doi: 10.17113/ftb.54.01.16.4041

16S rRNA *in situ* Hybridization Followed by Flow Cytometry for Rapid Identification of Acetic Acid Bacteria Involved in Submerged Industrial Vinegar Production

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Received: December 20, 2014
Accepted: October 13, 2015

Summary

Acetic acid bacteria are involved in many biotechnological processes such as vitamin C, gluconic acid, miglitol or acetic acid production, and others. For a technologist trying to control the industrial process, the ability to follow the microbiological development of the process is thus of importance. During the past few years hybridization in a combination with flow cytometry has often been used for this purpose. Since vinegar is a liquid, it is an ideal matrix for flow cytometry analysis. In this work we have constructed a specific probe for highly acetic acid-resistant species of the acetic acid bacteria and a protocol for *in situ* hybridization, which in combination with flow cytometry enables direct monitoring of bacteria producing vinegar with >10 % of acetic acid. The approach was successfully applied for monitoring microbiota during industrial vinegar production.

Key words: microbiota, vinegar, acetic acid bacteria, *Acetobacter*, *Gluconobacter*, *Gluconacetobacter*, *Komagataeibacter*, 16S rRNA probe, *in situ* hybridization, flow cytometry

Introduction

Acetic acid bacteria are involved in various biotechnological processes, among them the most traditional being the acetic acid production during making of different types of vinegar (*1*). In some countries, this process has been traditionally performed in wooden vessels with naturally present microbiota that has been identified as *Acetobacter aceti* and/or *Acetobacter pasteurianus* (*2*). In contrast to this process, a quick submerged bioprocess was developed in 1950s, with two main benefits: very short oxidation cycle and the production of vinegar with very high acetic acid volume fraction (>10 %) (*3*). In the extreme conditions of such bioprocesses only very well adapted strains of acetic acid bacteria survive, which according to the present knowledge belong to the following species: *Komagataeibacter europaeus*, *Komagataeibacter intermedius*, *Komagataeibacter oboediens* and *Gluconacetobacter entanii* (*4–10*). A typical characteristic of these species is a resistance to high percentage of acetic acid in contrast to the species of *Acetobacter* genus (*7*). Species of the genus *Acetobacter* may cause a delay of the oxidation cycle in submerged bioprocess. Therefore, the ability to follow the highly productive and highly acetic acid-resistant bacteria during industrial vinegar production is of importance.

Vinegar is an ideal matrix for flow cytometry analysis of the microbiota dispersed in liquid. We have already ap-

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plied this technique for enumeration of bacteria in vinegar, the approach which showed that the majority of the acetic acid bacteria in the submerged bioprocess are not in the cultivable state under the presently known *in vitro* growth conditions (*8*). In this report we extend the flow cytometry approach for monitoring of specific bacteria, *K. europaeus*, *K. intermedius* and *K. oboediens* during the production of vinegar with high acetic acid percentage.

Materials and Methods

Construction of specific DNA probe

A specific oligonucleotide Komag (5′-GAACCTTTC-GGGGTTAGTG-3′, position on 16S rDNA: 70–88 nt, numbered according to *Komagataeibacter medellinensis*, acc. no. NC_016027, locus GLX_r0010) was constructed by comparing all available 16S rRNA gene sequences of acetic acid bacteria, available through National Center for Biotechnology Information (NCBI), consisting of GenBank/EMBL/DDBJ databases. The specificity of the oligonucleotide was tested in a standard PCR reaction in combination with the oligonucleotide EUB338rev (5′-GCTGCCTC-CCGTAGGAGT-3′) using the following PCR amplification conditions: initial denaturation of DNA at 94 °C for 3 min, 30 cycles at 94 °C for 30 s, at 68 °C for 30 s and at 72 °C for 30 s, and a final extension at 72 °C for 7 min followed by cooling at 4 °C. The amplification was performed in a TProfessional Basic Cycler (Biometra, Göttingen, Germany) in 20-μL reaction mixture containing 10 ng of DNA, 2.5 mM of $MgCl_2$, 20 pmol of each primer, 0.5 U of *Taq* DNA polymerase (Thermo Fisher Scientific, Waltham, MA, USA), 0.2 mM of dNTP (Thermo Fisher Scientific) and 2 μL of 10× *Taq* buffer (Thermo Fisher Scientific). The specific PCR product is 247 bp in length. Based on the sequence of primer Komag, a DNA probe Komag-fluorescein isothiocyanate (FITC) (5′-CACTAACCCCGAAAGGTTC-3′) was constructed. All primers and a DNA probe were provided by MWG Genomics (Munich, Germany). The specificity of the probe Komag-FITC was tested *in silico* using the RDP probe match (*11*) and also practically with reference strains (Table 1) that were obtained from the BCCM/LMG (Belgian Coordinated Collections of Microorganisms, Brussels, Belgium) culture collection, DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) culture collection and Sugar Research Institute (SRI, Mackay, Australia).

In situ hybridization

A protocol developed by Lipoglavšek and Avguštin (*12*) for *in situ* hybridization of ruminal samples was optimized for acetic acid bacteria as follows. The bacteria were grown in liquid reinforced acetic acid and ethanol (RAE) medium (*13*) in glass tubes at 30 °C by shaking in 10-litre benchtop shaking incubator (Domel, Železniki, Slovenia) at 15×*g* until logarithmic growth phase was achieved ($A_{600\,nm}$=0.6–1.0). Then the biomass was harvested in a centrifuge Centric 400R (Domel) at 2000×*g* and 4 °C for 10 min. The biomass was washed with phosphate-buffered saline (PBS, pH=7.4) and fixed in 900 μL of 4 % (by mass per volume) paraformaldehyde at 4 °C for 12 h. Subsequently, the cells were washed with PBS buffer (pH=7.4) and suspended in 500 μL of ethanol (96 %) and 500 μL of PBS buffer (pH=7.4). If the hybridization did not proceed immediately, the samples were stored at –20 °C. The fixed cells (50 μL) were washed and suspended in 12 μL of PBS buffer (pH=7.4). A volume of 100 μL of hybridization buf-

Table 1. List of strains used for the analysis of the specificity of probe Komag-FITC. As a control, a probe EUB338-FITC was applied

Strain designation	Signal with the probes	
	EUB338-FITC	Komag-FITC
Acetobacter aceti LMG 1261^T	+	–
Acetobacter pasteurianus LMG 1262^T	+	–
Acetobacter pasteurianus LMG 1607	+	–
Acetobacter pomorum LMG 18848^T	+	–
Acetobacter cerevisiae LMG 1625^T	+	–
Acetobacter cibinongensis LMG 21418^T	+	–
Acetobacter estunensis LMG 1626^T	+	–
Acetobacter malorum LMG 1746^T	+	–
Acetobacter oeni LMG 21952^T	+	–
Acetobacter orleanensis LMG 1583^T	+	–
Acetobacter peroxydans LMG 1635^T	+	–
Acetobacter tropicalis LMG19825^T	+	–
Acetobacter syzygii LMG 21419^T	+	+
Acetobacter lovaniensis LMG 1579^T	+	+
Acetobacter fabarum LMG 24244^T	+	+
Acetobacter ghanensis LMG 23848^T	+	+
Acetobacter okinawensis LMG 26457^T	+	+
Komagataeibacter europaeus DSM 6160^T	+	+
Komagataeibacter europaeus LMG 18494	+	+
Komagataeibacter europaeus DSM 13109	+	+
Komagataeibacter europaeus LMG 1510	+	+
Komagataeibacter hansenii DSM 5602	+	+
Komagataeibacter intermedius LMG 18809^T	+	+
Komagataeibacter nataicola LMG 1536^T	+	+
Komagataeibacter oboediens LMG 18849^T	+	+
Komagataeibacter oboediens LMG 1517	+	+
Komagataeibacter oboedines DSM 13111	+	+
Komagataeibacter saccharivorans LMG 1582^T	+	+
Komagataeibacter sucrofermentans LMG 18788^T	+	+
Komagataeibacter swingsii LMG 22125^T	+	+
Komagataeibacter xylinus LMG 1515^T	+	+
Komagataeibacter maltaceti LMG 1529^T	+	+
Komagataeibacter medellinensis LMG 1693^T	+	+
Gluconacetobacter liquefaciens LMG 1381^T	+	+
Gluconacetobacter liquefaciens SRI 244	+	+
Gluconacetobacter liquefaciens SRI 1957	+	+
Gluconacetobacter sacchari SRI 1794^T	+	+
Gluconobacter cerinus^T DSM 9533^T	+	–
Gluconobacter oxydans LMG 1356^T	+	–
Gluconobacter oxydans DSM 50049	+	–
Gluconobacter frateurii LMG 1365	+	–

+=positive signal, –=no signal

fer (0.01 % SDS, 20mM Tris-HCl, 0.9 M NaCl, pH=7.4) and 2 μL of the FITC-labelled probe (25 pmol/μL) (MWG Genomics) were added to the cells and hybridized at 54 °C for 12 h. This hybridization temperature was selected because the results showed that it enabled differentiation among the highest number of target species of the genus *Komagataeibacter* and the non-targeted species of genera *Acetobacter*, *Gluconacetobacter* and *Gluconobacter*. Unbound probe was washed away with 500 μL of PBS buffer (pH=8.0).

Flow cytometry analysis

Cells were stained with 1 μg/mL of Trypan Blue (Thermo Fisher Scientific) in PBS buffer (pH=8.0) for 10 min. After centrifugation (6000×*g*, 10 min, 4 °C) the cells were suspended in 700 μL of PBS buffer (pH=8.0) and analyzed by FACScan flow cytometer (Becton Dickinson, Heidelberg, Germany). Prior to analysis, the samples were diluted to the concentration of approx. 1000 cells per mL, which was measured by flow cytometer in one second. The measurements were performed by CELLQuest v. 3.1 software at Power Macintosh 7300 (Becton Dickinson). The signals were logarithmically amplified. Cells were differentiated from noise according to Trypan Blue staining and gates for all cells were created on red fluorescence (FL3-H)-side scatter (SSC-H) dot plot.

Results and Discussion

Monitoring the microbiological status in industrial vinegar production is hampered by difficulties in growing under laboratory conditions the strains of acetic acid bacteria responsible for the production of vinegar with high percentage of acetic acid. Although a progress in growing these strains has been made with description of a double-layer acetic acid and ethanol (AE) agar medium (*14*) and also RAE agar medium (*13*), the major part of the bacterial microbiota for industrial vinegar production remains currently in nonculturable form (*15*,*16*). Moreover, if better cultivation conditions or techniques were known, a problem of time length in growing of the acetic acid bacteria would remain. On the other hand, identification of acetic acid bacteria has been well established over the past few years by using 16S–23S rRNA gene ITS regions as a target for species identification (*8*,*15*). To overcome the critical point of isolation of bacteria from different matrices, a fluorescence *in situ* hybridization (FISH) with specific DNA probes has been proven to be a suitable approach for identification of diverse microbiota (*17*–*22*). Additionally, a combination of FISH technique with flow cytometry has often been used for identification of food microbiota, especially those originating from liquid matrices (*23*,*24*). Therefore, we aimed in this report to develop a specific DNA probe for targeting strains for production of vinegar with high percentage of acetic acid from real industrial matrix.

Since the 16S rRNA gene sequences of the acetic acid bacteria show very high similarity to each other, it was impossible to construct 100 % genus- or species-specific probe. We constructed a probe that is partially specific for genus *Komagataeibacter*: the probe Komag-FITC is useful for differentiation between the species of acetic acid bacteria with very high acetic acid resistance (genus *Komagataeibacter*) and the species of the genus *Acetobacter*, *Gluconacetobacter* and *Gluconobacter* on the other hand. The exceptions are species *A. lovaniensis*, *A. syzygii*, *A. ghanensis*, *A. fabarum* and *A. okinawensis*, which also bind the probe Komag-FITC but have not been identified in wine or apple cider vinegar, which are the predominant vinegars on the European market. The probe also targets another genus of the acetic acid bacteria, *i.e. Saccharibacter*, but this genus does not resist as low as 0.35 % of acetic acid and is thus not present in vinegar (*25*). The species of the genus *Acetobacter* typically show low acetic acid resistance, the feature which is not appreciated in submerged bioprocess during production of vinegar with high percentage of acetic acid (*7*).

To improve the fluorescent signal intensity a helper probe was used. This additional unlabelled oligonucleotide opened the rRNA structure and improved the accessibility of the rRNA molecule for the probe by binding upstream or/and downstream of the binding site of the labelled probe (*26*). Indeed, concomitant application of the FITC-labelled probe Komag-FITC and helper oligonucleotide Komag-up1 (5′-CGTTACTCACCCGTCCGC-3′), binding just upstream in a close proximity of the labelled probe, approximately doubled the intensity of the signal (green fluorescence, FL1-H; Fig. 1). As the next step we mixed pure cultures of *Acetobacter aceti* and *Komagataeibacter europaeus* and proceeded with the above described protocol in order to show that *in situ* hybridization in combination with flow cytometry successfully differentiated both genera of the acetic acid bacteria (Fig. 2). The established protocol was then applied on alcohol and wine vinegar samples from industrial settings (Fig. 3). Concomitantly with the FITC analysis in combination with flow cytometry, the samples were used for direct identification of microbiota using the restriction analysis of the PCR-amplified 16S–23S rRNA gene as described before (*8*). This approach proved that in both types of vinegar the microbiota is homogenous and composed of a single species (data not shown). However, as noticed in Fig. 3, the applied protocol did not successfully label the

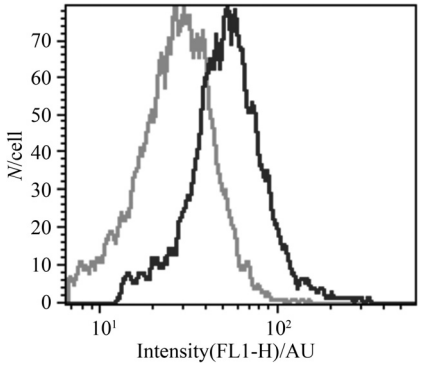

Fig. 1. Improvement of signal intensity of probe Komag-FITC with concomitant binding of the helper oligonucleotide. The analysis was performed with a pure culture of *Komagataeibacter europaeus*. Light grey line represents signal intensity of the labelled probe Komag-FITC and the dark grey line the signal intensity of the labelled probe Komag-FITC with bound helper oligonucleotide Komag-up1. FL1-H=green fluorescence

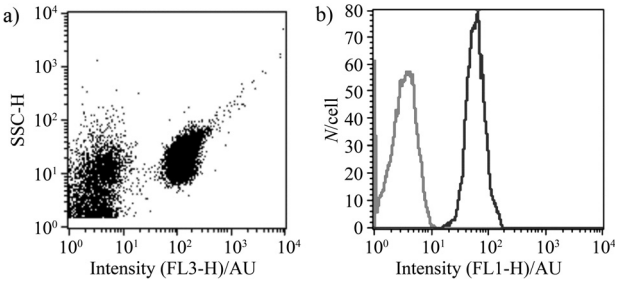

Fig. 2. Flow cytometry analysis of cells (*Acetobacter* and *Komagataeibacter*) after: a) treatment with Trypan Blue, and b) hybridization with probe Komag-FITC. Light grey line represents cells of *Acetobacter aceti*, dark grey line the cells of *Komagataeibacter europaeus*. FL1-H=green fluorescence, FL3-H=red fluorescence, SSC-H=side scatter

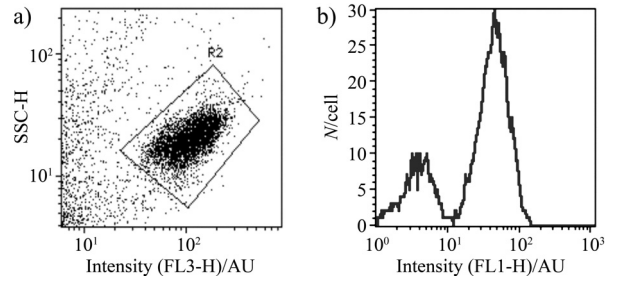

Fig. 3. Direct analysis of bacterial population from alcohol vinegar (10 %). Flow cytometric analysis of cells after treatment with: a) Trypan Blue, and b) after hybridization with probe Komag-FITC. FL1-H=green fluorescence, FL3-H=red fluorescence, SSC-H=side scatter

entire bacterial population, suggesting a technical problem that has to be improved in the future. Anyway, the data presented in this study suggest that the FITC analysis in combination with flow cytometry is a promising approach for monitoring the microbiota involved in industrial vinegar production by submerged culture.

Conclusions

In this work we established a protocol based on *in situ* hybridization in a combination with flow cytometry for quick following of high acetic acid resistance strains of the acetic acid bacteria responsible for an efficient industrial bioprocess of vinegar production. Because of high similarities among species of the genera *Acetobacter*, *Gluconacetobacter* and *Komagataeibacter*, a construction of a specific probe for a single genus was not possible. Nevertheless, the probe Komag-FITC binds to species *Komagataeibacter europaeus*, *Komagataeibacter oboediens* and *Komagataeibacter intermedius*, all typical species for industrial submerged processes for vinegar production with more than 6 % of acetic acid, but not to *Acetobacter aceti* and *Acetobacter pasteurianus*, which are typical species for production of vinegar with less than 6 % of acetic acid. The protocol for *in situ* hybridization followed by flow cytometry analysis enables a convenient microbiological monitoring of vinegar. Because of high 16S rRNA gene sequence similarities among species of the genera *Acetobacter*, *Gluconacetobacter* and *Komagataeibacter*, other target molecules should be tested in the future.

Acknowledgements

This work was supported by the Slovenian Research Agency of Science, and Food Industry Droga Kolinska (Atlantic Grupa d.d.), Ljubljana, Slovenia. We acknowledge the valuable advice of Mrs. Dunja Trebar.

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