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Acetobacter pasteurianus strain AB0220: cultivability and phenotypic stability over 9 years of preservation

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Abstract

Acetobacter species are members of the α-subclass of *Proteobacteria*, which harbors a large number of bacteria recalcitrant to cultivation. Strain AB0220 was isolated from a superficial acetification system and preserved for 9 years by short and long time methods. Under short time preservation it was estimated that 540.54 number of generations occurred, whereas in long time preservation conditions the number of generations was 17.40. Ethanol oxidation to acetic acid was stable and confirmed, as well as acetate assimilation during long time preservation. Cultivability checks showed persistence of phenotypic traits (growth on ethanol and methanol, growth on different carbon sources and cellulose production) over the extended preservation time. 16S rRNA gene sequences analysis showed 100% of similarity with *Acetobacter pasteurianus* (Accession number GQ240636). Stability of subcultures related to the culture age and subcultures frequency, tested by ERIC/PCR, confirmed the suitability of long term preservation at least over a period of 9 years.

Keywords: acetic acid bacteria, preservation, phenotypic stability, *Acetobacter pasteurianus*

Introduction

Acetobacter species are members of the α-subclass of *Proteobacteria*, which harbours a large number of bacteria recalcitrant to cultivation. Many strains are difficult to preserve especially those isolated from industrial acetification systems that are high acetic acid (AcOH) tolerant [10]. However, maintenance of “authentical” strains over the preservation time is a priority for microbial exploitation as bio-factory [17]. Acetic acid bacteria (AAB) preservation can be performed by short time and long time methods. By short time preservation (STP), cultures are maintained by renewals generally at +4°C, so they are cyclically on growing status with high probability of mutations inducing or not phenotypic changes [6]. Thus, phenotypic features of interest need to be periodically checked. To preserve phenotypic and genotypic traits of industrial strains, long time preservation (LTP) has been conducted by freeze-drying, ultrafreezing and ultralow-temperature of both pure and starter cultures.

On one side, LTP improves strains stability, but on the other side it strongly affect cell recovery [14, 15]. The stability of important traits has been widely studied within AAB, showing high frequency of phenotypic changes, mainly regarding ethanol (EtOH) oxidation and AcOH resistance. In the *Acetobacter pasteurianus* species, high rate of spontaneous mutants deficient in EtOH oxidation due to an inserted sequence of 1.665 bp were observed [19]; high frequencies of mutation was also reported for the strain NBRC 3283, where 74 copies of the same inserted sequence were found [2]. Other studies have been done to elucidate the mechanisms of AcOH resistance during exposure to stressors. In *Acetobacter* and *Gluconacetobacter* it was shown that genes *aarA* and *aarC* of the gene cluster *aarABC* are responsible for acetate assimilation [5]. Since *Acetobacter* and *Gluconacetobacter* members survive without assimilation of acetate under high concentration of AcOH, other mechanisms have been investigated proving that in *A. aceti* and *A. pasteurianus* species a proton motive force-dependent and ABC-transporter-like efflux pump systems occur for AcOH [12]. Moreover changes in membrane lipid composition have been reported as additional mechanism for AcOH resistance [20]. Strain stability can be evaluated on the basis of phenotype or genotype with different approaches. Phenotypic assay is dictated by specific traits of the organism. While, genotypic assays are based on a variety of DNA typing methods. Among them, ERIC/PCR has been described as fingerprinting tool providing specific pattern over the time from the same organism and applied to map insertion sequences, such as mutations caused by transposon insertions [9, 11, 21]. In this work, cultivability of the strain AB0220 was investigated with respect to phenotypic and genetic stability in STP and LTP conditions.

Materials and methods

Organisms and culture conditions

Strain AB0220 was isolated on GYC medium according to previous study [7]. STP was implemented by cultivation cycles on GYC plates incubated at +28°C and stored at +4°C; whereas LTP (-80°C) was conducted mixing 700 µl of glycerol at (50% (v/v)) and 300 µl of bacterial culture (Fig. 1a). Revitalization was done transferring aliquots (500 µl) on GYC tubes. Recovery was scored as

positive if at least one colony or visible biofilm was observed on plates after incubation at +28°C. Reference strains were cultivated according to manufacturer indications (DSMZ: Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). To determine the generation time, total number of cells was enumerated using Neubauer counting chamber (BRAND GMBH and CO KG, Germany). Generation number was calculated from the logarithmic equation *n=log (Nt final/Nt initial)/log2*, where *n* is the generation number, *Nt final* is the total number of cells at final time and *Nt initial* is the total number of cells at initial time.

Phenotypic characterization

Cell shape, gram and KOH tests, catalase production, formation of water-soluble brown pigments and cellulose production were tested as previously described [18, 22]. Oxidation of EtOH and acetate assimilation was tested by chalk-EtOH tests on Frateur and modified Carr and Passmore media [13]; colorimetric assay was performed by Carr medium [3]. Growth on GYC, AE, YPM and RAE was performed according to [7, 16]. Growth on D-glucose (20, 25, 30 (% w/v)) was tested on SM broth according to previous study [13]. Utilization of EtOH and methanol as carbon source was tested on salt agar medium [16]. Acetification ability was assayed on GYC at 2% of EtOH (v/v) for 168 h following by a scaling-up on sterile red wine (12% EtOH (v/v)). Acetic acid (g/L) was determined neutralizing samples at pH 7.2 with 0.1 N of NaOH.

Typing of subcultures by ERIC/PCR

ERIC/PCR was performed on genomic DNA (gDNA) from cultures by sodium dodecylsulfate (SDS) proteinase-cethyltrimethyl ammonium bromide (CTAB) treatment and preserved at -20°C as previously reported [7]. The value of reproducibility was estimated by analysis of two repeated DNA extracts. Two independent amplifications were performed using the conditions as described earlier [21] with modifications. PCR mix contained 30 ng/µL DNA, 1x Colorless GoTaq® buffer (Promega, USA), 4 mM $MgCl_2$ (Fermentas), 0.2 mM dNTPs (Takara Bio, Inc., Japan), 0.2 µL BSA (20 mg/mL, Fermentas), 0.4 pmol/µL of each primer and 0.06 U DNA polymerase (Takara Bio, Inc., Japan). Amplification and electrophoresis was performed as previously described [22]. A 100 bp DNA

molecular size marker (GeneRuler™ 100 bp Plus DNA Ladder, Fermentas) was loaded. Gels were stained in ethidium bromide, visualized under UV light and digital image captured using BioDocAnalyze analysis software (Biometra, Göttingen, Germany, version 2.1). Fingerprints were analyzed using the BioNumerics version 6.5 software (Applied Maths, Sint-Martens- Latem, Belgium). The similarity among digitized profiles was calculated using the Pearson correlation, and an average linkage (UPGMA) dendrogram was derived.

Partial 16S rRNA gene sequencing and accession number

gDNA extraction was performed as described for ERIC/PCR. Direct sequencing and analysis was done on amplicons according to previous study [8]. The nucleotide sequence have been deposited into EMBL databases under the accession number HE650905.

Results

Strain AB0220 was isolated in 2002 during a large spectrum of isolation work aimed to build up an AAB strains collection [7]. Starting from the isolation time, both STP and LTP were implemented. STP was performed on duplicates of AB0220 subcultures. To avoid selecting single colony, transferring from solid medium was performed by streaking part of the hold biofilm of the culture. Rapid growth (within 3 days) with a biofilm formation was observed when cultures were transplanted after 15 days of preservation. After 30 and 60 days of preservation, 5 incubation days to grow on GYC solid and broth were required. Subcultures from extended storage (3 months) grew only on GYC broth. After the first check, frequency between transfers along time was established each 60 days. The estimation of the number of generations during 9 years of STP was assessed by reconstruction of the 55 days of preservation. Within the 5-day incubation at +28 °C, cells number increased 20.45 fold, corresponding to 4.35 generations (SD = 0.01). The total cell number decreased 14.24 folds after 55 days of storage at +4°C corresponding to a loss of 5.66 generations (SD = 0.14). Subsequently 10.01 generations per passage during STP and thus 540.54 generations occurred. During LTP, viability check performed after 5 months from the first storage showed that 5 incubation days were required when subcultures were revitalized. After the first time, viability check was done

each 24 months. Phenotypic characterization was conducted on current subculture of STP and on subcultures of LTP (AB0220-1 and AB0220-3). Growth was observed on all media except for AE and RAE. No growth on methanol, EtOH and at 30% of D-glucose was observed (Table 1). Growth on solid media was observed mainly as a soft biofilm on the surface of plates, but differently from the growth modality of the strain DSMZ 2004 for which cellulose layer was visible. Recovery of isolated colonies was often no visible, instead common growth modality was as thin biofilm. In liquid both thin superficial layer and uniform turbidity was observed. Oxidation of EtOH to AcOH and acetate assimilation trialled by chalk-EtOH test was shown by a clear halo around bacterial growth following by a reappearance of opacity on the bottom of the *petri* dishes due to overoxidation of AcOH to CO_2 and H_2O. By colorimetric assay production of AcOH was shown by change of indicator from green to yellow and acetate assimilation by change from yellow to blue (Table 2). Amount of AcOH produced, tested using GYC at 2% of EtOH followed by periodical addition of wine, produced a gradual increase of AcOH until 68,26 ($\pm$0.26) g/L (Fig. 2). Typing by ERIC/PCR on gDNAs was performed on subcultures of STP (from 1 to 5) and LTP (AB0220-1, AB0220-2 and AB0220-3). The number of bands was ranging from 9 to 14 sizing from 2621 to 99 bp. Differences were mainly detected in bands intensity, in particular amplicons from 1000 to 200 bp of STP showed weaker bands respect to those of LTP. Macroscopically, the banding patterns appear very similar; this was confirmed by the dendrogram by which two major clusters were obtained. In particular, fingerprinting similarities showed that STP subcultures were most closely related to each other (97%); slightly lower similarities (96%) were observed within LTP cluster. The nearly complete 16S rRNA gene sequence of AB0220-3 was obtained (HE650905) and analyzed. Sequence showed 100% of similarity with *A. pasteurianus* (A. number GQ240636).

Discussion

Nowadays, there is a need to have cultivable and phenotipically stable AAB to be exploited as biocatalysts in a number of biotechnological applications. For AAB, the bottleneck is mainly due to the instability of key traits, such as: EtOH oxidation and AcOH resistance. Strain AB0220

(subculture AB0220-2) was successfully applied as selected starter culture, where it produced 60 g/L of AcOH [8]. In this study AcOH production and acetate assimilation, were assayed on AB0220-1 and AB0220-3 (1 year and 9 years of preservation, respectively) and on the current subculture of STP. By chalk-EtOH tests results were unambiguous concerning to EtOH oxidation, while the further oxidation of AcOH required 7 days for LTP subcultures and it taken more time for STP ones. Using bromocresol medium, oxidation of AcOH was detected after 72 hours for the LTP subcultures and about 168 hours for STP. The acetate assimilation is distinctive for members of AAB providing useful information on phenotypic stability [4, 3]. Metabolically it is caused by increasing activity of Krebs cycle enzyme and also by increased NADH oxidase activity, as a sophisticated strategy to survive in acetate stress environment [12]. Since both traits can be lost during preservation, they should be routinely tested. Although stability of phenotypic traits was generally confirmed, subcultures of STP required more time and some misinterpretation occurred (as in the case of acetate assimilation). Previous works stated that characteristics of AAB are strongly affected by the "*history*" of strains [15]. For instance, tolerance to EtOH and AcOH decreased when isolates have been used as inocula kept for long time in STP. Whereas high tolerance was observed for strains used immediately after the isolation. This inconsistency has been correlated to the genetic instability of strains. To clarify the mutability, *A. pasteurianus* (NBRC 3283), which forms a multiphenotype cell complex, was subjected to genome DNA sequencing. The genome analysis revealed more than 280 transposons and five genes with hyper-mutable tandem repeats as common features in the genome that consists of a 2.9-Mb chromosome and six plasmids which were recognised as characteristics leading to the hyper-mutability [2]. In this study, stability of subcultures related to the culture age and subcultures frequency was tested by ERIC/PCR. Fingerprintings were stable in term of size and number of bands but in the case of STP subcultures, bands intensity was weakly respect to LTP subcultures over repetition assays. Reasons could be explained by genetic damage and mutations as described for wild type cells and mutants of *E. coli* for which decrease of bands intensity or disappearance of bands on modified DNA occurred when alterations were higher than 2% [1]. In the

case of AB0220 we estimated that 10.01 generations per passage during STP and thus 540.54 generations occurred with the 9 years that could be responsible for mutations events inducing phenotypic instability. Although the high similarities (above 96%) detected by ERIC profiles clustering, the extended response to assays and the decreases of bands intensity observed could be related to the instability generated by the high number of generations occurred along 9 years of STP. Instead phenotypic and genotypic data on LTP subcultures, for which the number of generation occurred was 17.40, confirms the suitability of LTP by at least over a period of 9 years for AAB belonging to *A. pasteurianus* species from superficial acetification processes.

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Legend to figures

Fig. 1 Design of STP and LTP (a) and dendrogram generated by ERIC/PCR (b)

(a) Subcultures of LTP were named AB0220-1, AB0220-2 and AB0220-3; **(b)** STP-1, STP-2, STP-3 replicates of STP subculture transplanted on June 2011; STP-4 and STP-5 replicates of STP subculture transplanted in November 2011; 3509^T *A. pasteurianus* (DSMZ).

Fig. 2. Acetic acid production of AB0220-3 in wine

Different volume of wine was added in correspondence of arrows.

The data are averages based on three trials, and error bars indicate the standard deviation (SD).

Table 1 Phenotypic characteristic of AB0220

Feature	[a]AB0220
Shape	rod
Gram reaction	-
KOH reaction	+
Catalase reaction	+
Water-soluble pigments	-
Production of acetic acid	+
Oxidation of acetic acid to CO_2 and H_2O	+
Growth on:	
[b]Ethanol	-
[b]Methanol	-
Growth on culture media:	
GYC	+
AE	-
YPM	+
RAE	-
Frateur medium	+
Modified Passmore and Carr	+
Carr medium	+
[c]Cellulose production	-
Growth on D-glucose (%)	
20	+
25	+
30	-

[a]Assays were conducted on AB0220-1 and AB0220-3 subcultures
[b]DSMZ 11825^T (*A. pomorum*) was used as control
[c]DSMZ 2004 (*Ga. xylinus*) was used as control

Table 2 Oxidation of ethanol and acetic acid

Strain	Frateur medium			Modified Carr and Passmore medium			Carr medium		
	Growth	AcOH formation	AcOH assimilation	Growth	AcOH formation	AcOH assimilation	Growth	AcOH formation	AcOH assimilation
AB0220-3	24	24	120	24	24	96	24	24	72
[a]3509^T	48	72	168	48	72	168	24	24	72
[b]3503^T	48	48	-	48	48	-	24	24	-

[a]DSMZ 3509^T (*A. pasteurianus*);.
[b]DSMZ 3503^T (*Gluconobacter oxydans*)

Fig. 1 Design of STP and LTP (a) and dendrogram generated by ERIC/PCR (b)

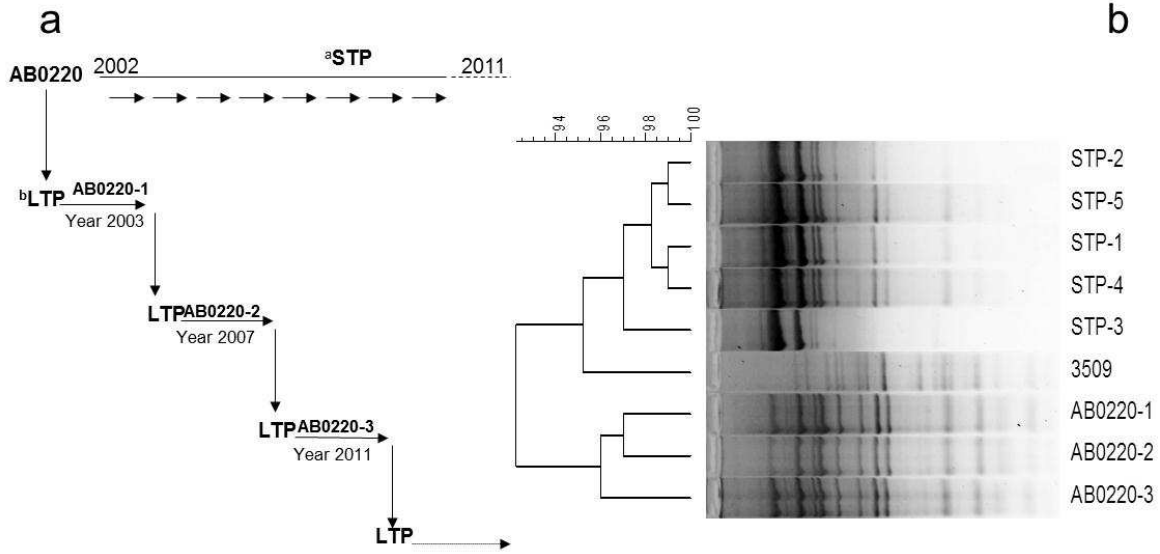

Fig. 2 Acetic acid production of AB0220-3 in wine