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ORIGINAL PAPER

The highly tolerant acetic acid bacterium *Gluconacetobacter europaeus* adapts to the presence of acetic acid by changes in lipid composition, morphological properties and PQQ-dependent ADH expression

Janja Trček · Katarina Jernejc · Kazunobu Matsushita

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Abstract The strain of acetic acid bacterium, *Gluconacetobacter europaeus* V3, previously isolated from industrial vinegar-producing bioreactor, tolerates extremely high acetic acid concentrations of up to 10% (v/v). Increased concentration of acetic acid changed the total fatty acid composition of cells by increasing the concentration of a major unsaturated fatty acid, the *cis*-vaccenic acid. Among the phospholipids, the most significant change was observed for phosphatidylglycerol with 7.3-fold increase and phosphatidylethanolamin with 2.7-fold decrease in the presence of 3% (v/v) of acetic acid. The sizes of cells analyzed with scanning electron microscopy changed from short to long rods in the presence of acetic acid. The cells were covered with spongy layer. The increase of acetic acid concentration from 1 to 2% (v/v) induced the expression of PQQ-dependent alcohol dehydrogenase, but the regulation could not be demonstrated at the transcriptional level. All together, our results suggest that *Ga. europaeus* activates several adaptive mechanisms to resist the stress of acetic acid.

Keywords Acetic acid bacteria · *Gluconacetobacter europaeus* · *Acetobacter* sp. · Acetic acid resistance · Lipid composition · Cell morphology · PQQ-dependent alcohol dehydrogenase

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J. Trček (✉)
Limnos, Podlimbarskega 31, 1000 Ljubljana, Slovenia
e-mail: janja.trcek@guest.arnes.si

J. Trček · K. Matsushita
Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, 753-8515 Yamaguchi, Japan

K. Jernejc
Department of Biotechnology, National Institute of Chemistry, Hajdrihova 19, 1000 Ljubljana, Slovenia

Introduction

Acetic acid is a well-known food preservative that inhibits growth of most microorganisms at concentrations as low as 0.5% (v/v) (Conner and Kotrola 1995). This is due to its uncoupling effect which disrupts the membrane proton gradient and causes acidification of the bacterial cytoplasm. Acetic acid bacteria (members of the family *Acetobacteraceae*) are the most prominent acetic-acid-resistant microorganisms (Sievers and Teuber 1995). However, among the ten currently recognized genera, substantial differences in tolerance to acetic acid exist, e.g. the genera *Asaia* and *Saccharibacter* do not tolerate acetic acid at all (Jojima et al. 2004; Yamada et al. 2000). The highest tolerance against acetic acid was so far reported for *Gluconacetobacter europaeus*, *Gluconacetobacter intermedius*, *Gluconacetobacter oboediens* and *Gluconacetobacter entanii* (Boesch et al. 1998; Schüller et al. 2000; Sievers and Teuber 1995; Sokollek et al. 1998). All these species were isolated from submerged industrial bioreactors with extremely high-acid concentrations (>10%, v/v). Other two species also involved in vinegar production, *Acetobacter aceti* and *Acetobacter pasteurianus*, are mainly used in traditional processes for vinegar production where the concentration of acetic acid does not exceed 6% (v/v).

The acetic acid bacteria are exposed to two different acid-resistance phases (Matsushita et al. 2005b): (a) the ethanol oxidation phase which is characterized by oxidation of ethanol to acetic acid and (b) the over-oxidation phase which is characterized by oxidation of acetic acid to

water and carbon dioxide. However, the over-oxidation occurs only in certain genera of acetic acid bacteria, i.e. *Acetobacter*, *Gluconacetobacter* and *Acidomonas* (Kersters et al. 2006; Sievers and Swings 2005).

The acetic-acid-resistant mechanisms were so far studied in *Acetobacter* spp. An over-expression of the genes encoding the enzymes of the tricarboxylic acid cycle was observed, suggesting their involvement in acetic acid consumption during over-oxidation phase (Fukaya et al. 1990; Nakano et al. 2004; Steiner and Sauer 2001). Recently, two types of transporters were described: a proton motive force-dependent efflux system (Matsushita et al. 2005a) and an ABC transporter (Nakano et al. 2006). These acetic acid efflux systems might be one of the main acetic-acid-resistance mechanisms during the ethanol oxidation phase. This phase is especially important for industrial vinegar production in contrast to the one during the catabolic conversion of acetic acid, which is of more ecological significance and probably favours the surviving of acetic acid bacteria in nature (Matsushita et al. 2005b).

Due to the proven unique high tolerance towards acetic acid, *Ga. europaeus* is an interesting subject for studying the adaptive mechanisms during vinegar production. The high acetic-acid-resistance during ethanol oxidation in high-acid containing industrial bioreactors represents one of the most appreciated technological characteristics. During this phase, the cells are exposed to two stressors (ethanol and acetic acid) at the same time and therefore it is difficult to separate the effect of acetic acid from the effect of ethanol. Besides, in the semi-continuous industrial vinegar production, each oxidizing cycle starts with a substrate, which already contains a substantial amount of acetic acid.

Recently, it was shown that *Ga. europaeus* when compared to *A. pasteurianus* exhibited a substantially reduced lag phase, an increased PQQ-dependent alcohol dehydrogenase (ADH) activity and a higher acetic acid stability of the purified enzyme (Trček et al. 2006). In the present study, we investigated the effect of acetic acid on lipid composition, morphological properties and ADH-expression in *Ga. europaeus* cells.

Materials and methods

Microorganisms

All strains (Table 1) used in this study were isolated from industrial vinegar bioreactors. Vinegars and the procedures of isolation were described previously (Trček et al. 2000, 2006). Under laboratory conditions, the strains were kept on a modified acetate-ethanol (AE) agar medium (Sievers and Teuber 1995) composed of 3 g/l yeast extract, 4 g/l peptone, 7.5 g/l glucose, 8 g/l agar, 3% (v/v) acetic acid and 3% (w/v) ethanol. Bacteria were grown at 30°C and 92–96% relative air humidity and were transferred onto fresh medium every 2 weeks. For long term preservation, strains were harvested at exponential growth phase from RAE broth (40 g/l glucose, 10 g/l peptone, 10 g/l yeast extract, 1.37 g/l citric acid and 3.38 g/l $Na_2HPO_4 \times 2H_20$), containing 1% (v/v) of acetic acid and 1% (w/v) of ethanol, re-suspended in 25% (v/v) glycerol and stored at –70°C.

Table 1 Strains used in this study and their maximum acetic acid tolerance

Strain	Concentration of acetic acid (w/v) (%)
Ga. europaeus V3 (LMG 18494)	10
Ga. europaeus JK2 (DSM 13109)	8
Ga. intermedius JK3 (DSM 13111)	6
A. pasteurianus (KKP 584)	6

DSM Deutsche Sammlung von Mikroorganismen und Zellkulturen; *LMG* Laboratorium voor Microbiologie, Gent; *KKP* Kolekcja Kultur Przemysłowych, Warsaw

Sample preparation

For fatty acids and lipids analysis, *Gluconacetobacter europaeus* V3 was grown in RAE broth (for composition, see above) without ethanol and acetic acid or with 1% (v/v) of acetic acid and 1% (w/v) of ethanol as appropriate for individual experiments. An amount of 500 µl culture with the density of around 70 Klett units (corresponding to $A_{600} = 0.5$) was used as an inoculum in 500 ml baffled flask containing 50 ml RAE broth with defined concentration of acetic acid and ethanol. In the case of lipids analysis, 6 ml of the culture was used as an inoculum for a bioprocess performed in 3 l baffled flask containing 600 ml RAE broth with defined concentration of acetic acid and ethanol. In both cases, the cells were rapidly harvested from the mid-exponential growth phase by centrifugation at 4°C and washed twice in buffer (Tris 50 mM, EDTA 1 mM, pH 8). About 10 mg and 2 g of wet biomass for FAME and lipids analysis, respectively, was harvested, lyophilized and kept at –20°C before the analysis. In all experiments, the flasks were incubated at 30°C and 200 rpm. The growth was monitored by measuring the optical density with a Klett–Summerson colorimeter using a red filter (640–700 nm). The acidity of the culture medium was measured with 1 N NaOH and phenolphthalein as indicator. The remaining glucose and ethanol concentration in medium were measured spectrophotometrically at 600 nm in reaction with glucose and alcohol dehydrogenases, respectively. The reactions were done with phenazine methosulfate (PMS) coupled with 2,6-dichlorophenol indophenol (DCIP) as an

artificial electron acceptor in potassium phosphate buffer (pH 7.0) (Ameyama 1982). High acid-containing samples were neutralized with 1 N NaOH before the analysis.

Extraction of lipids

Lyophilized cells of *Ga. europaeus* V3 were extracted with chloroform:methanol (2:1, v/v) according to Folch et al. (1957) and after the evaporation of solvents, the amount of total lipids was determined gravimetrically.

Separation of lipid classes

Total lipids were separated on silica columns (10 × 100 mm) to neutral lipids, glycolipids and phospholipids by elution with chloroform, acetone and methanol, respectively (Jernejc et al. 1989).

Separation of phospholipids

Individual phospholipids were separated by two dimensional thin layer chromatography (TLC) on Silicagel 60 TLC plates using chloroform:methanol:25% ammonia (65:35:5, v/v) as the first developing phase, and chloroform:aceton:methanol:acetic acid:water (50:20:10:10:5, v/v) as the second one. They were visualized by iodine vapours, scrapped off the TLC plate and quantified according to phosphorous content (Broekhuyse 1968).

FAME analysis

The total fatty acids analysed by gas chromatography as fatty acid methyl esters (FAMEs) were identified by Microbial ID, Inc. (Newark, DE) using MIDI Sherlock® system.

SEM analysis

Cells were grown in RAE medium containing the appropriate concentration of ethanol and acetic acid as described for sample preparation in the case of fatty acids analysis (see above). Biomass was harvested from the late logarithmic phase (determined from ethanol consumption) washed twice with 50 mM potassium phosphate buffer (KPB) pH 6.5 and re-suspended in 1 ml of 2.5% glutaraldehyde (in 50 mM KPB, pH 6.5). After 1 h of incubation at room temperature, the cells were three times washed with 50 mM KPB (pH 6.5), re-suspended in 1 ml of 50% ethanol and incubated for 15 min at room temperature. The cells were further dehydrated with 60, 70, 80, 90, 95, 99 and 100% of ethanol. The samples were transferred into filter pockets, and incubated in t-butylalcohol at 37°C twice for 1 h. The samples were dried by freeze-drying, mounted on metallic stubs and coated with gold by using an ion sputter apparatus. The samples were observed with scanning electron microscope (JEOL JSM 6100) operating at 10 kV.

Expression analysis of PQQ-dependent ADH

Cells were harvested from AE medium by centrifugation at 6,500 rpm for 10 min and washed twice with ice-cold 50 mM potassium phosphate buffer (pH 5.8). The washed cells were re-suspended at 0.1 g of wet weight per 3 ml in the same buffer and passed twice through a French pressure cell at 15,000 lb/in^2. The cell debris and the remaining intact cells were removed by centrifugation at 9,000 rpm for 10 min and the obtained supernatant was centrifuged at 40,000 rpm for 90 min to separate the soluble fraction from the membrane fraction. The membrane fraction was homogenized with 10 mM potassium phosphate buffer (pH 6.0) and the proteins were separated in SDS-PAGE without reducing agent. The amount of alcohol dehydrogenase (ADH) produced was visualized by tetramethylbenzidine staining of heme-associated peroxidase activity as previously described (Thomas et al. 1976). The ferricyanide reductase activity of ADH was measured colorimetrically with potassium ferricyanide as an electron acceptor at 660 nm (Ameyama 1982). The reaction mixture containing the enzyme fraction, ethanol and potassium ferricyanide was prepared in McIlvaine buffer pH 4.0. One unite of enzyme activity was defined as the amount of enzyme catalyzing the oxidation of 1 μmol of ethanol in 1 min. The protein contents were measured by a modified Lowry's method (Dulley and Grieve 1975) with bovine serum albumin as a standard protein. Errors were calculated as standard deviations.

Results

Effect of acetic acid on lipid composition of *Gluconacetobacter europaeus* V3

The amount of total lipids decreased in the presence of acetic acid (3%, v/v) for about 14% in comparison to the control strain grown in the medium with ethanol (3%, v/v) but without acetic acid (data not shown). The major class of lipids in cells harvested from all three types of growth medium was phospholipids (Fig. 1). In medium with 3% (v/v) of acetic acid, the most significant difference was observed in the content of glycolipids, which increased from 8.8 to 14.7% of the total lipid content (Fig. 1). The relative content of neutral lipids increased from 24.8 to 31.7% and phospholipids decreased from 66.4 to 53.6% (Fig. 1).

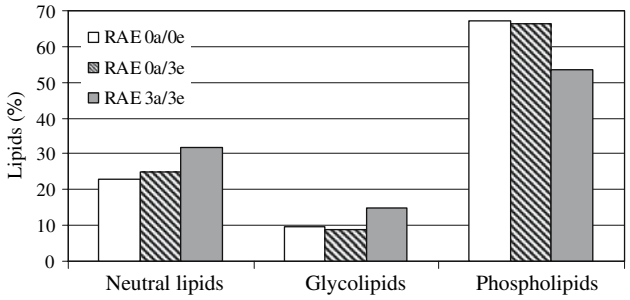


Fig. 1 Amount of neutral lipids, glycolipids and phospholipids in total lipids fraction extracted from cells of *Ga. europaeus* V3 grown in RAE medium containing no ethanol and no acetic acid (RAE 0a/0e), 3% (v/v) of ethanol (RAE 0a/3e), and 3% (v/v) of ethanol and 3% (v/v) of acetic acid (RAE 3a/3e)

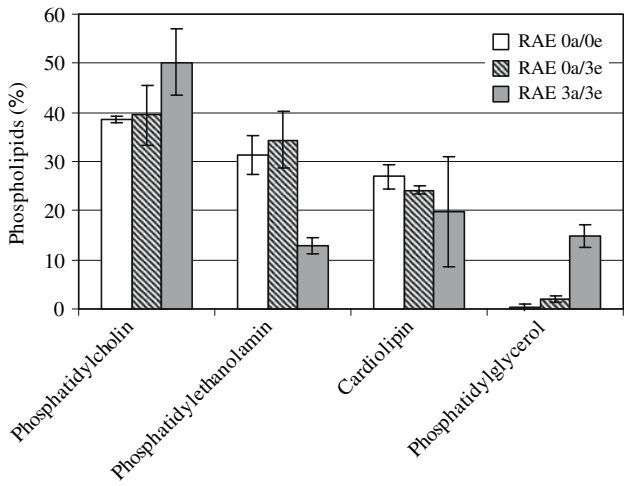


Fig. 2 Relative percentage of major phospholipids in cells of *Ga. europaeus* V3 grown in RAE medium containing no ethanol and no acetic acid (RAE 0a/0e), 3% (v/v) of ethanol (RAE 0a/3e), and 3% (v/v) of ethanol and 3% (v/v) of acetic acid (RAE 3a/3e)

Major phospholipids in *Ga. europaeus* V3 were cardiolipin (CL), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylcholine (PC) while phosphatidylinositol (PI), phosphatidylserine (PS) and phosphatidic acid (PA) were only present in amounts below 0.5%. The presence of 3% (v/v) of acetic acid most significantly affected the PG content, which increased 7.3-fold, while PE content decreased 2.7-fold (Fig. 2). The PC content also increased 1.3-fold in the presence of 3% (v/v) of acetic acid (Fig. 2).

The major fatty acids in total lipids of strain *Ga. europaeus* V3 were *cis*-vaccenic (C18:1w7c), palmitic (C16:0), 2-hydroxy palmitic (C16:0 2OH) and 2-hydroxy myristic acid (C14:0 2OH) (Fig. 3). Myristic acid (C14:0), 3-hydroxy palmitic acid (C16:0 3OH) and stearic acid (C18:0) were minor components detected in the range between 0.9 and 4.2%. No branched-chain or cyclopropane fatty acids were found. Among the major fatty acids, the most pronounced effect of acetic acid was noticed for *cis*-vaccenic acid which increased significantly (Fig. 3).

Effect of acetic acid on cell morphology

Comparison of the cell morphology between the cells of *Ga. europaeus* V3 and *Acetobacter pasteurianus* KKP 584 showed differences in size and in cell surface (Fig. 4). Both species formed small rods in RAE medium without acetic acid and ethanol although *Ga. europaeus* V3 formed cells of irregular shape and covered with spongy material. In contrast to the cells of *Ga. europaeus* V3, the cells of strain *A. pasteurianus* KKP 584 formed a very smooth surface. When strain *A. pasteurianus* KKP 584 grew in RAE medium with ethanol and acetic acid, the topology did not change in contrast to *Ga. europaeus* V3 which formed longer rods with pits in their outer membrane or surface (Fig. 4). With increased amount of acetic acid (7%) in RAE medium, an increased number of longer and thinner rods was observed.

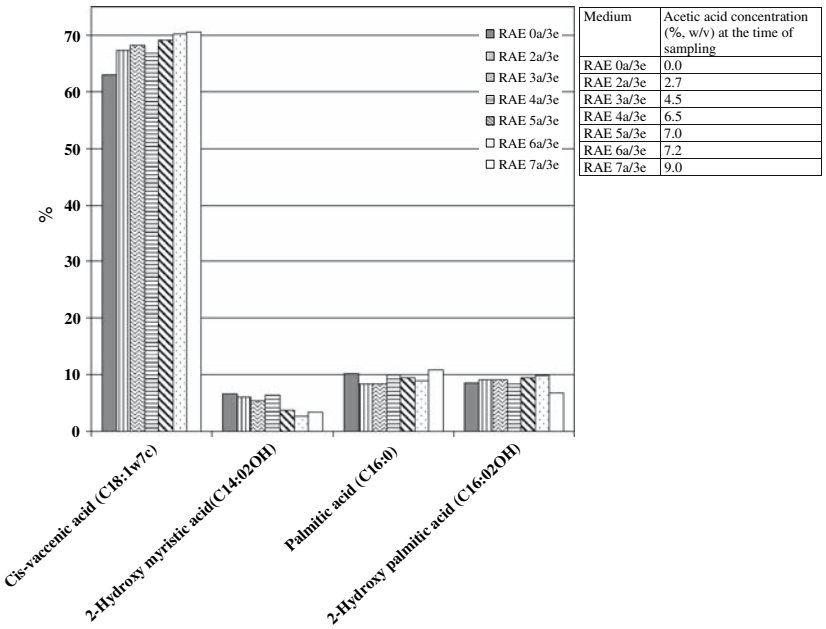
Effect of acetic acid on expression of PQQ-dependent ADH

By increasing the acetic acid concentration from 1% to 3% (v/v) and at constant ethanol concentration of 1% (v/v), the expression of PQQ-dependent alcohol dehydrogenase (ADH) in *Ga. europaeus* V3 was followed by tetramethylbenzidine staining analysis as well as by measuring the enzyme activity. The following control strains were used: *Gluconacetobacter europaeus* JK2, *Gluconacetobacter intermedius* JK3 and *A. pasteurianus* KKP 584. In all the analysed strains, the ADH activity substantially increased after raising the concentration of acetic acid in the growth medium from 1 to 2% (Fig. 5). However, when the acetic acid concentration was further raised to 3%, a decrease of ADH-activity in comparison to ADH-activities in medium with 2% of acetic acid was observed. Besides, the ADH-activities of *Gluconacetobacter* spp. were in all cases higher than the ADH-activity of *A. pasteurianus* KKP 584. Performing the RT-PCR with primers targeting *adhA* and also the *gyrase B* as a control gene, the regulation of ADH expression could not be demonstrated at the transcriptional level (data not shown).

Discussion

Acetic acid influences the phospholipids composition in *Gluconacetobacter europaeus* V3

Changes in the membrane composition are generally recognized as an adaptive response of microbial cells to toxic



Medium	Acetic acid concentration (%, w/v) at the time of sampling
RAE 0a/3e	0.0
RAE 2a/3e	2.7
RAE 3a/3e	4.5
RAE 4a/3e	6.5
RAE 5a/3e	7.0
RAE 6a/3e	7.2
RAE 7a/3e	9.0

Fig. 3 Relative percentage of major fatty acids in cells of *Ga. europaeus* V3 grown in RAE medium containing 3% of ethanol (3e) and 0–7% of acetic acid (3a to 7a)

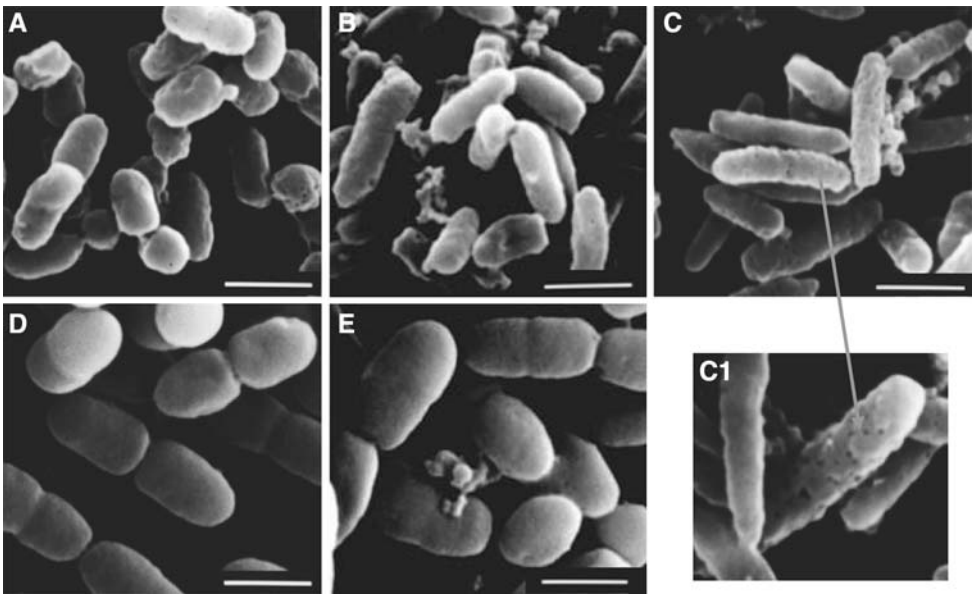


Fig. 4 Scanning electron micrograph of *Ga. europaeus* V3 grown in RAE medium without acetic acid and ethanol (A), with 3% (v/v) of ethanol and 3% (v/v) of acetic acid (B), and with 3% (v/v) of ethanol and 7% (v/v) of acetic acid (C). Photograph C1 represents magnification of the cells presented in photograph C. The *panel below* shows cells of *A. pasteurianus* KKP 584 grown in RAE medium without acetic acid and ethanol (D) and in RAE medium with 3% (v/v) of ethanol and 3% (v/v) of acetic acid (E). The *bar indicates* the length of 1 μm

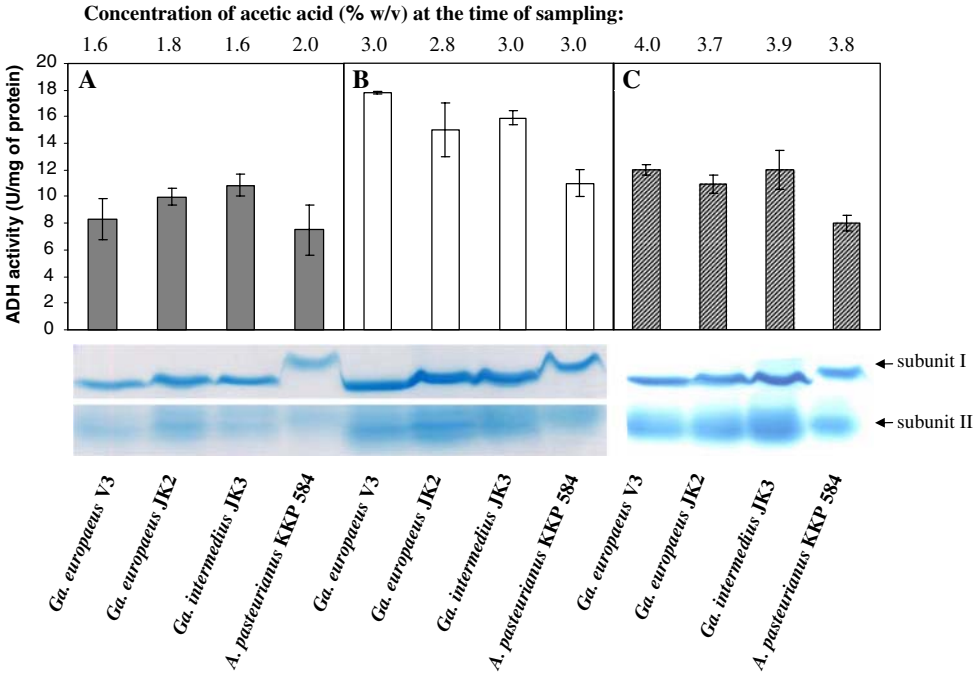


Fig. 5 Comparison of the amount and activity of alcohol dehydrogenase (ADH) produced in the membranes of *Ga. europaeus* V3, *Ga. europaeus* JK2, *Ga. intermedius* JK3 and *A. pasteurianus* KKP 584. The amount of ADH produced was visualized by tetramethylbenzidine staining of heme-associated peroxidase activity in SDS-PAGE. Each lane contains 60 µg of protein. Starting amount of acetic acid in AE medium was 1% (*A*), 2% (*B*) and 3% (*C*)

compound in the environment (Denich et al. 2003). In the case of acetic acid, the protonated, uncharged form of acid easily penetrates the microbial membrane and dissociates inside the cell at the higher pH of the bacterial cytoplasm (Russell and Diez-Gonzalez 1998). The products, particularly the released H^+, but also CH_3COO^-, are toxic for the cell (Russell 1992). During vinegar production, cells are not only exposed to acetic acid present already as one of the components in the growth medium, but additionally to the acetic acid produced from ethanol. The oxidation process is performed by enzymes bound to the outer surface of the inner cytoplasmic membrane. The produced acetic acid passively diffuses into the cytoplasm and is probably transported out of the cell by an efflux pump (Matsushita et al. 2005a). Next to this efflux system(s), the cell must additionally adapt to the high concentrations of acetic acid in its membrane and additionally prevent the diffusion of acetic acid into the cytoplasm. Since phospholipids are an essential component of the cell membrane by regulating the membrane permeability, being a matrix for membrane proteins and also acting as regulatory molecules (Cronan 2003), we were interested to know whether phospholipid composition changed as a response to acetic acid. The major phospholipid in the strain *Ga. europaeus* V3 was PC what corresponded to the results obtained with some other strains of the acetic acid bacteria representing the genera *Acetobacer*, *Gluconobacter* and *Gluconacetobacter* (Hanada et al. 2001; Tahara et al. 1976). This phospholipid is usually produced by methylation of PE (Kaneshiro and Law 1964), the biochemical route which was demonstrated also in *Acetobacter* sp. (Hanada et al. 2001). The increase of PC with concomitant decrease of PE is thus an expected effect. The other major phospholipid in *Ga. europaeus* V3 cultivated in the presence of acetic acid was PG. The PG is among the major anionic phospholipids and as such it is not involved only in cell membrane integrity but also in other cell processes. For example, in vivo evidence exists for its involvement in the initiation of DNA replication in *Escherichia coli* (Xia and Dowhan 1995) and also in ATP-dependent protein translocation across the inner membrane of *E. coli* (Lill et al. 1990; Hendrick and Wickner 1991). Recently, two different types of acetic acid efflux pump have been proposed for *Acetobacter* sp. (Matsushita et al. 2005a; Nakano et al. 2006), and we can speculate that PG might be also involved in transport across the membrane in *Ga. europaeus*. Further studies on the molecular-biological levels should elucidate the exact involvement of PG in tolerance to acetic acid in *Ga. europaeus*.

In the cells of *Ga. europaeus* V3 grown in the presence of acetic acid, the relative content of phospholipids substantially decreased, which might decrease the area available for a passive transport of lypophilic molecules such as acetic acid. Similar results were demonstrated also for ethanol-grown *E. coli* cells which contained roughly half the phospholipids of control membranes resulting in a decrease of phospholipids/protein ration and in more rigid membranes (Dombek and Ingram 1984). The content of nonpolar glycolipids increased in cells of *Ga. europaeus* V3 grown in the presence of acetic acid (3%, v/v), which might result in strengthening the cell's hydrophobic barrier.

Acetic acid influences the fatty acid composition in *Gluconacetobacter europaeus* V3

The cells of *Ga. europaeus* V3 show a very high content of unsaturated fatty acids with *cis*-vaccenic acid as major component. The cells are additionally enriched in *cis*-vaccenic acid as a result of acetic acid addition or production. *Cis*-vaccenic acid represents a major fatty acid (31–80%) in almost all genera of acetic acid bacteria (Franke et al. 1999; Greenberg et al. 2006; Jojima et al. 2004; Loganathan and Nair 2004; Urakami and Komagata 1987; Yamada et al. 1981) with the exception of the genus *Saccharibacter* with 2 hydroxy palmitic acid as a predominant fatty acid (Jojima et al. 2004). Interestingly, this genus also does not tolerate as low as 0.35% of acetic acid (Jojima et al. 2004).

In bacteria, unsaturated fatty acids are produced either by anaerobic or aerobic pathway (Fulco 1974; Keweloh and Heipieper 1996; Magnuson et al. 1993). The presence of *cis*-vaccenic acid, a characteristic fatty acid for anaerobic pathway, suggests that acetic acid bacteria produce fatty acids mainly by oxygen independent pathway.

The increase of unsaturated fatty acids (*cis*-vaccenic acid) leading to a more fluid membrane is an unexpected reaction to the presence of acetic acid, since its accumulation presumably fluidises the cell membrane (Sinensky 1974). The same kind of effect was described also for *E. coli* exposed to ethanol, although the toxic effect of ethanol differs from that of acetic acid (Dombek and Ingram 1984; Ingram 1990).

The study of Tahara et al. (1976) showed that the *cis*-vaccenic acid in *Gluconobacter cerinus* represents 80% of all fatty acids in PG. The increase of *cis*-vaccenic acid in *Ga. europaeus* V3 growing in the presence of acetic acid might thus be an effect of the substantial increase of relative PG content.

Acetic acid changes the cell morphology in *Gluconacetobacter europaeus* V3

Changes in the cell morphology as a response to toxic solvents have been recently reported for the cells of *Pseudomonas putida* and *Enterobacter* sp. (Neumann et al. 2005; Veeranagouda et al. 2005). The cells of *Ga. europaeus* responded in a similar way: acetic acid caused an increase in cell size and thus the reduction of the relative area responsible for the passive diffusion of acetic acid into a cell. In contrast to *Ga. europaeus*, the cells of *A. pasteurianus* did not change the morphology after the exposure to acetic acid. The spongy material which covers the surface of *Ga. europaeus* might represent the extensions or remnants of the outer membrane albeit changes in the membrane structure may be due to sample preparation and may not accurately represent the true native structure.

Acetic acid induces expression of PQQ-dependent ADH

We have already reported that the PQQ-dependent alcohol dehydrogenase (ADH) activity of *Ga. europaeus* V3 and *Ga. intermedius* JK3 reached at least twice as high values as that of *A. pasteurianus* KKP 584 and the purified enzymes had similar activities but were expressed at different levels (Trček et al. 2006). The results of this study additionally showed that the acetic acid (2%, v/v) induced expression of PQQ-dependent ADH in all analysed strains of acetic bacteria but the differences in the expression could not be demonstrated at the transcriptional level suggesting the regulation of PQQ-dependent ADH-expression in acetic acid bacteria at the posttranscriptional level.

Concluding remarks

Besides our recent study on correlation between acetic acid resistance and the characteristics of PQQ-dependent ADH (Trček et al. 2006), this is the only study toward understanding the acetic acid resistance in *Ga. europaeus* species. We have shown that the acetic acid changes total fatty acid composition of *Ga. europaeus* V3 by increasing the content of *cis*-vaccenic acid. In addition, a significant increase in phosphatidylglycerol was observed. The acetic acid caused also an increase in cell length and influenced the expression level of PQQ-dependent ADH in *Ga. europaeus* V3. Further studies on a wider range of *Ga. europaeus* strains are necessary in order to find out whether these characteristics are strain or species specific or perhaps general characteristics of the genus *Gluconacetobacter*.

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