NATURAL ZEOLITE INFLUENCE ON THE SURFACE MOTILITY OF ACINETOBACTER BAUMANNII

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ABSTRACT
Acinetobacter baumannii is a human pathogen, emergence of which in hospital acquired infections increased dramatically over the last decade, both in Croatia and worldwide. Resistance of *A. baumannii* to antibiotics, disinfectants, and adverse conditions lead to its long persistence in hospitals. Twitching and swarming surface motility together with biofilm formation are virulence factors that contribute to its pathogenesis. Inhibition of twitching motility as a prerequisite for biofilm formation is promising tool to suppress the virulence of *A. baumannii*. Influence of micronized natural zeolitized tuff (NZ) on twitching and swarming surface motility of 17 isolates of *A. baumannii* was tested. The NZ at concentration 1-3% reduced or completely blocked the twitching motility of *A. baumannii* on polystyrene due to the immobilization of bacterial cells onto NZ particles. The swarming motility on the surface of semisolid medium was not reduced. Micronized NZ could find application in control of the adherence and subsequent biofilm formation of pathogenic bacteria on abiotic surfaces.

Keywords: bacteria; immobilization; twitching; swarming; zeolitized tuff.

INTRODUCTION
Acinetobacter baumannii is an emerging hospital pathogen. Globally distributed isolates of *A. baumannii* are designated as international clones (IC) 1-3, while some isolates still remain nonclonally related. In Croatian hospitals *A. baumannii* belonging to IC1 was evidenced from 2002 and isolates belonging to IC2 from 2009 [1], while nonclonal isolates also persist in some hospitals. Environmental isolates of *A. baumannii* related to clinical isolates were found in wastewaters [2,3], Seine River [4] , and in acid paleosol from Croatia [5]. *A. baumannii* expresses the resistance to multiple antibiotics (MDR) as well as disinfectants, and survives in adverse conditions, leading to long-term persistence in the hospital environment [6]. Additionally, virulence factors that influence the success of *A. baumannii* as a pathogen are its surface motility on solid/semisolid media and ability to form biofilm on abiotic or biotic surfaces. Due to the lack of flagella *A. baumannii* is unable to swim in liquid media. Motility of some isolates is mediated by polar type IV pili [7,8]. Two distinct forms of phenotypic surface motility of *A. baumannii* are recognized: twitching defined as attachment to the solid surfaces and swarming defined as surface translocation on the semisolid media. Twitching motility is an important step in colonization and subsequent biofilm formation on medical devices such as ventilator tubing and catheters, which are one of the main sources of hospital infections with *A. baumannii*. Inhibition of twitching motility as a prerequisite for biofilm formation is promising tool to suppress the virulence of *A. baumannii*. Motility of *A. baumannii* was found to be inhibited by blue light illumination and iron limitation [9,10].
Particles of natural zeolitized tuff (NZ) were shown to display a high affinity for the immobilization of *A. baumannii* cells [11]. Therefore, it was presumed that the addition of NZ

into semisolid medium will result in immobilization of *A. baumannii* cells onto NZ particles, thus hindering their twitching motility. The effect of NZ on the surface motility was studied in the same system.

EXPERIMENTAL

In total 17 isolates of *A. baumannii* which displayed both twitching and swarming motility were chosen for study (Table 1). Eight clinical and 9 environmental isolates possessed different clonality, MDR profile and ability to form biofilm. Biofilm formation was quantified in polypropylene tubes after 24h of static incubation at 37°C by crystal violet staining, ethanol solubilisation and measurement of absorbance at 550nm [7,8].

For surface motility assay micronized (5-10μm) particles of NZ from quarry located at Donje Jesenje, Croatia were used. The mineralogical analysis revealed that NZ sample consisted mostly of clinoptilolite (50-55%) with major constituents being celadonite, plagioclase feldspars and opal-CT (10-15% each). Analcime (another zeolite group mineral) and quartz were present in traces. The isoelectric point of used NZ was at pH = 6.4 [12]. In the autoclaved Luria Bertani medium containing 0.5% agarose 0%, 1% and 3% of dry-autoclaved NZ was added, homogenized, and poured into Petri dishes. Overnight bacterial cultures were suspended in phosphate-buffered saline and inoculated with a pipette tip to the bottom of polystyrene Petri dish. Plates were tightly closed with parafilm to prevent drying and incubated in humid atmosphere at 37°C/24h. Swarming motility was observed at the air-agarose interface. Twitching motility was assessed by removing the agarose layer, staining the dishes with 0.5% crystal violet for 10min, and measuring the longest diameter of motility. Experiments were performed in duplicate with mean values presented. Statistical analyses were carried out using Statistica software 10 (StatSoft, Inc.) and decisions were made at a significance level of $p < 0.05$.

Table 1. Mean features of *A. baumannii* isolates used in study. * resistant to 3 or more classes of antibiotics.

Bacterial isolate	Source	International clonal lineage	Multi-drug resistant*	Biofilm formation (A_{550})
RUH134	reference strain	IC 2	no	1.277
RUH2037	reference strain	IC 1	no	1.474
ST91	wound	IC 2	no	1.250
ST98	eye swab	IC 2	yes	1.417
ST142	catheter swab	IC 2	yes	1.580
ST156	respiratory tract	IC 2	yes	1.561
P25	respiratory tract	IC 1	no	1.387
ŠI125	respiratory tract	non-clonal	no	1.131
Durn	paleosol	IC 1	yes	0.941
IN13	raw sewage	non-clonal	yes	0.979
IN14	raw sewage	non-clonal	yes	0.823
IN16	raw sewage	non-clonal	yes	1.060
IN18	raw sewage	non-clonal	yes	1.560
EF2	treated sewage	non-clonal	yes	1.255
EF4	treated sewage	non-clonal	yes	0.725
EF5	treated sewage	non-clonal	yes	1.594
EF6	treated sewage	non-clonal	yes	0.943

RESULTS AND DISCUSSION

All examined isolates of *A. baumannii* were good biofilm formers, exhibiting the proposed $A_{550} \geq 0.4$ (Table 1). Without addition of NZ the intensity of twitching motility (Table 2) showed significantly positive correlation with biofilm formation ($r = 0.598$), while being independent on the swarming motility. This suggests that the twitching motility is an important prerequisite for biofilm formation of *A. baumannii*. No significant differences in antibiotic resistance profiles, biofilm formation, twitching and swarming surface motility were observed among the clinical and environmental isolates, which suggest that the pathogenic potential of *A. baumannii* does not depend on the strain source.

The addition of NZ (Table 2) decreased the twitching motility of *A. baumannii* isolates, but in the same time increased their swarming motility ($r = -0.307$; $p < 0.05$). By addition of 1% and 3% of NZ reduction of twitching motility averaged 47 and 60%, respectively, while increase of swarming motility averaged 47 and 81%, respectively. Reduction of twitching motility by NZ was significantly higher for environmental isolates (76%) than for clinical isolates of *A. baumannii* (28%). Addition of 1% of NZ in the growth medium completely inhibited the twitching motility of 4 environmental isolates, and addition of 3% of NZ blocked the twitching motility of 6 environmental isolates. The effect of NZ on the swarming motility was not significant among environmental and clinical isolates. Addition of 3% of NZ increased the swarming motility of 4 clinical and 1 environmental isolates to the maximum diameter of 85mm.

Table 2. Effects of different concentrations of NZ on twitching and swarming motility of *A. baumannii* isolates. Percentages of reduction of twitching motility and increase of swarming motility are given in brackets. Minimum diameter of twitching zone was 0mm; maximum diameter of swarming zone was 85mm.

Bacterial	Twitching motility (mm)			Swarming motility (mm)		
isolate	0% NZ	1% NZ	3% NZ	0% NZ	1% NZ	3% NZ
RUH134	26	22 (15)	20 (23)	42	55 (31)	85 (102)
RUH2037	31	27(13)	25 (19)	32	34 (6)	55 (72)
ST91	31	27 (13)	24 (23)	49	60 (22)	64 (31)
ST98	29	25 (14)	20 (31)	71	78 (10)	85 (20)
ST142	32	24 (25)	21 (34)	36	60 (67)	70 (94)
ST156	27	21(22)	16 (41)	40	73 (83)	85 (113)
P 25	45	34 (24)	21 (53)	42	54 (29)	54 (29)
ŠI125	35	25 (29)	12 (66)	56	72 (29)	85 (52)
Durn	32	29 (9)	20 (38)	34	62 (82)	65 (91)
IN13	29	0 (100)	0 (100)	23	31 (35)	38 (65)
IN14	26	10 (62)	0 (100)	37	42 (14)	58 (57)
IN16	31	0 (100)	0 (100)	12	40 (233)	42 (250)
IN18	50	2 (96)	0 (100)	31	52 (68)	60 (94)
EF2	27	0 (100)	0 (100)	77	81 (5)	85 (10)
EF4	20	8 (60)	5 (75)	18	21 (17)	52 (189)
EF5	57	52 (9)	44 (23)	20	25 (25)	25 (25)
EF6	20	0 (100)	0 (100)	49	50 (2)	52 (6)

The clinoptilolite content in the used NZ was relatively low, but similar to 50% in other NZ samples from the same deposit in Croatia [13]. However, the clinoptilolite content in NZ was proved not to be the prevailing factor for the immobilization of bacteria [14]. 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 [12]. The extent of immobilization onto micronized NZ will surely be greater, since the number of immobilized bacteria increase with decrease of particle size [13]. Immobilization of *A. baumannii* onto micronized NZ particles in this study was confirmed microscopically after staining with carbol fuxin dye, but was not quantified. However, the reduction of twitching motility is explained by immobilization of *A. baumannii* onto NZ. Obviously, cells of *A. baumannii* were rather attached onto NZ particles than to the surface of polystyrene Petri dish. Proportion of bacteria which did not migrate to the down part of semisolid medium (where the NZ was more abundant) remained on the surface and swarmed faster than in medium without NZ.

CONCLUSION

Micronized particles of NZ at concentration of 1-3% reduced or completely blocked the twitching motility of *A. baumannii* on polystyrene. Bacterial cells were immobilized onto NZ particles, thus hindering the twitching motility of *A. baumannii*. The swarming motility on the surface of semisolid medium was not reduced by NZ. Micronized NZ is a promising nontoxic material for prevention of adherence of pathogenic bacteria onto abiotic surfaces, and consequently control of the biofilm formation.

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