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ORIGINAL PAPER

Bacterial chitin utilisation at extremely haloalkaline conditions

D. Y. Sorokin · T. P. Tourova · M. V. Sukhacheva · A. V. Mardanov · N. V. Ravin

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Abstract Chitin is produced in large amounts in hypersaline habitats with neutral pH due to the high biomass production of brine shrimp *Artemia*. Recently, a high abundance of *Artemia* was also noticed in hypersaline soda lakes in the Kulunda Steppe (Altai, Russia), which prompted us to survey the possibility of microbial chitin utilization at extremely haloalkaline conditions in soda brines. Most active chitin utilisation-supporting microbial growth was found at anaerobic conditions at pH 10 and up to 3.5 M total Na^+. At aerobic conditions, the degradation of chitin was slower, mostly incomplete and active at <2 M total Na^+, although very slow partial degradation was possible up to 4 M Na^+. Anaerobic enrichments at pH 10 yielded two different groups of obligately haloalkaliphilic fermentative anaerobes, exclusively specialized to utilise insoluble chitin as the only growth substrate. One group was represented by a single strain growing at moderate salinity, and another comprised multiple isolates growing up to 3.5 M Na^+. These groups represent two novel bacterial phyla not closely related to any other cultured bacteria. Aerobic enrichments from the lake sediments were dominated by several obligately haloalkaliphilic members of the genus *Marinimicrobium* in the *Gammaproteobacteria*. They were less specialised than the anaerobes and grew with chitin and its monomer and oligomers at a pH of 10 up to 2.5 M Na^+. Furthermore, several strains of haloalkaliphilic Gram-positive chitinolytics belonging to bacilli and actinobacteria were isolated from soda lake sediments and surrounding soda soils. In general, the results indicate the presence of an active and diverse haloalkaliphilic chitinolytic microbial community in hypersaline soda habitats.

Keywords Chitin · Chitinolytic · Haloalkaliphilic · Hypersaline soda lakes

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Introduction

Chitin, a natural polymer of *N*-acetyl-glucosamine with a β-1,4 linkage, is second in abundance to cellulose carbohydrate polymers on the planet, mostly due to its structural function in terrestrial insects and aquatic crustaceans. Partially de-acetylated chitin is present in the form of chitosan in the fungal cell wall. The microbial degradation of chitin, in many respects, resembles the degradation of cellulose with the participation of a complex of chitinolytic enzymes, which exhibit exo- or endo-activities, and chitobiohydrolase (Gooday 1990; Karlsson and Stenlid 2008). The ability to hydrolyse chitin and the respective genes is wide spread among the bacteria and fungi and is also present in some archaea (Karlsson and Stenlid 2008; Gao et al. 2003; Andronopoulou and Vorgias 2004). Among bacteria, most active chitinolytics are classified in

Firmicutes, such as actinobacteria, bacilli (aerobic) and clostridia (anaerobic). The environmental importance of microbial chitin degradation (Gooday 1990) and the biotechnological potential of products of enzymatic chitin hydrolysis (Sarethy et al. 2011; Wanga et al. 2010) have made it an active research field. However, little is known about the potential of microbial chitin degradation and its utilisation as a growth substrate at extreme conditions, particularly at high salt and high pH. To our knowledge, despite the well-recognised presence of enormous populations of the chitin-producing brine shrimp *Artemia* in hypersaline habitats, only two reports exist in the literature on the presence of halophilic chitin-utilising bacteria in solar salterns (Liaw and Mah 1992; Hau-Heredia et al. 2009). The presence of a chitinase in an extremely halophilic archaeon, *Halobacterium salinarium*, has been reported (Hatori et al. 2005, 2006). It is not, however, clear yet whether the haloarchaea are able to actually grow on chitin as a sole substrate and how widely the chitinolytic potential is distributed among these extreme halophiles. With respect to high pH, there are few reports that characterise alkalitolerant chitinases produced by non-halophilic alkalitolerant actinomycetes (Tsujibo et al. 2003; Bansode and Bajekal 2006), by *Bacillus* spp. (Bhushan and Hoondal 1998; Sampei et al. 2004; Bansode and Bajekal 2006), and by a marine alkalitolerant micromycete *Beauveria bassiana* (Suresh and Chandrasekaran 1999).

Attempts have also been made to survey haloalkaliphilic chitinolytics and alkalitolerant chitinases in the haloalkaline Mono Lake in California (LeCleir and Hollibaugh 2006; LeCleir et al. 2007). However, in our opinion, the obtained results were not very conclusive and, especially in the primary survey (LeCleir and Hollibaugh 2006), mostly incorrectly interpreted. In particular, all organisms obtained from an enrichment with chitin, except for two *Vibrio* isolates, were obviously not chitinolytic. And even for the *Vibrio* isolates, the evidence of actual growth on chitin in liquid culture and the pH/salt profiles for chitinolysis were not presented. Furthermore, we believe that the results of measurements of chitinolytic activity with soluble oligosaccharides (chitobiose and chitotriose) directly in the environmental samples and in the isolates for which growth on insoluble chitin was not shown need to be considered with caution. While this can confirm the activity in pure cultures or in isolated chitinases for which action on insoluble chitin has already been demonstrated, it cannot be considered as proof of chitinase activity, since the true chitinase complex works on the insoluble polymer for which not only a catalytic hydrolase domain is necessary but also an insoluble substrate-binding domain. There are many examples of "parasitic" (opportunistic) organisms developing in co-culture with true chitinolytics (investors), which feed on released soluble chitin oligomers, but they cannot grow on chitin itself (Jagmann et al. 2010). Such "parasites" may even numerically dominate chitinoclastic cultures (our unpublished data), making the isolation of a true chitinolytic organism very difficult. Furthermore, direct enzymatic evidence shows that soluble chitin oligomers are substrates for specific members of the chitinolytics complex that cannot degrade polymeric chitin (Howard et al. 2003).

The obvious gap in knowledge about the possibility of microbial chitin utilisation at extremely haloalkaline conditions prompted us to undertake research on the isolation of haloalkaliphiles that are actually able to utilise insoluble chitin as the sole growth substrate. The obtained results revealed, unexpectedly, that most active chitin utilisation in soda habitats takes place under anoxic conditions by fermentative bacteria not related to any cultured bacterial species.

Methods

Samples

Samples from the top 10 cm of sediments from five hypersaline soda lakes and two soda solonchak soils were collected in the Kulunda Steppe (Altai, Russia) in July 2009 and 2010. The brine pH varied from 9.95 to 10.5, the total salinity from 100 to 250 g l^{-1}, and the soluble carbonate alkalinity from 1.1 to 2.5 M. The soils contained 5–7 % (w/w) of soluble salts with alkalinity of 0.2–0.5 M and pH of 10.1–10.2 in the water extract. Surface sediment samples from eight haloalkaline lakes in Wadi Natrun (Libyan Desert, Egypt) were obtained in 2000 and kept either aerobically or anaerobically under argon at 4 °C. The brine pH varied from 9.4 to 10.1, the total salinity from 200 to 360 g l^{-1} and the soluble carbonate alkalinity from 0.1 to 0.75 M. The individual sediment and soil samples from each area were mixed in equal proportions to obtain two cumulative sediments and a single soil samples used as inocula to enrich for haloalkaliphilic chitinolytics. Furthermore, dry soda mud samples collected near soda lakes Bogoria and Nakuru in Kenya in 1996 by Dr. B.E. Jones (pH of water extract from 10.05 to 10.2) and a soda solonchak soil from the north-eastern Mongolia (2001; pH of water extract 10.4) have been used for enrichment of aerobic haloalkaliphilic chitinolytics.

Chitin degradation rates in lake sediments

5 cm^3 of two mixed sediment samples from the Kulunda Steppe and Wadi Natrun were incubated in 20 ml of soda mineral buffers at pH 10 containing 0.6–4.0 M total Na^+ either aerobically with shaking at 200 rpm or anaerobically under argon with 0.5 mM sulfide as a reductant. Chitin was added at a final concentration of 0.5 g l^{-1}, and its

mineralisation was followed within 2 months by incubation at 28 °C by analysing the liberation of ammonia.

Enrichment and cultivation of chitinolytic haloalkaliphiles

The enrichment and cultivation of haloalkaliphilic chitinolytics were performed at 28 °C on mineral medium containing sodium carbonate buffer containing 0.6–4.0 M total Na^+ at pH 10 (stable after sterilisation) and 0.5 g l^{-1} of K_2HPO_4. After sterilisation at 120 °C for 30 min, the medium was supplemented with 10 mg l^{-1} of yeast extract, 1 mM $MgSO_4$, and 1 ml l^{-1} each of acidic trace metal solution and vitamin mix (Pfennig and Lippert 1966). The crab-shell chitin powder (Sigma-Aldrich) was washed several times in 0.1 M NaOH and then in water and finally sterilised as a 10 % (w/v) suspension. It was used at a final concentration of 1 g l^{-1}. Amorphous chitin was prepared from crystalline crab-shell chitin by dissolving in phosphoric acid, rehydration in 10 volumes of ice-cold distilled water and final washing of the suspension until neutralisation. It was sterilised as a 5 % (w/v) suspension and added to the medium at a final concentration of 1 g l^{-1}. Soluble carbohydrates were filter-sterilised as 10 % (w/v) solutions. The soluble chitin oligomer mixture containing 4–6 *N*-acetyl-glucosamine molecules was a gift from Dr. S. Lopatin (Centre "Bioengineering" RAS, Moscow). Amorphous chitosan was prepared from the commercial crystalline chitosan (Sigma-Aldrich) by dissolution in 5 % acetic acid with subsequent washing in ice-cold water up to neutral pH. Aerobic incubations were performed in 20–50 ml serum bottles filled with 5–10 ml medium and capped with rubber stoppers with shaking at 100 rpm. For anaerobic cultivation, the same types of bottles were filled to 80 % capacity, closed with butyl rubber stoppers and subjected to five cycles of evacuation-argon flushing with final addition of 0.5 mM HS^- as a reductant. Solid medium with amorphous chitin was prepared by a 1:1 mixing of the complete liquid medium and 4 % (w/v) washed agar at 50 °C. The plates for anaerobes were incubated in closed jars under argon with an oxygen-consuming catalyst (Oxoid).

The pH dependence for growth was examined with amorphous chitin as the substrate at a Na^+ content of 0.6 M, using the following filter-sterilised buffering systems: for pH 6–8, 0.1 M HEPES + NaCl/$NaHCO_3$, and for pH 8.5–11.0, a mixture of sodium bicarbonate/sodium carbonate containing 0.1 M NaCl. Since the pH was changing during cultivation (mostly at highest starting values), the final values at the end of the exponential growth phase were taken into consideration in the growth pH profiles. To study the influence of salt concentration on growth, sodium carbonate media at pH 10, containing 0.2 and 4.0 M of total Na^+, were mixed in different proportions.

Analyses

The biomass growth was followed by an increase in optical density at 600 nm after vigorous homogenisation of the cultures followed by chitin separation by low-speed centrifugation in 2 ml Eppendorf tubes at 1000× rpm for 10 s. The growth experiments were performed in duplicate. The growth was accompanied by visible chitin degradation. Fermentation products were analysed by HPLC anionic chromatography (BioRad, HPX-87-H column at 60 °C, an eluent containing 5 mM H_2SO_4 solution at 0.6 ml min^{-1}, and UV and RI detectors) after neutralisation of the supernatant. The production of H_2 was measured by gas chromatography [Chromateck Crystall 5000 (Russia), column Hayesep 80–100 mesh, 2 m × 3 mm, 40 °C; detector TCD, 150 °C; carrier gas argon 25 ml/min]. The NH_3 release from chitin in the chitin degradation experiments was analysed by the phenol-hypochlorite method (Weatherburn 1967). Chitinolytic activity in aerobic isolates and the influence of pH on the activity were qualitatively monitored by the agar-diffusion method. For this, the buffers mentioned above with pH 6–11.5 containing 0.6 M total Na^+ were supplemented with 0.1 % (w/v) amorphous chitin and solidified with 1 % (w/v) washed agar. The tests were performed in three variants: whole cells, culture supernatants and sonicated cells. The fractions were applied to the wells cut in the agar, and the whole cells were inoculated directly onto the agar surface. The clearance of chitin around the colonies or wells was taken as an indication of the chitinolysis. Phase contrast photomicrographs were obtained with a Zeiss Axioplan Imaging 2 microscope (Göttingen, Germany). For electron

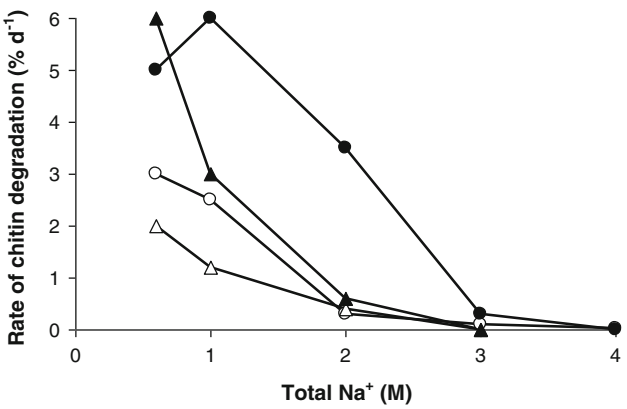


Fig. 1 Amorphous chitin degradation (0.5 g l^{-1}) in sediments from hypersaline alkaline lakes in the Kulunda Steppe (Altai, Russia) (*circles*) and Wadi Natrun (Egypt) (*triangles*) at aerobic (*open symbols*) and anoxic (*closed symbols*) conditions at pH 10 in sodium carbonate buffer with different salinity. 5 cm^3 sediment samples were incubated with 20 ml of buffer. The results are average values from duplicate incubations. The sediments without chitin served as the control

microscopy, the cells were transferred to neutral NaCl solutions with the same molarity as in the growth medium, fixed with glutaraldehyde (3 % v/v) for 2 h and positively stained with 2 % (w/v) neutralised phosphotungstic acid.

For molecular analysis, the DNA was extracted from the cells using alkaline SDS lysis at 60 °C and purified with the Wizard Preps Kit (Promega, USA). The nearly complete 16S rRNA gene was obtained using the general bacterial PCR primers 11f and 1492r (Lane 1991). The sequences of aerobic strains were aligned with sequences from GenBank using CLUSTAL W, and phylogenetic trees were reconstructed using the neighbour-joining algorithm in the TREECONW program package (van de Peer and de Wachter 1994). The alignment and phylogenetic reconstruction of the 16S rRNA genes of anaerobic strains were performed using MEGA 5.05 software (Tamura et al. 2011). The tree was calculated with the neighbour-joining method based on the maximum composite likelihood method. Bootstrap tests were performed with 100 resamplings. The 16S rRNA sequence of *Aquifex aeolicus* VF5 (AJ309733) was used as an outgroup.

Results and discussion

Chitin degradation in hypersaline soda lake sediments at pH 10

In the Kulunda Steppe sediment sample, both native and amorphous chitins were degradable aerobically within all

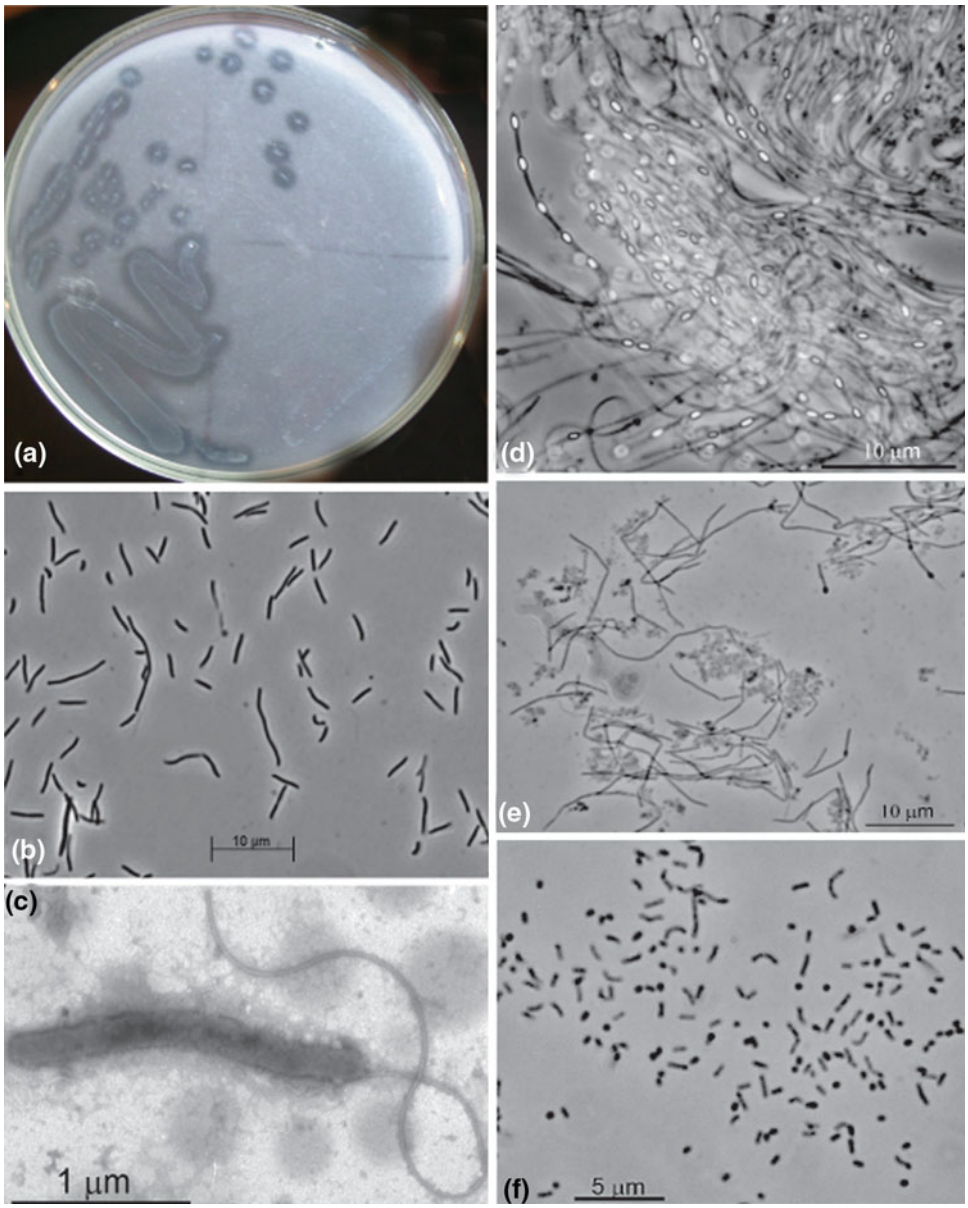


Fig. 2 Morphology of aerobic chitinolytics from Kulunda soda lakes and soda soils grown at pH 10 with amorphous chitin. **a–c** strain ABCh5: **a** clearing zones around colonies on chitin agar; **b** phase contrast photomicrographs; **c** electron photomicrographs. **d** strain ABCh1; **e** strain ABCh6; **f** strain ACB59

salinity ranges tested and up to 3 M Na^+ at anoxic conditions; while in the Wadi Natrun sample, the degradation was possible only up to a moderate salinity of 2 M Na^+. The anaerobic degradation started almost immediately, while at aerobic conditions, it was usually preceded by a lag phase of at least 10 days. Maximum rates were observed at moderate salinity and at anaerobic conditions in which chitin was eventually completely degraded, in contrast to the aerobic incubations in which maximally only half of the added substrate was decomposed (Fig. 1). The results indicated a presence of active moderately salt-tolerant alkaliphilic chitinolytic microbial community in hypersaline soda lakes.

Enrichment and isolation of aerobic haloalkaliphilic chitinolytics

Primary aerobic enrichments at pH 10 obtained in the degradation experiments were further transferred at 1:100 several times until stable growth was obtained followed by plating on chitin-containing agar media. In this way, enrichments from the Kulunda lake sediments gave positive results at salinity of up to 2 M Na^+, while the Wadi Natrun sample gave a stable culture only at low salinity of 0.6 M Na^+. The plating of the stable liquid enrichments revealed colonies that formed clearing zones, which were further purified (see example Fig. 2a). The Kulunda sediment cultures, both at low and high salt, were dominated by motile, long-curved rods with polar flagella (Fig. 2b, c), whereas the Wadi Natrun culture yielded a single isolate represented by filamentous rods with multiple endospores (Fig. 2d). The soda soil enrichment gave a stable culture at 0.6 M Na^+, which eventually resulted in the isolation of two different Gram-positive morphotypes—the nocardia-like rods fragmenting to coccoid cells (Fig. 2f) and the spore-forming filamentous rods (Fig. 2e). Direct plating of the dry soda soil sample on chitin-containing plates at pH 10 and 0.6 M Na^+ resulted in domination of actinomycetes, some of which formed moderate clearance around the colonies. From these plates, several actinomycetes strains were isolated in pure culture. Some of the characteristics of the aerobic isolates are presented in Table 1. All aerobic isolates were able to grow in liquid medium at pH 10 with amorphous chitin (and most also with its crystalline form) as the only substrate as well as with the monomer, *N*-acetyl-glucosamine. The most active growth with chitin was exhibited by the Gram-negative isolates ABCh4 and ABCh5 and by the spore-forming isolates

Table 1 Aerobic chitinolytic strains isolated from soda lakes and soda soils at pH 10

Strain	Source	Enriched at: Na^+ (M)	Growth in liquid media with chitin				Closest culturable relative (% of 16S-rRNA gene sequence similarity)
			Amorphous	Crystal-line	Max. Na^+ (M)	<pH 8	
ACht2	Kulunda soda lake sediments	2.0	Slow, incomplete degradation	−	2.5	+	*Marinimicrobium agarilyticum* (97 %)
ACht3		2.0					
ACht5-1		0.6					
ACht5-2		0.6					
ABCh4	Soda soils (Kulunda)	2.0	Fast, complete degradation	+	2.5	+	
ABCh5		0.6		+	3.0	−	
ABCh1	Wadi Natrun lake sediments	0.6	Fast, complete degradation	+	3.0	+	*Bacillus akibai* (97 %)
ABCh2	Kulunda soda lake sediments	0.6		+	3.0	+	
ABCh3				+	3.75	+	
ABCh6		2.0	Very slow, incomplete	−	2.5	+	*Bacillus firmus* (91 %)
ACB56	Soda soils (Kenya)	0.6	Fast, complete degradation	+	2.0	+	*Isoptericola halotolerance* (98 %)
ACB59							
ACB96	Soda soil (Mongolia)						
AACh1	Soda soils (Kulunda)	0.6	Fast, complete degradation	+	2.0	+	*Nocardiopsis* spp. (99 %)
AACh2					2.0		
AACh4					2.0		*Glycomyces harbinensis* (95 %)
ACPA51					1.75		*Nocardiopsis* sp. (99 %)
ACPA54					1.5		*Streptomyces sodiphilis* (99 %)
ACA32	Soda soils (Kenya)		Slow, complete degradation	+	1.5		*Streptomyces* sp. E-070

ABCh1, 2 and 3. Most of the aerobic isolates, except for ABCh5, were able to grow with chitin at neutral pH of 7–7.5 and up to pH 10.5 with an optimum around 9–9.5, thus belonging to the facultative alkaliphiles. The chitinolytics activity tested qualitatively on solid media was optimal at pH of 8–9 and in some isolates was still visible up to pH 11.5 (Supplementary Fig. S1). The activity was obviously associated with the cells, except for the Gram-negative strains ABCh4 and 5, in which a weak activity was also detectable in the culture supernatants. Most of the isolates showed moderate salt tolerance for growth on amorphous chitin in liquid culture (Table 1). The most salt-tolerant strains were ABCh3 (up to 3.75 M Na^+) and ABCh5 (up to 3.0 M Na^+) (Fig. 3).

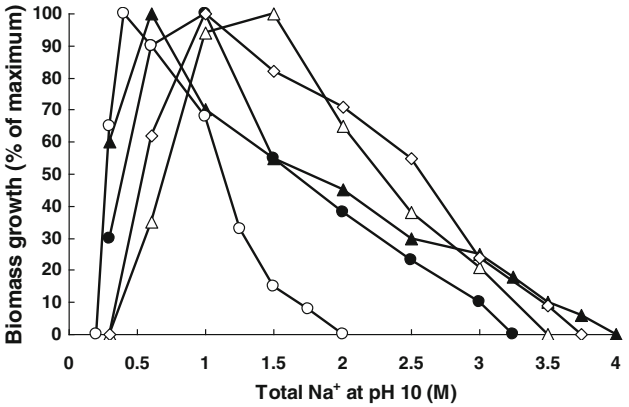


Fig. 3 Influence of salinity at pH 10 on growth of haloalkaliphilic chitinolytic isolates on amorphous chitin. *Closed symbols* aerobic bacteria (*circles* strain ABCh5; *triangles* ABCh3). *Open symbols* anaerobic fermentative bacteria (*circles* ACht6-1; *triangles* ACht1; *diamonds* ACht10)

Identification of aerobic chitinolytic isolates

Phylogenetic analysis based on 16S rRNA gene sequencing placed the aerobic isolates into three eubacterial phyla: the *Proteobacteria*, the *Firmicutes* and the *Actinobacteria*. The proteobacterial strains were closely related to each other and belonged to the genus *Marinimicrobium* in the Gammaproteobacteria, which includes marine and halophilic

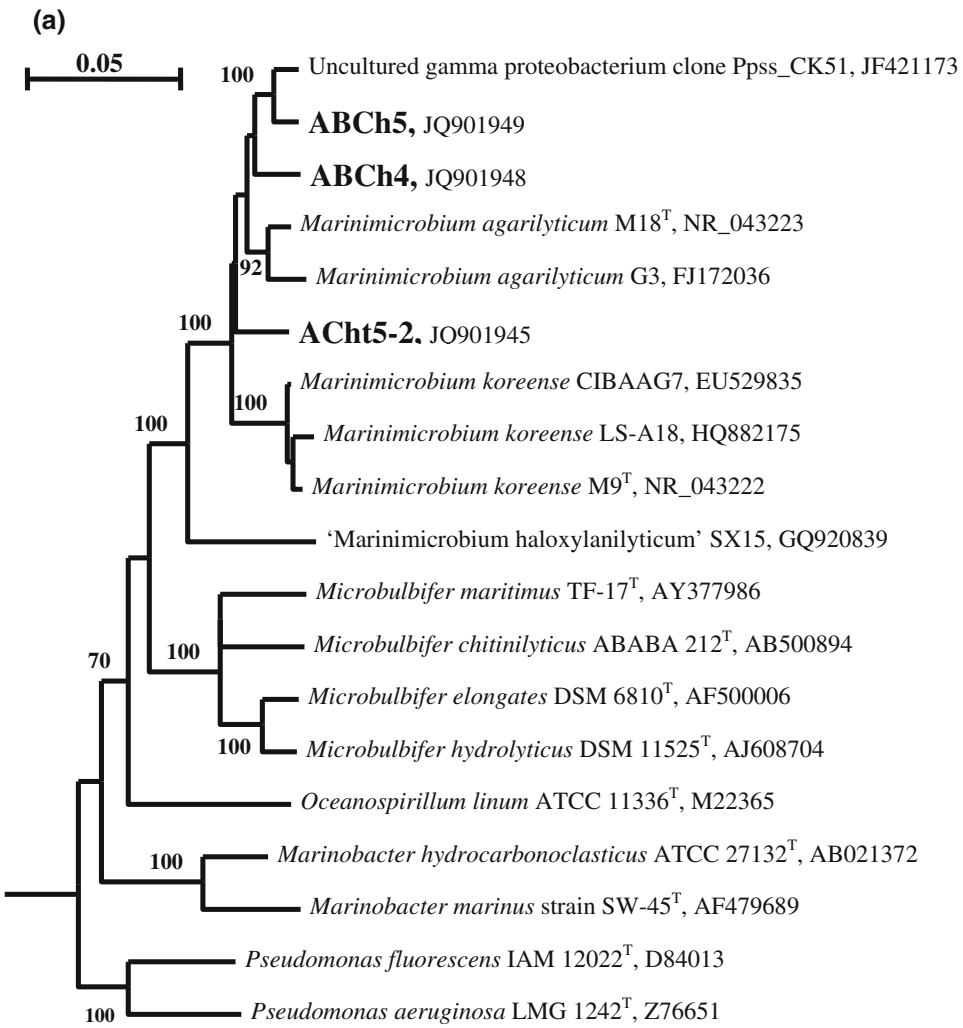
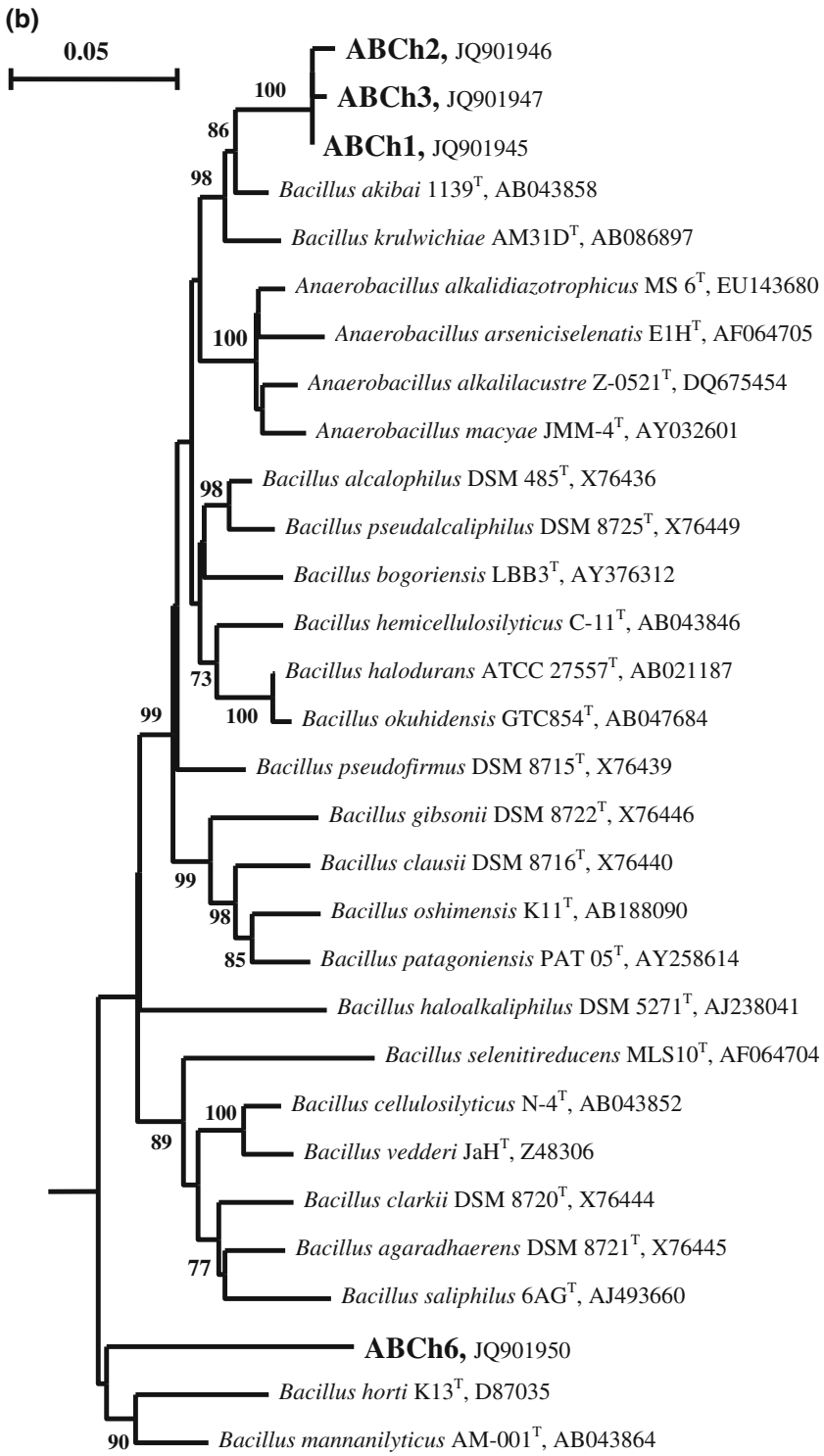


Fig. 4 Phylogenetic position of aerobic haloalkaliphilic chitinolytics based on 16S rRNA gene sequence analysis. **a** In the *Gammaproteobacteria*; **b** in the order *Bacillales* and **c** in the *Actinobacteria*. Tree topography and evolutionary distances were obtained by the neighbour-joining method with Jukes and Cantor distances. The *scale bar* represents nucleotide changes per 100 nucleotides. The *numbers on the nodes* indicate bootstrap values above 70 %

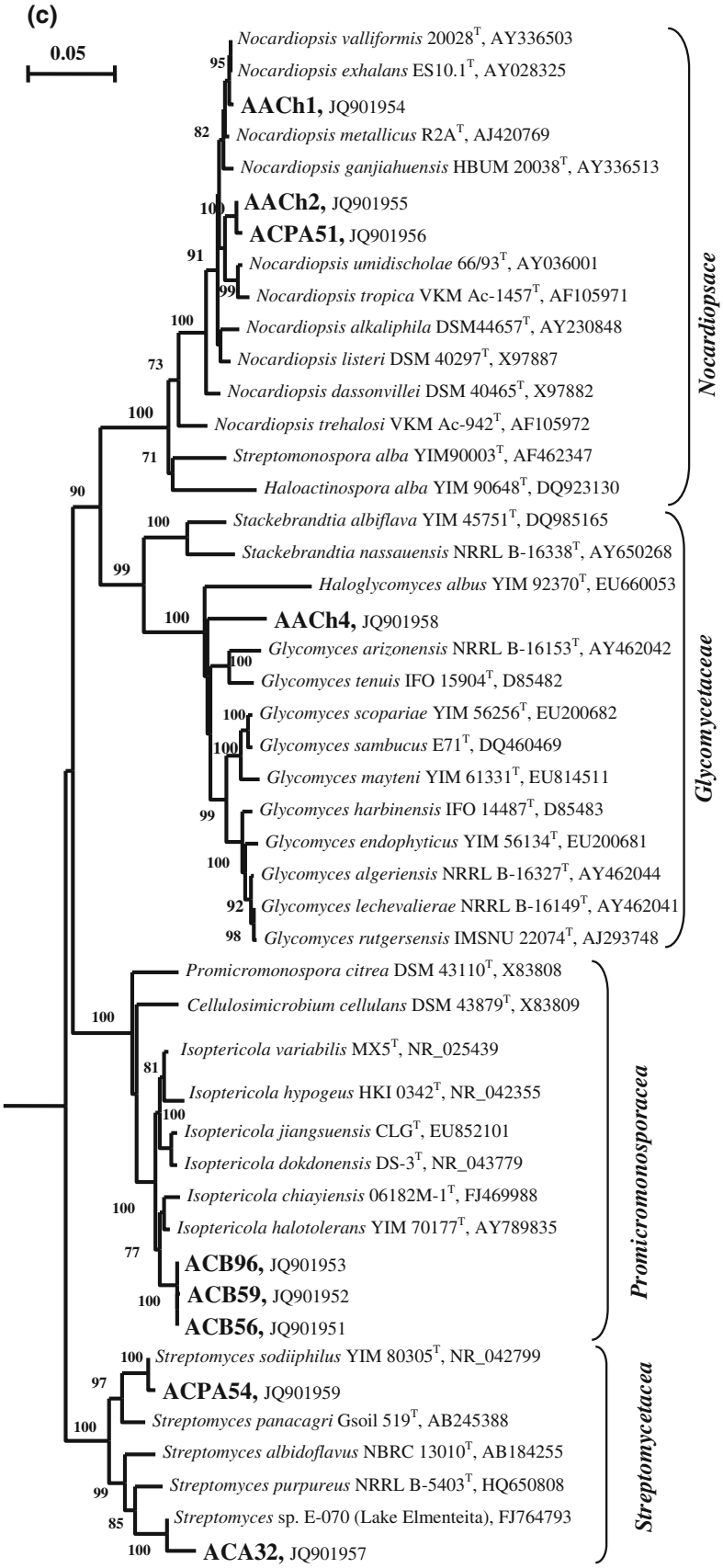
Fig. 4 continued



bacteria with prominent hydrolytic activities (Lim et al. 2006; Møller et al. 2010), although alkaliphily and chitinolysis have not been demonstrated in the so far described species. The haloalkaliphilic chitinolytic isolates probably represent two novel species within this genus (Fig. 4a).

The spore-forming isolates are members of the order *Bacillales*. Strains ABCh1, 2 and 3 were close to each other and also closely related to a number of alkaliphilic bacilli of the rRNA group 6, while strain ABCh6 represented a novel deep independent lineage in the order (Fig. 4b). The

Fig. 4 continued



actinobacterial isolates included three phylogenetic clusters: three strains belonged to the genus *Nocardiopsis*, two strains to the genus *Streptomyces*, one strain to the genus *Glycomyces* and three strains to the group *Cellulomonas-Isoptericola* (Fig. 4c). Both streptomycetes and cellulomonads are well-known hydrolytics, and they also contain many alkaliphilic representatives (Jones et al. 2005). Therefore, the affiliation of most of the aerobic chitinolytic isolates from soda habitats with these actinobacteria is not surprising.

Enrichment and isolation of anaerobic haloalkaliphilic chitinolytics

As mentioned above, enrichments with chitin under anaerobic conditions degraded crystalline chitin much faster than aerobic cultures, especially at moderate salinity of 2 M total Na^+. However, attempts to obtain colonies on solid media with amorphous chitin failed, and further purification and isolation of pure cultures had to be done by multiple dilution series with amorphous chitin. The primary enrichments were dominated by long, thin flexible rods, while, after several dilution series, motile vibrio-shaped cells attached to the chitin particles started to take over, and, eventually, it became apparent that the vibrio-shaped cells were the primary chitinolytics, while the rods belonged to the satellite microflora which utilized the products of chitin hydrolysis. The latter was confirmed for two enrichments: from the low-salt enrichment of Kulunda sediment mix, strain ACht4 (JF304646) was isolated in pure culture using the soluble chitin oligomer mixture. This organism grew and utilised chitin monomers and oligomers but not chitin itself and was identified as a member of the genus *Natronoflexus* described previously as a soda lake pectinolytic (Sorokin et al. 2011). The second satellite organism, strain ACht11 belonging to the genus *Amphibacillus* (Q901944), was isolated from the dry mud enrichment culture at 2 M Na^+. It was able to grow in saturated soda brines with the chitin monomer but not with oligomers or chitin itself.

Final purification on amorphous chitin at 0.6–2 M Na^+ and pH 10 resulted in the isolation of six pure cultures of obligately anaerobic fermentative haloalkaliphilic bacteria with vibrio-shaped cells (Fig. 5). The isolates shared several specific features: they were obligate anaerobes with a fermentative metabolism, they did not form colonies, they were super-specialised chitinolytics not able to utilise any other substrates for growth, including either chitin monomers or oligomers, and they attached firmly to chitin particles during the initial growth phase and lysed massively upon complete chitin degradation. All isolates were obligately alkaliphilic with optimal pH around 10 and pH maximum up to 10.5–10.6. Morphologically and phenotypically, the isolates can be divided into two groups. The major group included five strains with short motile vibrio-shaped cells, which had a tendency for aggregation (Fig. 5, in red). All strains in this group were able to grow at salinity above 2 M Na^+, and one strain grew up to 3.5 M (see Fig. 3). The second group included a single, low salt-tolerant isolate ACht6-1 from the Wadi Natrun lakes,

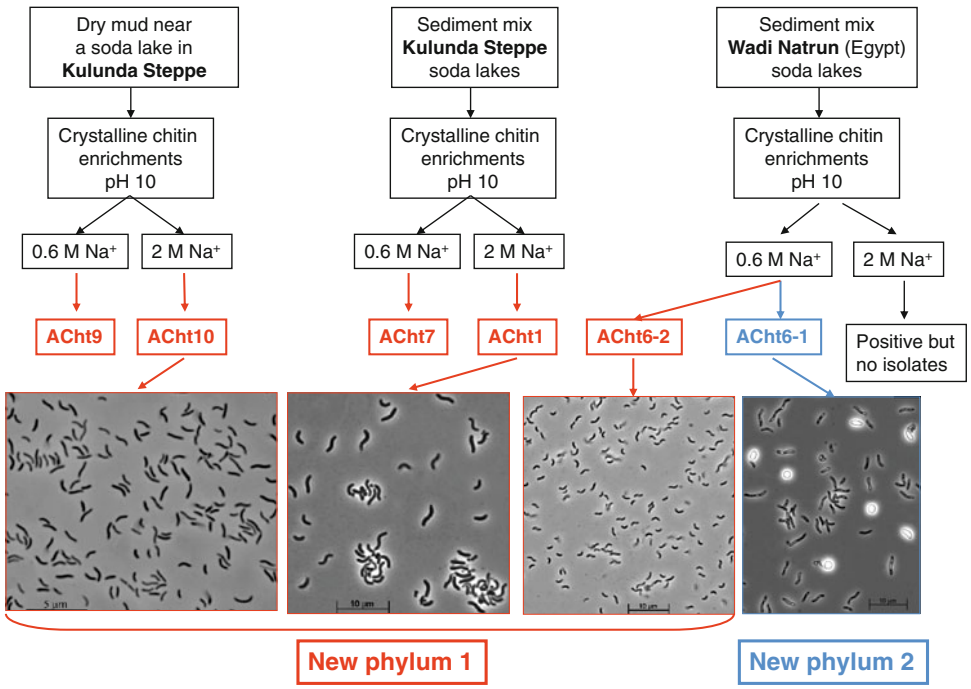


Fig. 5 Scheme of isolation and cell morphology of anaerobic fermentative haloalkaliphilic bacteria from enrichments with chitin

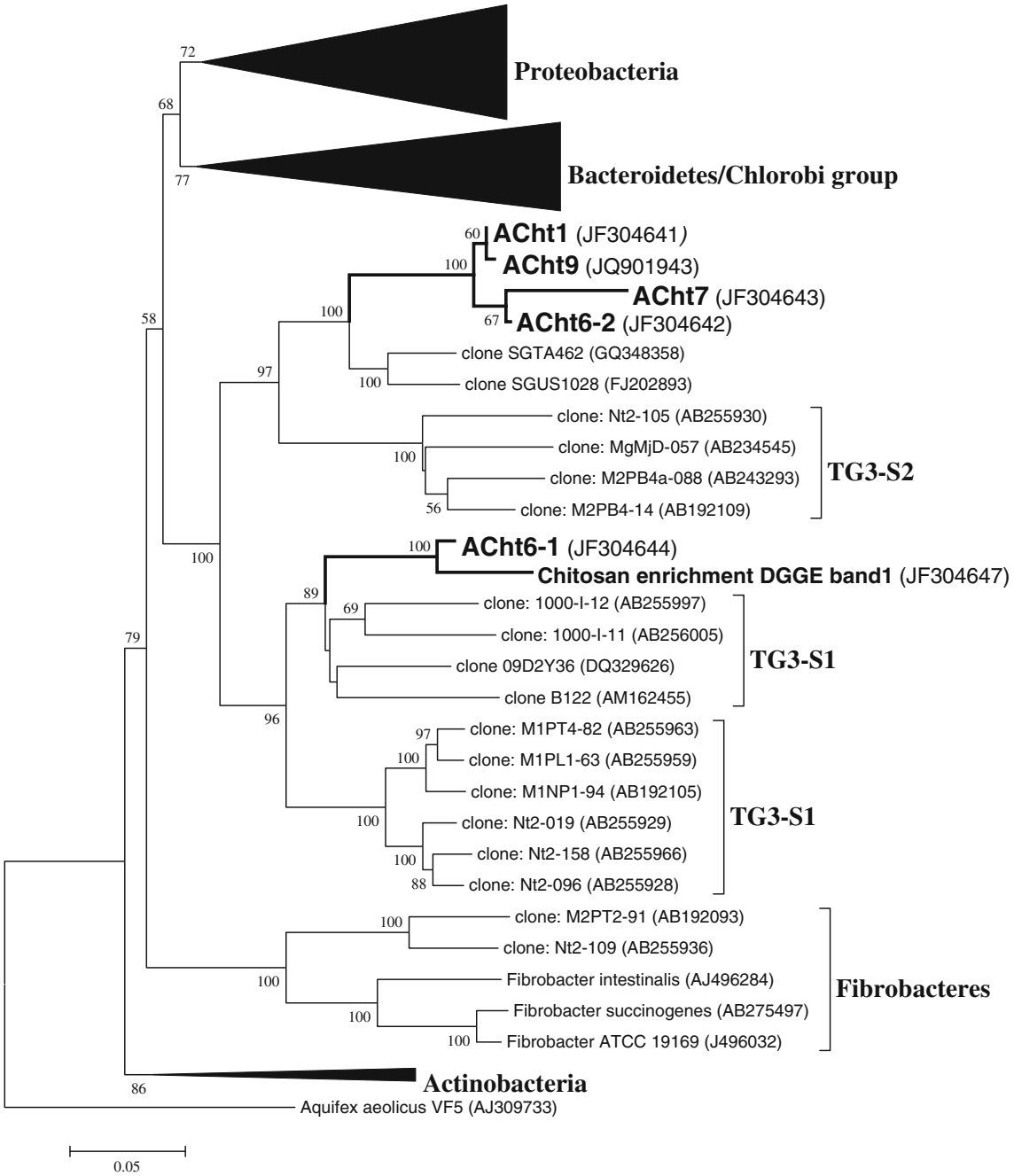


Fig. 6 Phylogenetic position of anaerobic haloalkaliphilic chitinolytics based on 16S rRNA gene sequence analysis. The tree was calculated with the neighbour-joining method based on maximum composite likelihood method. Bootstrap tests were performed with 100 resamplings. The 16S rRNA sequence of *Aquifex aeolicus* VF5 (AJ309733) was used as an outgroup

which had a peculiar life cycle with young cells represented by relatively long motile spirilla gradually becoming thicker and shorter with the accumulation of PHB-like refractive material and eventually turning to lipid (reaction with Nile Blue) cyst-like coccoids (Fig. 5). Phylogenetic analysis confirmed the separation of anaerobic chitinolytics into two distant groups. The vibrio-shaped strains had 96–99 % 16S rRNA gene sequence similarity between each other but only 81 % with strain ACht6-1, indicating that they belonged to two different phyla. Furthermore, phylogenetically, both groups were very distant from any cultured bacteria (79–82 % sequence similarity) and therefore represent two novel bacterial phyla (Fig. 6). These two phyla corresponded to two candidate subdivisions currently designated as TG3-S1 and TG3-S2 (Termite Group 3 subphylum 1 and 2), which include multiple

clones mostly associated with the termite gut (Hongoh et al. 2005, 2006; Hongoh 2010). Full characterisation of these novel bacteria and their genomes is under way.

Chitosan utilisation

Chitosan is a product of chitin deacetylation and consists mostly of glucose amine monomers. In contrast to chitin, the chitosan degradation at haloalkaline conditions was not so active. At aerobic conditions, the enrichments failed, while only a single positive enrichment at low salt conditions was obtained from the Wadi Natrun sample under anoxic conditions. Chitosan degradation in this culture was much slower compared to chitin, and it was not possible to isolate a pure culture. However, the dominant morphotype resembled strain ACht6-1 (also obtained from the Wadi Natrun sample) by the very characteristic lipid cyst formation. Indeed, DGGE analysis of the enrichment showed the presence of a relative of ACht6-1 as one of the two dominant organisms (Supplementary Fig. S2). These results suggest that chitosan is not a natural substrate for the haloalkaline habitats.

Overall results demonstrated the presence of active bacterial chitinolytic communities in soda habitats that are able to utilise chitin as a growth substrate at extremely high pH and up to nearly saturating soda concentrations. Especially interesting is the presence of very active haloalkaliphilic anaerobic fermentative bacteria exclusively specialised on chitin as a growth substrate that are not closely related to any other cultured bacteria.

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