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Virulence Factors of *Acinetobacter baumannii* Environmental Isolates and Their Inhibition by Natural Zeolite

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A B S T R A C T

Acinetobacter baumannii is an emerging human pathogen causing great concern in hospitals. There are numerous studies regarding the virulence factors that contribute to the pathogenesis of *A. baumannii* clinical isolates, whereas data regarding environmental isolates are missing. The virulence factors (biofilm formation at the air-liquid/solid-liquid interfaces and surface motility) of *A. baumannii* isolated from natural environment were determined. The influence of natural zeolite (NZ) on the expression of virulence factors was examined by addition of 1 and 10% of NZ into the growth medium. In total 24 environmental isolates of *A. baumannii* were recovered from different stages of the secondary type of municipal wastewater treatment plant. 14 isolates were multi-drug resistant, while 10 of them were sensitive to all antibiotics tested. Isolates sensitive to antibiotics were statistically significantly more hydrophobic and formed stronger biofilm and pellicles than multi-drug resistant isolates. Biofilm and pellicle formation were statistically significantly positive correlated with hydrophobicity of cells. Biofilm formation and twitching motility were significantly inhibited by the addition of 1% of NZ into the growth medium due to the immobilization of bacterial cells onto NZ particles, while pellicle formation and swarming motility were inhibited only by the addition of 10% of NZ. NZ is a promising material for the reduction of the *A. baumannii* virulence factors and could find application in control of the adherence and subsequent biofilm formation of this emerging pathogen on abiotic surfaces.

Introduction

Acinetobacter baumannii is an emerging human pathogen causing great concern in hospital environment over the last two decades. *A. baumannii* expresses the resistance to multiple antibiotics as well as disinfectants, and survives in adverse conditions, leading to long-term persistence in the hospital environment (Espinal *et al.*, 2012; Towner, 2009). Additionally, virulence factors that influence the success of *A. baumannii* as a pathogen are its surface motility on solid/semisolid media and the ability to form biofilm on abiotic or biotic surfaces (McConnell *et al.*, 2013).

Biofilm formation is considered as one of the main virulence factor in clinical isolates of *A. baumannii*. Biofilm is an assemblage of

microbial cells enclosed in an extracellular matrix, which can be formed on wide variety of solid surfaces (Antunes *et al.,* 2011). Biofilm provides protection from harsh environmental conditions, and therefore isolates which are strong biofilm producers survive longer in the environment (Espinal *et al.,* 2012). Highly organized types of biofilm formed at the air-liquid interface are called pellicles (Nait Chabane *et al.,* 2014). Pellicle formation is recognized as a feature of pathogenic strains of *A. baumannii* (Marti *et al.,* 2011).

Bacteria in the form of pellicle might contribute to their persistence in the environment. *A. baumannii* is considered to be non-motile in liquid media due to the absence of flagella, but surface motility on solid/semisolid media was described. Two distinct forms of phenotypic surface motility of *A. baumannii* are recognized: twitching defined as surface translocation on solid surfaces and swarming defined as surface translocation on the semisolid media (Antunes *et al.,* 2011; Eijkelkamp *et al.,* 2011a). Twitching motility is considered as an important step in colonization and subsequent biofilm formation on medical devices, which is one of the main sources of hospital infections with *A. baumannii*. Although the bacterial motility is generally linked to increased virulence, there is no confirmation of the influence of motility on the virulence of *A. baumannii*.

In order to suppress the factors that contribute to the persistence and epidemicity of *A. baumannii,* recently attempts are made to elucidate underlying mechanisms and to suppress the expression of its virulence factors. Motility and biofilm formation of clinical strain of *A. baumannii* were found to be inhibited by blue light illumination and iron limitation (Eijkelkamp *et al.,* 2011b; Mussi *et al.,* 2010). However, blue light illumination and iron limiting conditions are difficult to achieve in the environment in order to be used for the suppression of virulence factors of *A. baumannii*. Among different types of natural zeolitizied tuff (NZ), those containing clinoptilolite are usually used in scientific studies as well as in industrial applications(Wong, 2009)on the base of its widespread occurrence in nature, price-easily accessibility and feasibility, cost effectiveness, large surface area, rigidity, surface functionality, thermal, mechanical and radiation stability. Particles of nontoxic NZ were shown to display a high affinity for the immobilization of different bacterial species including the *Acinetobacter* spp. (Hrenovic *et al.,* 2005; Hrenovic *et al.,* 2009; Hrenovic *et al.,* 2011). Therefore, it was presumed that the addition of NZ into the growth medium will result in immobilization of *A. baumannii* cells onto NZ particles, thus hindering the expression of their virulence factors.

Due to its clinical relevance, *A. baumannii* is considered as an exclusive bacterium of the hospital environment. From 2010 onwards, continuous reports on the occurrence of *A. baumannii* outside hospital environment can be found. Multi-drug resistant (MDR) isolates of *A. baumannii* were found in hospital (Ferreira *et al.,* 2011; Zhang *et al.,* 2013)and municipal sewage (Goic-Barisic *et al.,* 2017;Hrenovic *et al.,* 2016), Seine River (Girlich *et al.,* 2010), and in soil influenced by human solid waste (Hrenovic *et al.,* 2014). However, to our knowledge there is no data on the phenotypic expression of the virulence factors that contribute to the pathogenesis in environmental isolates of *A. baumannii*. The aim of this study was to investigate the virulence factors of *A. baumannii* recovered from the natural environment, as well as the influence of NZ on the expression of biofilm and pellicle formation, swarming and twitching motility.

Materials and Methods

Isolation and characterization of *A. baumannii*

The samples of influent and effluent wastewater, fresh activated sludge, and sludge passed through the anaerobic mesophilic digestion were collected between September 2015 and March 2016 at the secondary type municipal wastewater treatment plant of the City of Zagreb, Croatia. The isolation of *A. baumannii* was performed according to Hrenović et al.(2016)at 42°C/48h on CHROMagar Acinetobacter (CHROMagar) supplemented with 15 mg/L of cefsulodin sodium salt hydrate(Sigma-Aldrich). Identification of isolates was performed by routine bacteriological techniques, Vitek 2 system (BioMerieux), and MALDI-TOF MS (software version 3.0, Microflex LT, Bruker Daltonics) on cell extracts (Sousa *et al.*, 2014). Susceptibility testing was done by Vitek 2 system and confirmed by gradient dilution E-test for colistin. Minimum inhibitory concentrations (MIC) were interpreted according to European Committee on Antimicrobial Susceptibility Testing (2016) criteria for all antibiotics with defined breakpoints for *Acinetobacter* spp., while for penicillins/β-lactamase inhibitors and minocycline Clinical and Laboratory Standards Institute (2013) breakpoints were used.

Bacterial hydrophobicity

Hydrophobicity of bacteria was measured via the bacterial adhesion to hydrocarbon (BATH) assay according to Rosenberg *et al.*, (1980)with slight modifications. The assay is based on the affinity of bacterial cells for an organic hydrocarbon such as hexadecane. More hydrophobic bacteria will migrate from aqueous suspension to the hexadecane layer, resulting in reduction of bacterial concentration in the water phase. Overnight bacterial culture was suspended in 5mL of phosphate-buffered saline (PBS), 0.5mL of n-hexadecane was added to the suspension, shaken for 10 min and left to stand for 2 min. The reduction in bacterial concentration was measured spectrophotometrically (DR/2500 Hach spectrophotometer) at absorbance of 410nm (OD_{410}) both before and after the addition of n-hexadecane.

Natural zeolitizied tuff

The NZ was obtained from quarries located at Donje Jesenje, Croatia. The main constituent of NZ is clinoptilolite (50-55%). Other major constituents (10-15% each) are celadonite, plagioclase feldspars and opal-CT, while analcime and quartz are present in traces (Hrenovic *et al.*, 2011). The NZ was crushed, sieved, and the size fraction less than 0.122mm was used. Prior to its usage in experiments, dry NZ was sterilized by autoclaving.

Biofilm formation

The ability to form biofilm in vitro was tested via the crystal violet assay (Kaliterna *et al.*, 2015). Overnight bacterial culture was diluted in nutrient broth (Biolife) to an absorbance of 0.1 at 600nm (OD_{600}). The suspension was distributed into the polypropylene tubes and then incubated at 37°C/48 h without shaking. After incubation, the planktonic bacteria were removed and the tubes were gently washed with PBS. Biofilm was stained with 0.5% (w/v) crystal violet at 37°C/20 min. After solubilisation with 96% ethanol at 37°C/20 min, biofilm was quantified at 550nm (OD_{550}). The estimated criteria used to interpret the biofilm formation were: OD_{550} value beneath 0.3 poor biofilm formers; value between 0.3 and 1 intermediate biofilm formers; value above 1.0 strong biofilm formers. The procedure was repeated with the

addition of 1% NZ into the bacterial suspension for all isolates, while 10% of NZ was added into the suspension of selected intermediate and strong biofilm formers.

Pellicle formation

Pellicle formation assay was performed according to the protocol described in Nait Chabane *et al.,* (2014). Overnight bacterial culture with the initial concentration of 0.01 at an OD_{600} was divided into the polystyrene tubes with 2mL of Mueller Hinton Broth (Biolife) and incubated at 25°C/72h. Pellicle formation was identified visually and its cohesion was examined by inverting the tubes. Cohesion of pellicles was divided into three categories: no pellicle formation (0); poor pellicle formation (1); strong pellicle formation (2). The procedure was repeated with the addition of 1 and 10%of NZ for isolates which formed poor and strong pellicles.

Swarming and twitching surface motility

Swarming and twitching surface motility was assessed according to Antunes et al. (2011). For surface motility study Luria-Bertani medium with 0.5% agarose was used, into which0, 1 and 10% of NZ was added. Overnight bacterial cultures were suspended in 1mL PBS and inoculated with a pipette tip to the bottom of the polystyrene Petri dish, tightly closed with parafilm and incubated in humid atmosphere at 37°C/24 h. Swarming motility was observed at the air-agarose interface by direct measuring of the longest diameter of motility. Twitching motility was determined after the removal of the agarose layer, staining the Petri dish with 0.5% crystal violet for 10 min and measuring the longest diameter of motility. Isolates were grouped into categories based on the average values of motility: < 25mm poor; 25-50mm intermediate; >50mm highly motile isolates.

To confirm the immobilization of bacteria onto NZ, particles of NZ were taken at the end of experiments on motility and biofilm formation. Particles were stained with carbol fuxin dye and examined under optical microscope (Olympus CX21) at magnification of 1000x.

Data analyses

All experiments were performed in biological and technical duplicate with mean values presented. Percentages of reduction were calculated for each isolate with addition of NZ as compared to the same isolate without NZ addition. Statistical analyses were carried out using Statistica software 12 (StatSoft, Inc.). The comparisons between samples were done by using the ordinary Student's *t*-test for independent variables. The correlation between variables was estimated by Pearson linear correlation analysis. Statistical decisions were made at a significance level of $p<0.05$.

Results and Discussion

Characterization of *A. baumannii* isolates

In total24 environmental isolates of *A. baumannii* have been isolated from 4 different stages of municipal wastewater treatment plant (6 per each stage of treatment): influent wastewater, effluent wastewater, fresh activated sludge and digested sludge. The list of recovered isolates, their origin, date of isolation, and MALDI-TOF MS score values are given in table 1.

The antibiotic resistance profile of isolates is shown in Table 2. From each stage of the wastewater treatment plant, isolates sensitive to all 12tested antibiotics (10 isolates), as well as MDR isolates (14 isolates) were chosen for study. MDR isolates shared the resistance to carbapenems and fluoroquinolones, but

sensitivity to colistin. The pan drug-resistant isolate EF7 has already been described in Goic-Barisic et al. (2017).

Significant hydrophobicity, estimated as migration of cells to hydrocarbon of 46% and higher, was observed for 2/6 isolates from influent wastewater, 1/6 isolates from effluent wastewater, 3/6 isolates from fresh activated sludge, and 3/6 isolates from digested sludge (Table 2). In total 9/24 isolates from wastewater treatment plant were hydrophobic. 7/9 hydrophobic isolates were sensitive to tested antibiotics, while 2 remaining hydrophobic isolates which were MDR showed the lower level of hydrophobicity than sensitive isolates. Isolates sensitive to all tested antibiotics were statistically significantly more hydrophobic than MDR isolates (p=0.000).

Biofilm formation

The results of biofilm formation of isolates are presented in Fig. 1. Great proportion (14/24) of isolates were intermediate biofilm formers (OD_{550} 0.3-1.0), whereas only 3/24 (IN41, D12, D13) formed poor biofilm. Among 7strong biofilm formers, the isolate IN58 stands out with an OD_{550} value of 2.5.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, Table 3).With the addition of 1% of NZ biofilm formation dropped significantly (p=0.003, Fig. 1). With the addition of 10% of NZ to selected isolates, biofilm formation dropped significantly even further (p=0.002).Average percentage of inhibition for isolates were 39±21% and 76±21% with the addition of 1 and 10% of NZ, respectively.

Pellicle formation

Majority (19/24) of isolates formed poor pellicles, while only isolate IN41 formed no pellicle. Isolates EF11, S9, D17 and especially IN58 formed strong pellicles (Table 2). Among 4 isolates which formed strong pellicles, 3 were hydrophobic and sensitive to antibiotics, while this does not stand only for isolate D17. Pellicle formation showed statistically significant positive correlation with cell hydrophobicity (r=0.433, p=0.002), as well as with the biofilm formation (r=0.682, p=0.000, Table 3). The addition of 1%of NZ did not influence the pellicle formation (data not shown).However, 10% of NZ decreased the consistency of pellicles from strong to intermediate consistency.

Swarming and twitching surface motility

The results presented in Figs. 2 and 3indicate that all examined environmental isolates of *A. baumannii* expressed the surface motility by swarming or twitching.10/24 isolates showed poor swarming, whereas 8/24 and 6/24 isolates showed intermediate and high swarming, respectively. Only 3/24 isolates showed poor twitching, whereas 11/24 and 10/24 isolates showed intermediate and high twitching, respectively. No connection of surface motility and sensitivity or MDR of isolates to antibiotics could be established. Swarming and twitching motility were not mutually linked parameters (r=-0.018, p=0.904) and showed no correlation with cell hydrophobicity, biofilm or pellicle formation (Table 3).

The addition of 1% of NZ significantly increased the swarming motility of isolates (47±21% increase), while the addition of 10% of NZ had no statistically significant influence on swarming (18±51% reduction, Fig. 2). Contrary to swarming, twitching

motility was significantly reduced by 1% of NZ (48±19% reduction, p=0.000) and10% of NZ reduced twitching even further (52±20% reduction, p=0.001, Fig. 3), but there was no statistically significant difference between addition of 1 and 10% of NZ. In order to elucidate the mechanism of significant reduction of biofilm formation and twitching motility by the addition of NZ, the particles of NZ were examined at the end of experiments for the immobilization of *A. baumannii*. Microscopic examination confirmed the immobilization of cells of *A. baumannii* onto NZ particles in high extent (Fig. 4).

Table1. Origin, date of isolation, MALDI-TOF MS score values, hydrophobicity values, and pellicle formation of *A. baumannii* isolates. Isolates with hydrophobicity higher than 46% are considered hydrophobic. Cohesionof pellicles was divided into three categories: no pellicle formation (0), poor pellicle formation (1); strong pellicle formation (2).

Isolate	Origin	Date of isolation	MALDI-TOF score	Hydrophobicity (% OD_{410})	Pellicle formation
IN31	Influent	23.9.2015	2.119	97.15	1
IN34	Influent	23.9.2015	2.066	0.66	1
IN36	Influent	23.9.2015	2.184	1.97	1
IN41	Influent	4.11.2015	2.068	0.00	0
IN47	Influent	18.11.2015	2.198	0.00	1
IN58	Influent	26.1.2016	2.205	92.76	2
EF7	Effluent	9.9.2015	2.150	0.00	1
EF8	Effluent	23.9.2015	2.180	0.00	1
EF11	Effluent	18.11.2015	2.173	80.00	2
EF13	Effluent	2.12.2015	2.074	0.00	1
EF22	Effluent	26.1.2016	2.149	0.00	1
EF23	Effluent	26.1.2016	2.189	0.00	1
S5	Fresh sludge	23.9.2015	2.178	2.54	1
S6	Fresh sludge	4.11.2015	2.247	78.04	1
S9	Fresh sludge	26.1.2016	2.063	80.00	2
S10	Fresh sludge	10.2.2016	2.025	1.69	1
S11	Fresh sludge	10.2.2016	2.079	0.00	1
S15	Fresh sludge	23.3.2016	2.000	78.84	1
D10	Digested sludge	23.9.2015	2.248	0.00	1
D11	Digested sludge	14.10.2015	2.103	46.36	1
D12	Digested sludge	14.10.2015	2.037	48.63	1
D13	Digested sludge	18.11.2015	2.048	0.00	1
D16	Digested sludge	26.1.2016	2.081	67.36	1
D17	Digested sludge	10.2.2016	2.253	1.40	2

Table.2 MIC values of tested antibiotics against isolates of *A. baumannii*.

Isolate	MIC values of antibiotics (mg/L)											
	MEM	**IMI**	**CIP**	**LVX**	**TOB**	**GEN**	**AMK**	**MIN**	**SAM**	**TIM**	**SXT**	**CST**
IN31	<0.25	<0.25	<0.25	<0.12	<1	<1	<2	<1	<2	<8	<20	≤0.5
IN34	>16^{R}	>16^{R}	>4^{R}	8^{R}	>16^{R}	>16^{R}	>64^{R}	>16^{R}	>32^{R}	>128^{R}	<20	≤0.5
IN36	<0.25	<0.25	<0.25	<0.12	<1	<1	<2	<1	<2	<8	<20	≤0.5
IN41	≥16^{R}	≥16^{R}	≥4^{R}	≥ 8^{R}	≤1	≤1	≥64^{R}	8^{I}	≥32^{R}	≥128^{R}	≥320^{R}	≤0.5
IN47	≥16^{R}	≥16^{R}	≥4^{R}	≥ 8^{R}	≥16^{R}	8^{R}	≥64^{R}	8^{I}	16^{I}	≥128^{R}	≥320^{R}	≤0.5
IN58	≤0.25	≤0.25	≤0.25	≤0.12	≤1	≤1	≤ 2	≤1	≤2	≤8	≤ 20	≤0.5
EF7	>16^{R}	>16^{R}	>4^{R}	>8^{R}	>16^{R}	>16^{R}	>64^{R}	8^{I}	>32^{R}	>128^{R}	>320^{R}	16^{R}
EF8	≥16^{R}	≥16^{R}	≥4^{R}	≥ 8^{R}	≥16^{R}	≥16^{R}	8	≥16^{R}	≥32^{R}	≥128^{R}	≤20	≤0.5
EF11	≤0.25	≤0.25	≤0.25	≤0.12	≤1	≤1	≤2	≤1	≤2	≤8	≤ 20	≤0.5
EF13	≥16^{R}	≥16^{R}	≥4^{R}	≥ 8^{R}	≥16^{R}	≥16^{R}	≥64^{R}	≥16^{R}	≥32^{R}	≥128^{R}	≤20	≤0.5
EF22	≥16^{R}	≥16^{R}	≥4^{R}	4^{R}	8^{R}	2	8	2	16^{I}	≥128^{R}	≥320^{R}	≤0.5
EF23	≥16^{R}	≥16^{R}	≥4^{R}	4^{R}	≥16^{R}	4	16^{I}	4	16^{I}	≥128^{R}	≥320^{R}	≤0.5
S5	>16^{R}	>16^{R}	>4^{R}	>8^{R}	>16^{R}	>16^{R}	>64^{R}	>16^{R}	>32^{R}	>128^{R}	<20	≤0.5
S6	≤0.25	≤0.25	≤0.25	≤0.12	≤1	≤1	≤2	≤1	≤2	≤8	≤20	≤0.5
S9	≤0.25	≤0.25	≤0.25	≤0.12	≤1	≤1	≤2	≤1	≤2	≤8	≤20	≤0.5
S10	≥16^{R}	≥16^{R}	≥4^{R}	≥ 8^{R}	≥16^{R}	≥16^{R}	≥64^{R}	8^{I}	16^{I}	≥	≥320^{R}	≤0.5
S11	≥16^{R}	≥16^{R}	≥4^{R}	≥ 8^{R}	4	≥16^{R}	≥64^{R}	8^{I}	16^{I}	≥128^{R}	≥320^{R}	≤ 0.5
S15	≤0.25	≤0.25	≤0.25	≤0.12	≤1	≤1	≤2	≤1	≤2	≤8	≤20	≤0.5
D10	0.5	<0.25	<0.25	<0.12	<1	<1	<2	<1	<2	<8	<20	≤0.5
D11	≥16^{R}	≥16^{R}	≥4^{R}	4^{R}	≤1	≤1	32^{R}	≤1	16^{I}	≥128^{R}	≤20	≤0.5
D12	≥16^{R}	≥16^{R}	≥4^{R}	4^{R}	≤1	≤1	16^{I}	≤1	16^{I}	≥128^{R}	≤20	≤0.5
D13	≤0.25	≤0.25	≤0.25	≤0.12	≤1	≤1	≤2	≤1	≤2	≤8	≤20	≤0.5
D16	≤0.25	≤0.25	≤0.25	≤0.12	≤1	≤1	≤2	≤1	≤2	≤8	≤20	≤0.5
D17	≥16^{R}	≥16^{R}	≥4^{R}	≥ 8^{R}	≥16^{R}	≥16^{R}	≥64^{R}	8^{I}	16^{I}	≥128^{R}	≥320^{R}	≤0.5

[a] carbapenems (MEM-meropenem, IMI-imipenem), fluoroquinolones (CIP-ciprofloxacin, LVX-levofloxacin), aminoglycosides (TOB-tobramycin, GEN-gentamicin, AMK-amikacin), tetracyclines (MIN-minocycline), penicillins/β-lactamase inhibitors (SAM-ampicillin/*sulbactam*,TIM-ticarcillin/clavulanic acid), SXT- trimethoprim/sulfamethoxazole, CST-colistin.^{R} - resistant, ^{I} - intermediate according to EUCAST or CLSI criteria.IN - influent wastewater, EF -effluent wastewater, S - fresh sludge, D - digested sludge isolates.

Table.3 Summary of the correlation parameters for the expression of virulence factors of *A. baumannii* isolates

	Hydrophobicity	Biofilm	Pellicle	Swarming	Twitching
Hydrophobicity	1.000	***r=0.425, p=0.003***	***r=0.433, p=0.002***	r=-0.142, p=0.335	r=0.249, p=0.088
Biofilm		1.000	***r=0.682, p=0.000***	r=-0.123, p=0.405	r=-0.049, p=0.740
Pellicle			1.000	r=0.096, p=0.518	r=-0.028, p=0.851
Swarming				1.000	r=-0.018, p=0.904
Twitching					1.000

Fig.1 Biofilm formation (OD_{550}) without natural zeolite (0% NZ), with 1% of NZ, and for selected isolates with 10% of NZ. Lines represent boundaries: OD_{550}<0.3 poor, OD_{550}0.3-1.0 intermediate, OD_{550}>1.0 strong biofilm formation

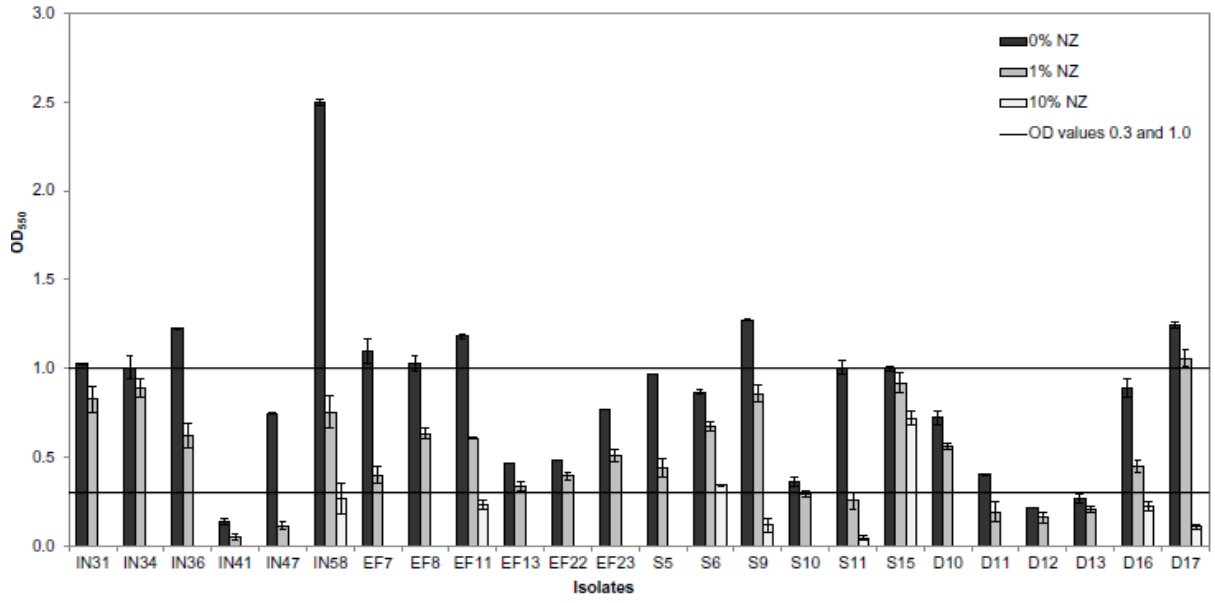


Fig.2 Swarming motility without natural zeolite (0% NZ), with 1% of NZ, and for selected isolates with 10% of NZ. Lines represent boundaries: <25mmpoor, 25-50mm intermediate, >50mm high swarming. Maximum diameter of swarming zone was 85mm; minimum diameter of swarming zone was estimated at 10mm

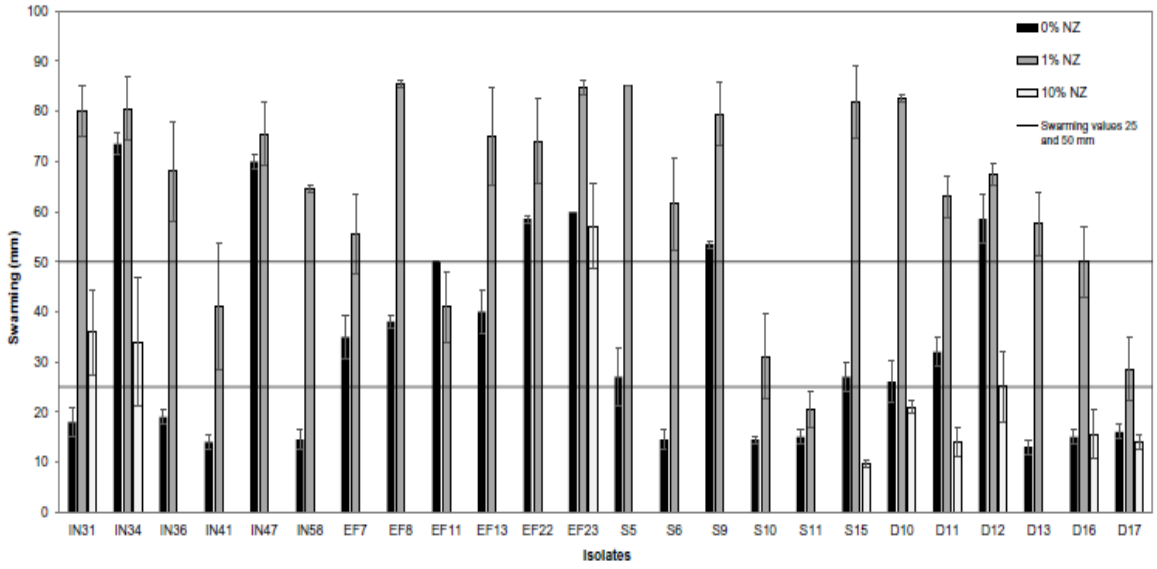


Fig.3 Twitching motility without natural zeolite (0% NZ), with 1% NZ, and for selected isolates with 10% of NZ. Lines represent boundaries: <25mmpoor, 25-50mm intermediate, >50mm high twitching. Maximum diameter of twitching zone was 85mm; minimum diameter of twitching zone was 0mm

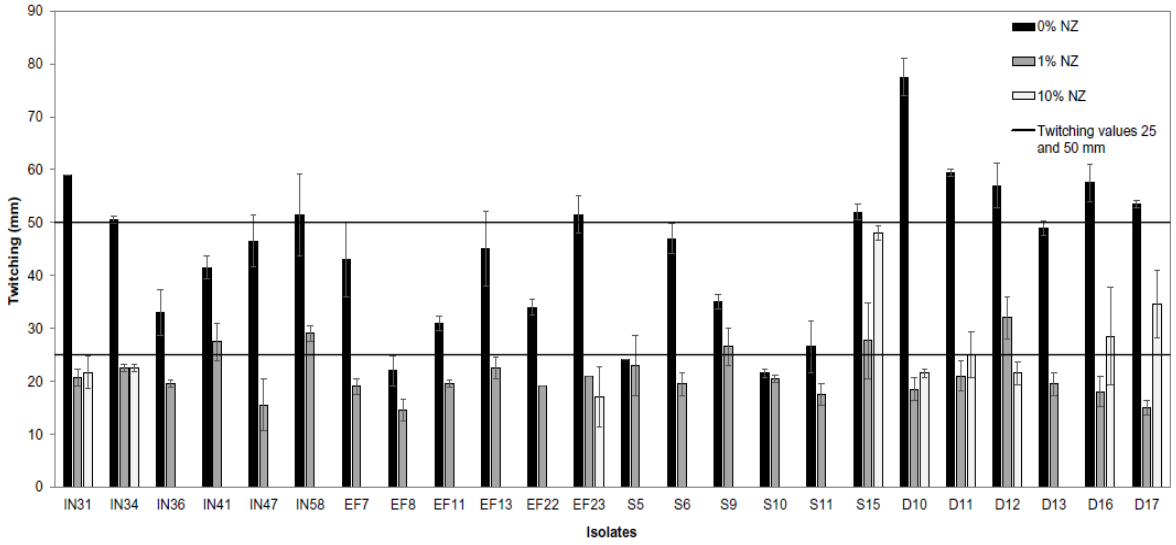
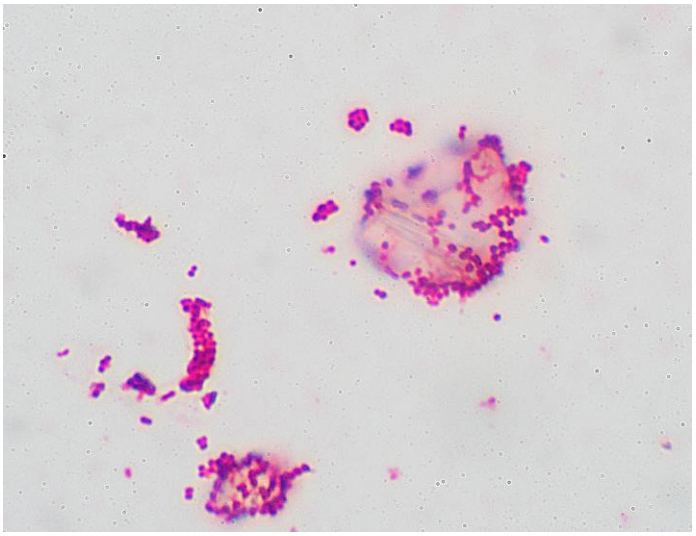


Fig. 4 Cells of *Acinetobacter baumannii* immobilized onto NZ particles.



The 24 isolates of *A. baumannii* recovered from different stages of secondary type municipal wastewater treatment, both antibiotics-sensitive and MDR, showed different expression of the virulence factors that may contribute to its pathogenesis and survival in the natural environment. Ability of the expression of biofilm and pellicle formation, swarming and twitching motility was comparable to those of clinical isolates described in many literature reports(Antunes *et al.,* 2011; Eijkelkamp *et al.,* 2011a; Espinal *et al.,* 2012; Marti *et al.,* 2011; Nait Chabane *et al.,* 2014).

The 38% (9/24) of all isolates showed marked hydrophobicity in BATH assay, suggesting the wastewater as more suitable ecological niche for hydrophilic isolates. Statistically significantly higher hydrophobicity of antibiotics-sensitive isolates as compared to MDR isolates indicates the cell hydrophobicity as a possible protection mechanism against different emerging chemical compounds present in wastewater. Hydrophobicity of cells was statistically significantly positively correlated with biofilm formation at solid-liquid and air-liquid interfaces. Clinical strains of *A. baumannii* that were more hydrophobic also formed stronger biofilm (Kempf *et al.,* 2012) and pellicles (Nait Chabane *et al.,* 2014).Obviously more hydrophobic bacteria form stronger biofilms in order to protect themselves from aqueous medium.

Biofilm formation at solid-liquid and air-liquid interfaces of environmental isolates of *A. baumannii* was significantly positively correlated and mutually linked parameters. Statistically stronger biofilm formation at solid-liquid and air-liquid interfaces was confirmed for antibiotics-sensitive isolates as compared to MDR isolates. This observation is in accordance with statements published for clinical isolates of *A. baumannii* that isolates sensitive to antibiotics form stronger biofilm (Kaliterna *et al.,* 2015; Perez *et al.,* 2015; Qi *et al.,* 2016). Biofilm protects sensitive isolates from the harmful effect of antibiotics, while MDR isolates have already developed mechanisms to protect themselves from antibiotics and therefore do not tend to assemble cells in biofilm. The majority of the examined environmental isolates showed intermediate or high swarming (14/24or 58%

of isolates) and twitching (21/24 or 88% of isolates) motility. Swarming and twitching motility were confirmed to be independent phenotypic parameters. Twitching motility was found to be a common trait of clinical strains of *A. baumannii* which are high biofilm formers (Eijkelkamp *et al.*, 2011a). In this study no correlation of twitching motility and biofilm formation was detected. Twitching motility and biofilm formation on solid surface were shown to depend on the source of clinical isolates of *A. baumannii*, where blood isolates were more motile and sputum isolates formed stronger biofilm (Vijayakumar *et al.*, 2016). Due to the absence of any significant correlation with the examined parameters, it seems that the surface motility of environmental isolates of *A. baumannii*is strain dependant.

Biofilm formation and twitching motility on solid surfaces of environmental isolates of *A. baumannii* were significantly reduced by the addition of 1% of NZ into the growth medium, and addition of 10% of NZ resulted in further suppression of these parameters. Biofilm formation and twitching motility could be reduced up to 85 and 76%, respectively by the addition of only 1% of NZ. Although the addition of 10% of NZ did not significantly increase the reduction of twitching motility, it reduced the biofilm formation up to 96%. The effect of NZ addition was evident in experiments on the biofilm formation and twitching motility on solid surfaces where bacteria were in direct contact with the NZ particles. In experiments on the pellicle formation and swarming motility, NZ particles were located at the bottom of the tube or Petri dish and were not in direct contact with bacteria. This explains the lower efficiency of NZ addition on the reduction of pellicle formation and swarming motility.

The reduction of biofilm formation and twitching motility is explained by the immobilization of *A. baumannii* onto NZ particles. The clinoptilolite content of the NZ used in this study was relatively low (50-55%). However, the clinoptilolite content in NZ was proved not to be the prevailing factor for the immobilization of bacteria (Hrenovic *et al.*, 2009). Another species of the genus *Acinetobacter*, *A. junii*, was immobilized in high numbers (1.27×10^{10} CFU/g) onto same NZ of particle size 0.122-0.263mm (Hrenovic *et al.*, 2011). The extent of the immobilization onto NZ of particle size <0.122mmwould surely be greater, since the number of immobilized bacteria increase with decrease of particle size (Hrenovic *et al.*, 2005). Immobilization of *A. baumannii* onto NZ particles in this study was not quantified, but is confirmed microscopically. Therefore, the reduction of biofilm formation and twitching motility is explained by the immobilization of *A. baumannii* onto NZ particles. Obviously, cells of *A. baumannii* were rather attached onto NZ particles than to the surface of polypropylene and polystyrene surfaces of tubes and Petri dish. The affinity of bacteria for NZ could be explained by the rough surface of NZ particles compared 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 and twitching motility.

Inhibition of expression of the virulence factors of *A. baumannii* by NZ is promising in control of this emerging human pathogen. 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.

In conclusion environmental isolates of *A. baumannii* express the virulence factors comparable to the clinical isolates. Isolates sensitive to antibiotics form stronger biofilm

and pellicles than MDR isolates. Cell surface hydrophobicity is an important feature which determines biofilm and pellicle formation, while swarming and twitching motility seem to be strain dependant. The addition of 1% of NZ into the growth medium effectively reduced the twitching motility and biofilm formation due to the immobilization of bacterial cells onto NZ particles, while pellicle formation and swarming motility were 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 adherence and subsequent biofilm formation of this emerging pathogen on abiotic surfaces.

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