REDUCTION OF ACINETOBACTER BAUMANNII BIOFILM FORMATION BY NATURAL ZEOLITE

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ABSTRACT
Acinetobacter baumannii is an emerging human pathogen. Biofilm formation is an important virulence factor which contributes to the pathogenesis of *A. baumannii*, but data regarding the isolates recovered from the natural environment are missing. The biofilm formation at the solid-liquid/air-liquid interfaces of environmental isolates of *A. baumannii*, as well as the influence of natural zeolitizied tuff (NZ) on its formation was determined. In total, 24 environmental isolates of *A. baumannii* (14 multi-drug resistant, 10 sensitive to antibiotics) were recovered from different stages of the secondary type of municipal wastewater treatment plant. Isolates sensitive to antibiotics were statistically significantly more hydrophobic and formed stronger biofilm than multi-drug resistant isolates. Biofilm formation was significantly positive correlated with hydrophobicity of cells. Biofilm formation at solid-liquid interface was significantly inhibited by the addition of 1% of NZ into the growth medium due to the immobilization of bacterial cells onto the NZ particles. Biofilm formation at air-liquid interface was inhibited only by the addition of 10% of NZ. NZ could find application in control of the biofilm formation of this pathogen on abiotic surfaces.

Keywords: *Acinetobacter baumannii*, biofilm, pellicle, immobilization, natural zeolite

INTRODUCTION
Acinetobacter baumannii is an emerging human pathogen. It is resistant to multiple antibiotics as well as disinfectants and survives in harsh environmental conditions [1,2]. One of the main reasons for the success of *A. baumannii* as a pathogen is its ability to form biofilm on biotic and abiotic surfaces[3]. Biofilm is an assemblage of cells enclosed in an extracellular matrix which can be formed on different solid-liquid interfaces[4]. Another highly organized form of biofilm are pellicles which form at the air-liquid interface. Both forms of biofilm contribute to better survival and pathogenicity of *A. baumannii* [2,5]. There are numerous studies regarding the biofilm formation of clinical isolates of *A. baumannii*, whereas data regarding environmental isolates are missing. Previous studies have demonstrated the immobilization of *A. junii* onto the particles of natural zeolitizied tuff (NZ) [6–8]. The goal of this study was to examine the influence of NZ on the biofilm and pellicle formation of environmental isolates of *A. baumannii*.

EXPERIMENTAL
The samples of influent and effluent wastewater, fresh and digested activated sludge from the secondary type municipal wastewater treatment plant of the city of Zagreb were collected between September 2015 and March 2016. The isolation and identification of *A. baumannii* cells was performed on CHROMagar Acinetobacter[9]. Antibiotic resistance profiles were interpreted according to the EUCAST[10] and CLSI[11] criteria. Hydrophobicity of bacteria was measured via the bacterial adhesion to hydrocarbon[12]. Biofilm formation was tested according to the crystal violet assay[13]. After the staining of biofilm with crystal violet, it was solubilized in ethanol and quantified by absorbance at 550nm. Biofilm formation was estimated according to these criteria: A_{550} <0.3 poor; A_{550} 0.3-1.0 intermediate; A_{550} >1.0 strong biofilm formation.

Pellicle formation was tested visually[5] and divided into three categories: no (0); poor (1); strong pellicle formation (2). The experiments for both biofilm and pellicle formation were repeated in duplicate with the addition of 1% of NZ into the growth medium for all isolates, and with 10% of NZ only for strong biofilm and pellicle formers. The NZ was obtained from quarries located at Donje Jesenje, Croatia. The composition of NZ is: clinoptilolite (50-55%), celadonite, plagioclase feldspars and opal-CT (10-15% each), analcime and quartz in traces[8]. The NZ was crushed, sieved, and the size fraction less than 0.122mm was used. Prior to its usage, dry NZ was sterilized by autoclaving. To confirm the immobilization of bacteria onto NZ, particles of NZ were taken at the end of experiments, stained with carbol fuxin dye and examined under the optical microscope. Statistical analyses were carried out using Statistica 12 software and decisions were made at a significance level of $p<0.05$.

RESULTS AND DISCUSSION

In total, 24 environmental isolates of *A. baumannii* were isolated from influent and effluent wastewater, fresh and digested activated sludge (Table 1). 14 isolates were multi-drug resistant (MDR, resistant to three or more classes of antibiotics), while 10 isolates were sensitive to 12 antibiotics tested. 9/24 isolates from wastewater treatment plant were hydrophobic (Table 1). Isolates sensitive to antibiotics were statistically significantly more hydrophobic than MDR isolates ($p=0.000$).

Great proportion (14/24) of isolates were intermediate biofilm formers, 7/24 were strong biofilm formers, whereas only 3/24 formed poor biofilm (Table 1). Isolates sensitive to antibiotics formed stronger biofilm than MDR isolates ($p=0.005$). Biofilm formation showed statistically significant positive correlation with hydrophobicity of cells ($r=0.425$, $p=0.003$). By the addition of 1% of NZ (Table 1) biofilm formation was significantly reduced (39±21%, $p=0.003$). By the addition of 10% of NZ to selected isolates, biofilm formation was significantly reduced even further (76±21%, $p=0.002$).

Majority (19/24) of isolates formed poor pellicles, only isolate IN41 formed no pellicle, while 4/24 isolates formed strong pellicles (Table 1). Pellicle formation showed statistically significant positive correlation with cell hydrophobicity ($r=0.433$, $p=0.002$), as well as with biofilm formation ($r=0.682$, $p=0.000$). Clinical strains of *A. baumannii* that were more hydrophobic also formed stronger biofilm[14] and pellicles[5]. The addition of 1% of NZ did not influence the pellicle formation. However, 10% of NZ decreased the consistency of pellicles from strong to intermediate consistency.

In order to elucidate the mechanism of significant reduction of biofilm formation by the addition of NZ, the particles of NZ were examined at the end of experiments for the immobilization of bacteria. Microscopic examination confirmed the immobilization of cells of *A. baumannii* onto NZ particles in high extent (Figure 1). The reduction of biofilm formation is explained by the immobilization of *A. baumannii* onto the NZ particles. Cells of *A. baumannii* were rather attached onto the rough surface of NZ particles than to the smooth plastic surfaces. The proportion of bacteria captured by NZ resulted in lower bacterial abundance as compared to the medium without NZ, therefore bacteria were not available for biofilm formation. In experiments with pellicle formation, NZ particles were located at the bottom of the tube and were not in direct contact with the bacteria. This explains the lower efficiency of NZ on the reduction of pellicle formation. The clinoptilolite content of the NZ used in this study was relatively low (50-55%), but it was proved earlier that clinoptilolite content is not the prevailing factor for the immobilization of bacteria[7]. Another species of the genus *Acinetobacter*, *A. junii*, was immobilized in high numbers (1.27×10^{10} CFU/g) onto the same NZ of particle size 0.122-0.263mm[8]. The extent of the immobilization onto the NZ of particle size <0.122mm would surely be greater, since the number of immobilized bacteria increase with decrease of particle

size [6]. NZ particles could find the application in cleaning products, where *A. baumannii* could be captured by NZ and then easily removed from the contaminated environment.

Table 1. Origin, antibiotic resistance profile, hydrophobicity, pellicle, biofilm formation values and percentages of biofilm reduction by NZ of *A. baumannii* isolates. IN-influent, EF-effluent, S-fresh sludge, D-digested sludge, MDR-multi-drug resistant. Isolates with hydrophobicity higher than 46% are considered hydrophobic.

Isolate	Antibiotic resistance	Hydrophobicity (%)	Pellicle formation	Biofilm formation (A_{550})	Biofilm reduction 1% NZ	Biofilm reduction 10% NZ
IN31	sensitive	97	1	1.024	19	-
IN34	MDR	1	1	1.006	12	-
IN36	sensitive	2	1	1.225	49	-
IN41	MDR	0	0	0.138	65	-
IN47	MDR	0	1	0.745	85	-
IN58	sensitive	93	2	2.497	70	89
EF7	MDR	0	1	1.098	64	-
EF8	MDR	0	1	1.026	38	-
EF11	sensitive	80	2	1.180	49	80
EF13	MDR	0	1	0.468	29	-
EF22	MDR	0	1	0.483	19	-
EF23	MDR	0	1	0.766	34	-
S5	MDR	3	1	0.971	55	-
S6	sensitive	78	1	0.868	22	61
S9	sensitive	8	2	1.274	33	91
S10	MDR	2	1	0.364	20	-
S11	MDR	0	1	1.005	75	96
S15	sensitive	79	1	0.998	8	28
D10	sensitive	0	1	0.723	23	-
D11	MDR	46	1	0.402	52	-
D12	MDR	49	1	0.217	26	-
D13	sensitive	0	1	0.267	23	-
D16	sensitive	67	1	0.891	50	75
D17	MDR	1	2	1.244	15	91

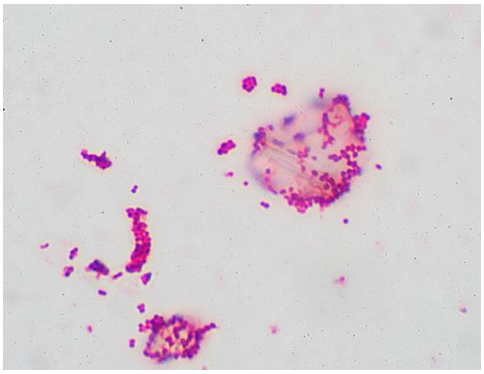

Figure 1. Cells of *Acinetobacter baumannii* immobilized onto NZ particles.

CONCLUSION

Environmental isolates of *A. baumannii* express the virulence factors comparable to the clinical isolates. Cell surface hydrophobicity is an important feature which determines biofilm and pellicle formation of *A. baumannii*. Isolates sensitive to antibiotics form stronger biofilm and

pellicles than MDR isolates. The addition of 1% of NZ into the growth medium effectively reduced biofilm formation due to the immobilization of bacteria onto the NZ particles, while pellicle formation was inhibited only by the addition of 10% of NZ. NZ is a promising material for the reduction of *A. baumannii* virulence factors and could find application in control of the biofilm formation of this emerging pathogen on abiotic surfaces.

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