Cell Surface Characteristic of *Asaia bogorensis* – Spoilage Microorganism of Bottled Water

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Abstract

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The ability of bacteria to attach to a surface and develop a biofilm has been of considerable interest for many groups in the food industry. Biofilms may serve as a chronic source of microbial contamination and the research into biofilms and cells interactions might help to improve general understanding of the biofilm resistance mechanisms. Multitude of factors, including surface conditioning, surface charge and roughness and hydrophobicity, are thought to be involved in the initial attachment. Hydrophobic interactions have been widely suggested as responsible for much of the adherence of cells to surfaces. Cell-surface hydrophobicity is an important factor in the adherence and subsequent proliferation of microorganisms on solid surfaces and at interfaces. In the present study, we have estimated the cell-surface characteristics of *Asaia bogorensis* – isolated contamination of flavoured bottled water and compared its ability to colonise surfaces which are typical in the beverage production – stainless steel, glass and plastic materials.

Keywords: hydrophobicity; hydrocarbon; adherence; *Asaia*; MATH

A Gram-negative microorganism *Asaia bogorensis* was isolated from a batch of fruit-flavoured bottled water, which was spoilt as a result of bacterial overgrowth. The spoilage isolate belonged to the recently described genus which is a member of the acetic acid bacteria (Katsura & Kawasaki 2001; Moore *et al*. 2002; Tuuminen *et al*. 2006; Yamada & Yukphan 2008; Horsakova *et al*. 2009).

It has been supposed that *Asaia bogorensis* displays a broad spectrum of resistance to antimicrobial agents because the isolate of the bacterium (from polyethylene tube) was able to survive the process of sanitation (chlorine dioxide) used in the beverage production. The resistance may be rather due to the biofilm formation on many types of surfaces which are commonly used in food processing, i.e. stainless steel, plastic materials – polyethylene (PE), polystyrene (PS), and glass. In this process, bacterial hydrophobicity appears to be of major importance. Much debate has existed as to which is the best method to measure bacterial surface hydrophobicity (Palmer *et al*. 2007). The most popular method, i.e. Bacterial Adherence To Hydrocarbons (BATH), which is now generally called MATH test (microbial adhesion to hydrocarbons), is a general method and a simple rapid technique for determining, the cell-surface hydrophobicity (Rosenberg 2006). As an assay for the cell surface hydrophobicity, the adhesion to liquid hydrocarbons has an undeniable advantage – there is no need for special equipment, apart from the vortex and spectrophotometer. This technique was employed to demonstrate the correlation between the adherence of bacteria to hydrocarbons and their attachment to the surfaces.

MATERIAL AND METHODS

Bacterial isolates. *Pseudomonas fluorescens* (DBM 3113 BHA) and *Escherichia coli* (DBM 3125 BHA) were obtained from the Department of Biochemistry and Microbiology (DBM) Collection of the Institute of Chemical Technology Prague); *Klebsiella* spp. and *Acinetobacter* spp. were kindly provided by Doc. Ing. Miroslav Marek, CSc., and *Asaia bogorensis* was isolated from spoilage fruit-flavoured bottled water.

***Isolation of** Asaia bogorensis*. A Gram-negative microorganism was isolated from a batch of fruit-flavoured bottled water, which had spoilt as a result of bacterial overgrowth. The spoilage isolate was difficult to identify. The contamination was able to survive the process of sanitation commonly used in the beverage production. By isolation on Plate Count Agar (Oxoid, Basingstoke, UK) at 30°C for 48 h, we obtained pure culture consisting of small, pale pink colonies. A single colony was purified and by sequencing the bacterial 16S rDNA gene (Beckmann Coulter CEQ8000, Department of Biochemistry and Microbiology, Institute of Chemical technology Prague, Czech Republic), the recently described genus and member of the acetic acid bacteria, *Asaia bogorensis*, was identified.

Bacterial growth condition. *Asaia bogorensis* was grown in standard nutrient broth I (Merck, Darmstad, Germany) with 2% glucose, 0.5% yeast extract powder, and was harvested in the stationary phase after 48 h incubation at the temperature of 25°C under shaking. The cells were centrifuged (3000 g, 10 min) and washed three times with PBS (phosphate buffered saline, pH 7.2, 0.01mM). Subsequently, the cells were resuspended in sterile PBS at OD_{600} 0.324 ± 0.007 (standardised suspension; required cell count 10^8 colony forming units, CFU/ml).

Acinetobacter ssp. was grown in nutrient broth (Difco Laboratories, Detroit, USA) supplemented with 0.5% NaCl and harvested in the stationary phase after overnight incubation at 30°C under shaking. The centrifugation, washing, and diluting were carried out in the same way as in the case of *A. bogorensis*.

BATH – bacterial adherence to hydrocarbon test. The BATH assay was performed as described previously (Rosenberg 1984; Matz & Jürgens 2001). In the BATH test, an aliquot of 1 ml of the standardised bacterial suspension in PBS was added to *n*-hexadecane, *n*-heptane or *p*-xylene (20, 50, 100 and 150 µl) and vortex a mixed at constant speed for 120 second. Following 15 min equilibration, the bottom aqueous layer was carefully removed and its absorbance was measured at 400 nm. Hydrophobicity was expressed as the percentage of the applied cell suspension absorbance which had been excluded from the aqueous phase.

The percentage expression of bacterial affinity to organic phase (the degree of hydrophobicity) was calculated as :

$$(1 - A/A_0) \times 100\ (\%)$$

where:
A_0 – OD_{400} water phase before vortex homogenisation
A – OD_{400} water phase after vortex homogenisation

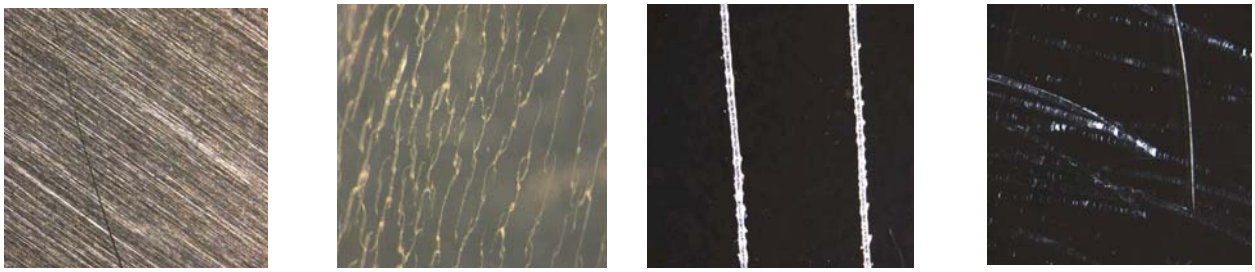
Adhesion to polystyrene. In the preliminary experiments, a direct correlation was found between the bacterial strains showing a high affinity for liquid hydrocarbons and the ability of these strains to adhere to polystyrene. Similarly, the bacterial strains which did not adhere to hydrocarbons could be easily displaced from the polystyrene surface by washing (Buckingham-Meyer *et al*. 2007; Shi & Zhu 2009; Simões et al. 2009).

100 µl of the standardised bacterial suspension (OD_{600} = 0.32 (10^8 CFU/ml) was pipetted on the sterile polystyrene microtiter plate. The plate was incubated for 30 min (*E. coli* 37°C, *P. fluorescens*, *A. bogorensis* 25°C), then rinsed 3 times with standard sterile nutrient broth and stained with crystal violet. The covered plate was left for 1 h then it was washed 6 times with sterile buffer (pH 7.01) and finally dried in air. The absorbance was measured at 590 nm and the results were statistically evaluated using the software NCSS-PASS 1997

The ability to adhere to surfaces. 1 ml of standardised bacterial suspension (OD_{600} = 0.32 (10^8 CFU/ml) was pipetted on the treated area of stainless steel, glass, and plastic materials. After 3 h, 6 h and 24 h waiting period in an incubator without shaking each treated area was washed 3 times with sterile distillated water to remove the cells which were not firmly bound. The area was swabbed and after 1 min shaking with vortex-mixer, the viable adherent cells were grown on nutrient agar plate.

Surfaces

– Stainless steel – AIS 304 (40 × 40 mm, treated area 13 × 13 mm).



stainless steel (SS) 16 x magnified | polyethylene (PE) 25 x magnified | polystyrene (PS) 16x magnified | Glass (G) 13 x magnified

Figure 1. Treated surfaces (Images were taken by SZX12 Olympus stereomicroscope with digital camera Camedia 5050)

– Glass – microscopic glass (76 × 26 mm, treated area 13 × 13 mm).
– Polyethylene (PE) – tube of sanitation system (Ø 6 mm), treated area 15 mm.
– Polystyrene (PS) – sterile Petri dishes (treated area 13 ×13 mm).

RESULTS AND DISCUSSION

The bacteria causing spoilage of fruit-flavoured bottled water were isolated from a polyethylene tube (part of the sanitation system), purified, and identified by sequencing the bacterial 16S rDNA gene as *Asaia bogorensis* – a recently described genus and member of the acetic acid bacteria species. Its ability to form resistant biofilms has been confirmed (Figure 2) by Scanning Electron Microscopy.

The cell-surface hydrophobicity of the treated microorganisms is presented in Table 1. It is believed that the ability to adhere to hydrocarbons is a characteristic predominantly possessed by hydrocarbon-degrading microorganisms such as *Klebsiella* spp. and *Acinetobacter* spp. (commonly used for the biodegradation of some hydrocarbons). Regarding the percentage affinity to *n*-hexadecane, the bacteria are classified by the scale < 10% hydrophilic, 10–29% medium hydrophilic, 30–54% medium hydrophobic, > 55% highly hydrophobic. We have shown the hydrophobicity of common spoilage microorganisms occurring in food processing (*P. fluorescens*, *E. coli*), and the hydrophobicity of *A. bogorensis*.

The values of hydrophobicity of bacterial cells and the values of surface tension of the treated materials (the surface tension was in order PE > PS > G > SS, however, this was the aim of a different study and the methodology is not presented here) were used to compare the ability of the tested microorganisms to colonise the surfaces typical for the food processing and beverage production (Figure 3). The results were statistically evaluated using the software NCSS PASS97.

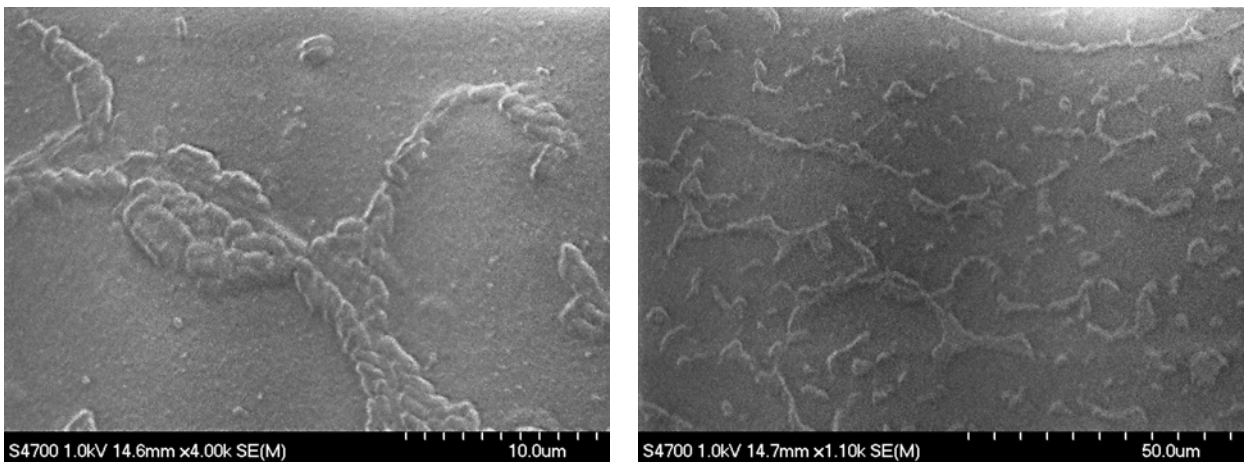


Figure 2. The SEM micrograph of Asaia bogorensis biofilm

Table 1. The cell-surface hydrophobicity of tested microorganisms

Microorganism	BATH (*n*-hexadecane) (%)[a]	Polystyrene absorbance (590 nm)	Hydrophobicity
Asaia bogorensis	7.064 ± 1.991	0.032 ± 0.010	hydrophilic
Acinetobacter spp.	31.976 ± 3.062	0.128 ± 0.001	hydrophobic
Escherichia coli	2.085 ± 0.861	0.046 ± 0.006	hydrophilic
Klebsiella spp.	45.439 ± 5.757	0.169 ± 0.008	hydrophobic
Pseudomonas fluorescens	10.218 ± 0.336	0.087 ± 0.001	medium hydrophilic

[a]mean ± standard deviation based on five measurements for duplicate cultures

Asaia bogorensis and *Escherichia coli* have been estimated as bacteria with hydrophilic cell surface. Therefore, their interaction with the materials tested should be weaker than the binding of *Pseudomonas fluorescens* which is medium hydrophilic. However, the determined values of hydrophobicity were not significantly different, so we could not expect a huge difference between the counts of the adhered bacterial cells. From the results in Figure 4, it is obvious that the bacteria with hydrophobic cell surface (*Acinetobacter* spp.) colonise plastic materials better than the hydrophilic bacteria during the first period (3 h) of adhesion. After 24 h, all microorganisms tested showed almost the same values of the adhered bacterial cells. Although *Asaia bogorensis* is more hydrophobic than *Escherichia coli*, we suppose that it can be easily removed from the polystyrene surface by simple washing due its low value of polystyrene adhesion as shown in Table 1.

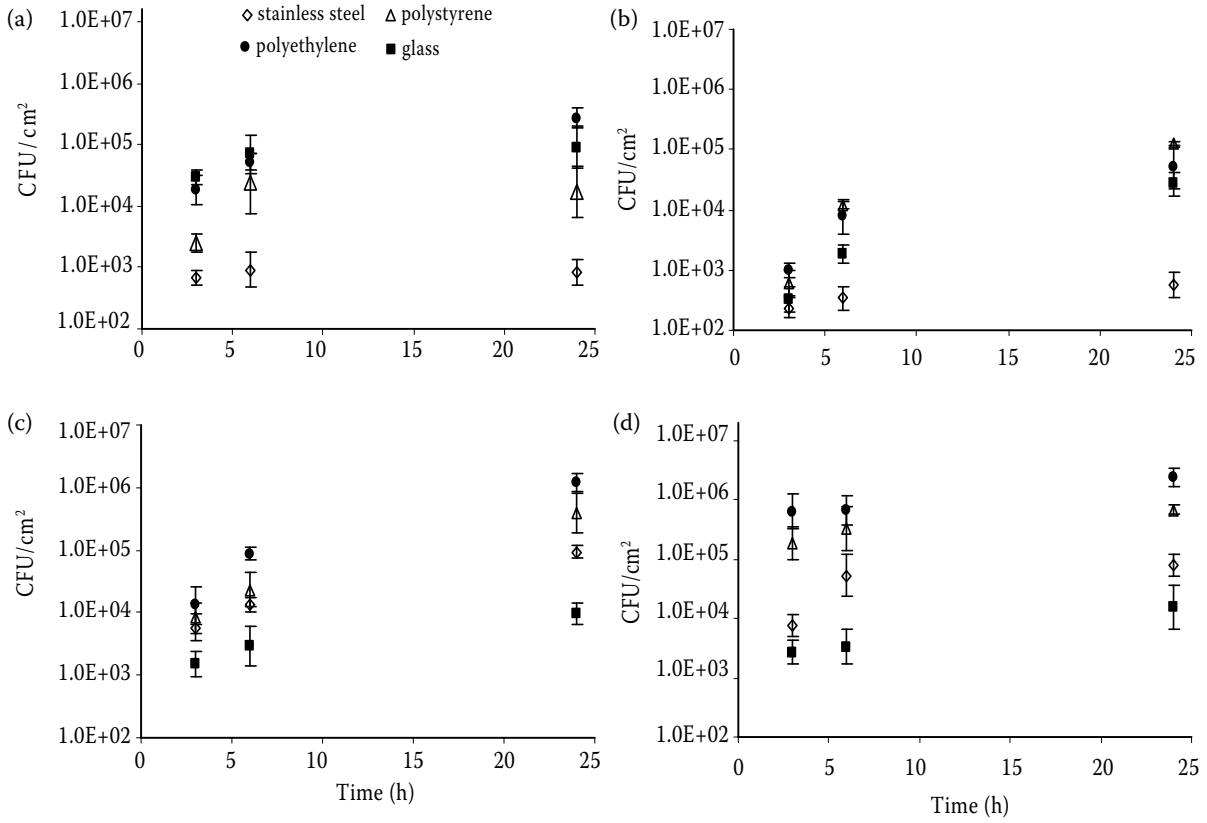


Figure 3. The bacterial adhesion of *A. bogorensis* (a), *E. coli* (b), *P. fluorescens* (c), and *Acinetobacter* spp. (ad to stainless steel, glass, polystyrene and polyethylene. The results are expressed as CFU/cm^2 after 3, 6 and 24 h incubation at the 25°C (*A. bogorensis*) and 37°C (the other tested microorganisms)

CONCLUSION

We have found that *Asaia bogorensis* is a bacterium with hydrophilic cell surface which is able to colonise the surfaces generally used in food processing and beverage production such as stainless steel, glass, polyethylene, and polystyrene. The highest adhesion was observed to stainless steel and glass materials. Due to its low ability to adhere to polystyrene, it can be removed from this surface more efficiently than from the other materials tested by SEM we have confirmed, its ability to form strong biofilms. It can be supposed that via the biofilm the matrix bacteria are more resistant than planktonic cells and this may be the reason why *Asaia bogorensis* is able to survive the sanitation process involving chlorine dioxide. The contamination of the technological equipment such as polyethylene tubes is always a serious problem because of the impossibility to use the common sanitation procedures at high temperatures, acetic based sanitisers, or mechanical treatment. In the beverage production, the application of an appropriate CIP and a perfect system of the control points is the best that can be recommended.

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