involvement of the four putative chitinases ($ChiA_{Hme}$, $ChiB_{Hme}$, $ChiC_{Hme}$, and $ChiD_{Hme}$) in chitin degradation, RT-PCR was performed to examine their transcripts under the growth conditions with different carbon sources, including glucose, colloidal chitin, and powdered chitin. The results showed that all of the four chitinase genes were transcribed when colloidal or powdered chitin was provided as the sole carbon source, but not when glucose was used as the carbon source (Fig. 4a). These results indicated that these four chitinase genes were induced by colloidal or powdered chitin and might participate in chitin catabolism. Moreover, the RT-PCR results suggested that $chiA_{Hme}$, $chiB_{Hme}$, and $chiC_{Hme}$ were cotranscribed, while $chiD_{Hme}$ was transcribed alone (Fig. 4b). This is a very interesting transcriptional profile. But the transcriptional regulation mechanism of the four chitinase genes remains to be clarified.

Genetic determination of the involvement of $ChiABCD_{Hme}$ in chitin utilization

Based on the transcriptional pattern of the four chitinase genes, three knockout mutants were constructed using *H. mediterranei* DF50 (Liu et al. 2011) as the host strain. These mutants included the knockout of cotranscribed $chiA_{Hme}$, $chiB_{Hme}$, and $chiC_{Hme}$ in combination, the knockout of $chiD_{Hme}$, and the knockout of the gene cluster of $chiABCD_{Hme}$, which were named $\Delta chiABC_{Hme}$, $\Delta chiD_{Hme}$, and $\Delta chiABCD_{Hme}$, respectively. A growth curve was constructed by plotting the CFU per milliliter versus time. As shown in the growth curves, *H. mediterranei* DF50 was able to grow well with colloidal or powdered chitin as the sole carbon source, while the two knockout mutants, $\Delta chiABC_{Hme}$ and $\Delta chiD_{Hme}$, could hardly grow on the powdered chitin and $\Delta chiABCD_{Hme}$ could grow on neither powdered nor colloidal chitin (Fig. 5a). These results suggested that the four chitinases were indeed involved in the degradation of colloidal and powdered chitin by *H. mediterranei*, and both $ChiABC_{Hme}$ and $ChiD_{Hme}$ were indispensible for the utilization of powdered chitin.

As PHBV is deposited as a kind of intracellular carbon and energy storage compound in the presence of excessive carbon source, the PHBV accumulation capabilities could to some degree reflect the carbon source utilization capabilities of the strains. Thus, the final PHBV accumulation of *H. mediterranei* DF50 and the chitinase knockouts at the stationary phase (maximum PHBV production) were investigated when chitin

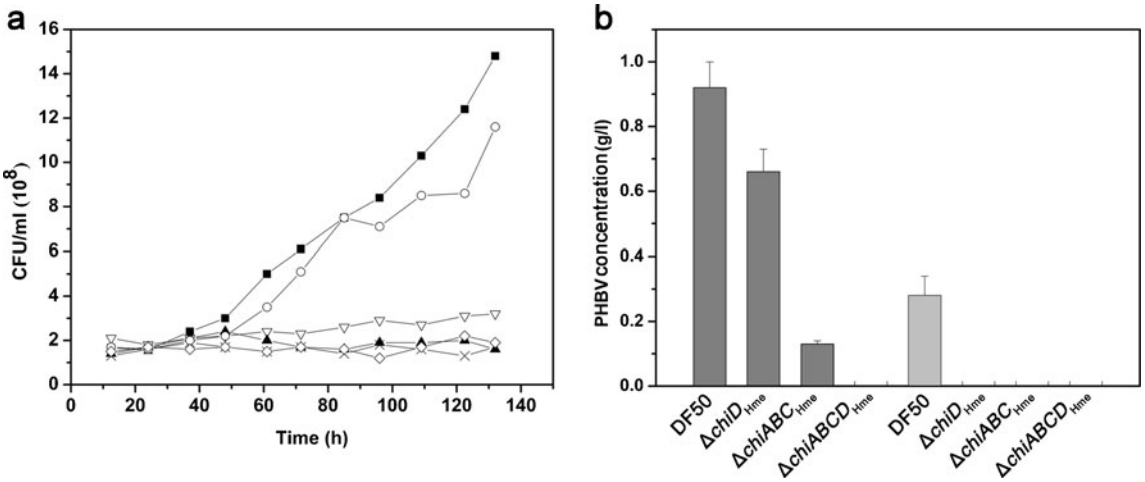

Fig. 5 Utilization of colloidal or powdered chitin for growth and PHBV accumulation by *H. mediterranei* DF50 and the recombinant strains. **a** The growth curves of the *H. mediterranei* strains (DF50, Δ*chiABC*$_{Hme}$, Δ*chiD*$_{Hme}$, and Δ*chiABCD*$_{Hme}$) grown with colloidal (Col) or powdered (Pow) chitin as the sole carbon source were constructed by plotting the CFU per milliliter versus time. DF50 with Pow, *filled square*; DF50 with Col, *empty circle*; Δ*chiABC*$_{Hme}$ with Pow, *empty inverted triangle*; Δ*chiD*$_{Hme}$ with Pow, *filled triangle*; Δ*chiABCD*$_{Hme}$ with Col, *empty diamond*; Δ*chiABCD*$_{Hme}$ with Pow, *cross*. **b** The final PHBV concentration of *H. mediterranei* strains (DF50, Δ*chiABC*$_{Hme}$, Δ*chiD*$_{Hme}$, and Δ*chiABCD*$_{Hme}$) at the stationary phase (maximum PHBV production) from colloidal (*dark gray columns*) or powdered (*light gray columns*) chitin. The data in **b** represent mean values±SD of three independent experiments

was used as the sole carbon source. It was found that when colloidal chitin was used as the sole carbon source, Δ*chiABC*$_{Hme}$ and Δ*chiD*$_{Hme}$ strains accumulated less PHBV than DF50. Specifically, knockout of *chiD*$_{Hme}$ resulted in a 28 % decrease in the final PHBV concentration from colloidal chitin compared with DF50, and the final PHBV concentration in Δ*chiABC*$_{Hme}$ was reduced by 86 % (Fig. 5b). As for the Δ*chiABCD*$_{Hme}$ strain, no PHBV was detected, which was consistent with no observed growth of this strain on colloidal chitin (Fig. 5a, b). When powdered chitin was used as the sole carbon source, PHBV was only detected in the DF50 strain but not in the three chitinase knockouts, which was in agreement with the result that the three chitinase knockouts could not grow on powdered chitin at all (Fig. 5a, b). These results showed that ChiD$_{Hme}$ and the combination of ChiA$_{Hme}$, ChiB$_{Hme}$, and ChiC$_{Hme}$ could degrade colloidal chitin individually, but the chitin degradation efficiency of ChiD$_{Hme}$ was lower than the combination of ChiA$_{Hme}$, ChiB$_{Hme}$, and ChiC$_{Hme}$. Furthermore, the presence of all the four chitinases was more efficient in degrading colloidal chitin than the combination of ChiA$_{Hme}$, ChiB$_{Hme}$, and ChiC$_{Hme}$, suggesting a combined action of these four chitinases in colloidal chitin degradation. Additionally, only the combined action of the four chitinases could produce enough usable carbon from powdered chitin for growth and PHBV accumulation. Therefore, the growth curves and PHBV accumulation analysis both confirmed the significance of the four chitinases in chitin degradation.

Enzyme activity of the four chitinases and detection of the colloidal chitin hydrolysis products

To biochemically demonstrate the functions of the four chitinases in chitin degradation in *H. mediterranei*, the chitinase activity was assayed with colloidal or powdered chitin as the substrate. The four chitinase genes, depleted of the signal sequence for intracellular expression, were introduced individually under the *hsp5* promoter (Lu et al. 2008a) into the host strain Δ*chiABCD*$_{Hme}$. The resulting strains were cultivated with glucose as the sole carbon source, and under this condition, the chitin catabolism-related genes except the introduced *chiA*$_{Hme}$, *chiB*$_{Hme}$, *chiC*$_{Hme}$, or *chiD*$_{Hme}$ were not transcribed. The crude extracts were subject to the chitinase assay. All of the four crude extracts with chitinases showed hydrolytic activities toward both colloidal and powdered chitin (Fig. 6a). The crude extracts with ChiD$_{Hme}$ showed a specific activity of 253.2 U/g crude protein toward colloidal chitin and 140.1 U/g crude protein toward powdered chitin, and ChiC$_{Hme}$ showed a specific activity of 135.6 U/g crude protein toward colloidal chitin and 49.7 U/g crude protein toward powdered chitin. The specific activity of the crude extracts with ChiB$_{Hme}$ was 90.4 U/g crude protein with colloidal chitin and 13.6 U/g crude protein with powdered chitin, respectively. As for the crude extracts containing ChiA$_{Hme}$, it exhibited a specific activity of 31.6 U/g crude protein with colloidal chitin and 4.5 U/g crude protein with powdered chitin (Fig. 6a). In addition, the reaction products

from hydrolysis of colloidal chitin were also detected using HPAEC. Hydrolysis of colloidal chitin by the four chitinases produced a mixture of GlcNAc, $GlcNAc_2$, and $GlcNAc_3$, with the diacetylchitobiose $GlcNAc_2$ being the dominant product, whereas no hydrolysis product was generated in the chitinase-negative control (Fig. 6b). Therefore, the role of the four chitinases in chitin degradation was further confirmed by these in vitro results.

The role of Dac_{Hme} and $GlyA_{Hme}$ in chitin utilization

In addition to the four chitinases, the uncharacterized LmbE-like deacetylase (Dac_{Hme}, HFX_5027) and the glycosidase ($GlyA_{Hme}$, HFX_5029) might also play important roles in chitin utilization for *H. mediterranei*. To determine the involvement of dac_{Hme} and $glyA_{Hme}$ in chitin utilization by *H. mediterranei*, RT-PCR analysis was performed at first. The results showed that the expression of dac_{Hme} and $glyA_{Hme}$ was induced by colloidal or powdered chitin, but not by glucose (Fig. 7a), indicating that dac_{Hme} and $glyA_{Hme}$ might be involved in chitin utilization. Additionally, the knockout mutants of dac_{Hme} and $glyA_{Hme}$ were constructed to examine their capability to utilize chitin, which could be reflected by the PHBV accumulation capability from chitin, as mentioned above. Hence, the PHBV concentration of the strains of DF50, Δdac_{Hme}, and $\Delta glyA_{Hme}$ at the stationary phase (maximum PHBV production) was obtained when chitin was used as the sole carbon source. The results showed that deletion of dac_{Hme} led to dramatic decreases in the final PHBV concentration from colloidal chitin (a reduction of 81 %) and powdered chitin (a reduction of 91 %) compared with DF50 (Fig. 7b). A small amount of PHBV was still produced from colloidal or powdered chitin by the Δdac_{Hme} strain, which may be due to the presence of a small number of deacetylated units of GlcN in the chitin chain (Mathur and Narang 1990) or the presence of other proteins with the same function as Dac_{Hme}. Additionally, the $\Delta glyA_{Hme}$ strain could only produce a very small amount of

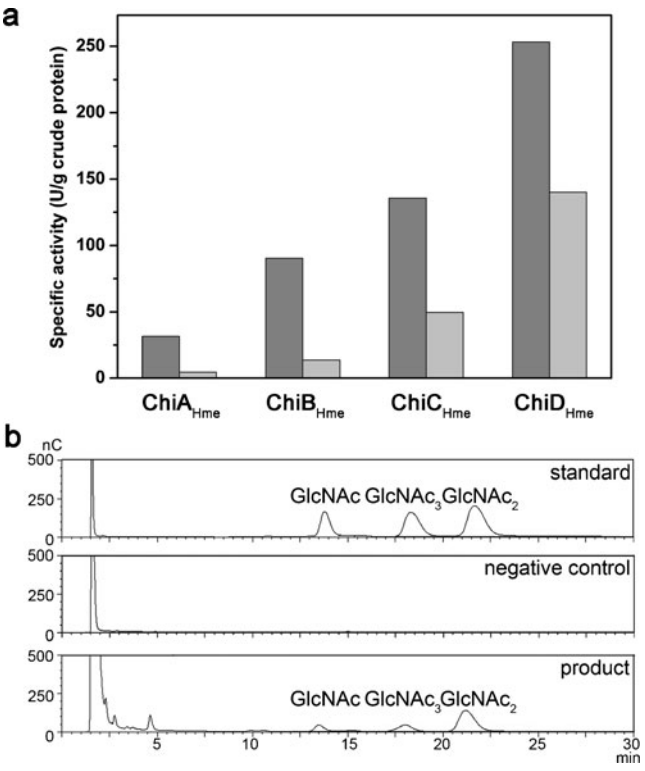

Fig. 6 Biochemical determination of the four chitinases. **a** The specific activities of the four chitinases toward colloidal (*dark gray columns*) or powdered (*light gray columns*) chitin. The experiment was performed in triplicate and the representative data are shown here. **b** HPAEC detection of the colloidal chitin hydrolysis products. The product was the hydrolysis product of colloidal chitin catalyzed by the mixture of crude extracts with $ChiA_{Hme}$, $ChiB_{Hme}$, $ChiC_{Hme}$, and $ChiD_{Hme}$. The spectrum of standards of GlcNAc, $GlcNAc_2$, and $GlcNAc_3$ is shown. The product from the assay with colloidal chitin and the crude extracts of $\Delta chiABCD_{Hme}$ harboring pWL502 was set as negative control

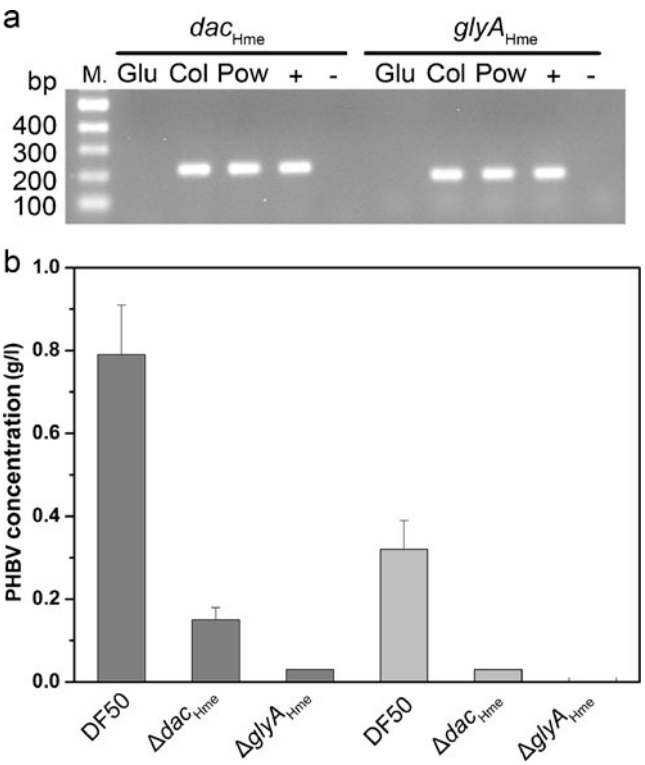

Fig. 7 Demonstration of the involvement of dac_{Hme} and $glyA_{Hme}$ in chitin utilization by *H. mediterranei*. **a** RT-PCR analysis of the expression of dac_{Hme} and $glyA_{Hme}$ under different culture conditions. *Lane M* marker; *lanes Glu*, *Col*, and *Pow* the reverse transcripts of the total RNA from *H. mediterranei* grown with glucose (Glu), colloidal chitin (Col), and powdered chitin (Pow), respectively, as the template; *lanes* + genome as the template, positive control; *lanes* − negative control. The primers used here are listed in Table S1. **b** The final PHBV concentration of *H. mediterranei* strains (DF50, Δdac_{Hme}, and $\Delta glyA_{Hme}$) at the stationary phase (maximum PHBV production) from colloidal (*the columns in dark gray*) or powdered chitin (*the columns in light gray*)

PHBV from colloidal chitin (approximately 0.03 g/l) and none from powdered chitin (Fig. 7b). These results indicated the important roles of Dac_{Hme} and $GlyA_{Hme}$ in chitin utilization by *H. mediterranei*. But the function of Dac_{Hme} and $GlyA_{Hme}$ in the degradation of chitin remains to be determined. Identification of these key enzymes in chitin utilization would advance our understanding of the chitin catabolism in haloarchaea.

Discussion

In this study, we report that *H. mediterranei* is able to efficiently degrade colloidal and powdered chitin for growth and PHBV accumulation and reveal the key enzymes involved in the chitin catabolism pathway. This is the first study of chitin catabolism and bioconversion in haloarchaea.

The characterized or annotated archaeal chitinases so far are listed in Table S4, except the three identified chitinases from *T. chitonophagus* with no sequences in NCBI GenBank (Andronopoulou and Vorgias 2004). These chitinases were mainly from *Euryarchaeota*, including eight species within the class *Halobacteria*, four *Thermococci* (*T. chitonophagus* included), and three *Methanomicrobia*. Besides, one species of *Crenarchaeota* (*S. tokodaii*) also possessed a chitinase, though it is not the classical GH18 family or GH19 family chitinase (Staufenberger et al. 2012). This indicated the potential of archaea for chitin bioconversion. The capability of *H. mediterranei* to degrade powdered chitin has ecological and economical significance because powdered chitin does not have to be pretreated with acid. In some microorganisms, a type of oxidative enzyme classified as CBMs is responsible for disrupting the highly crystalline structure of powdered chitin (Vaaje-Kolstad et al. 2010). Bioinformatic analysis revealed that this type of enzyme was present in the haloarchaeon of *Halomicrobium mukohataei* (accession number YP_003177200) but was not present in other archaea with available genome sequences, including *H. mediterranei*. ChtBD is able to disrupt the hydrogen bonds of the chitin chains (Nakamura et al. 2008), and the role of PKD in powdered chitin degradation has also been reported in bacterial chitinases (Orikoshi et al. 2005). Therefore, the ChtBD and PKD of the chitinases from *H. mediterranei* may act together to disrupt the hydrogen bonds of powdered chitin.

So far, archaeal chitin catabolism pathway is established only in *T. kodakaraensis* KOD1. Chitin is first broken down into $GlcNAc_2$ by $ChiA_{Tk}$. $GlcNAc_2$ is deacetylated by Dac_{Tk} to generate GlcN-GlcNAc and then cleaved into GlcN and GlcNAc by $GlmA_{Tk}$. The generated GlcNAc is also deacetylated by Dac_{Tk} to produce GlcN (Tanaka et al. 2004). In this study, we identified the key enzymes for chitin catabolism in *H. mediterranei* including four chitinases ($ChiABCD_{Hme}$), LmbE-like deacetylase (Dac_{Hme}), and glycosidase ($GlyA_{Hme}$). When compared with the chitin degradation pathway in *T. kodakaraensis* KOD1, the LmbE-like deacetylase exhibited 31 % identity to Dac_{Tk} and was assumed to be the deacetylase of *H. mediterranei*. Nevertheless, the homolog of $GlyA_{Hme}$ was not found in *T. kodakaraensis* KOD1, and the homolog of the key enzyme for chitin degradation in *T. kodakaraensis* KOD1, $GlmA_{Tk}$, was also not found in *H. mediterranei*. Further analysis revealed that $GlmA_{Tk}$ was a member of GH family 42, while $GlyA_{Hme}$ belonged to GH family 3. The enzymes of GH family 3 have broad substrate specificity and exhibit diverse catalytic properties, including β-glucosidases, β-xylosidases, β-*N*-acetylhexosaminidases, and even the combination of them (Faure 2002). Additionally, there have been some reports about the enzymes of GH family 3 being involved in chitin catabolism, such as the β-*N*-acetylglucosaminidase of the marine bacterium *Vibrio furnissii* CIP 102972 (accession number ZP_05880052) (Keyhani and Roseman 1996). $GlyA_{Hme}$ showed 34 % identity to the β-*N*-acetylglucosaminidase from *V. furnissii* CIP 102972 and might have the catalytic activity of β-*N*-acetylglucosaminidase. These results indicate that Dac_{Hme} and $GlyA_{Hme}$ might be involved in the further catabolism of $GlcNAc_2$ for both cell growth and PHBV biosynthesis, although their exact function remains to be demonstrated. It is noteworthy that horizontal gene transfer might occur in the archaeal chitin catabolism pathway because archaea represents an ancient form of life, while chitin is mainly present in highly evolved organisms (Tanaka et al. 1999). Hence, it will be intriguing to discover more novel chitin degradation pathways in archaea and discuss the evolutionary process of different chitin degradation pathways operating in bacteria, eukarya, and archaea.

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Conflict of interest The authors declare that they have no conflict of interest.

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