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Research article

A rapid method for the detection of motility in *Acinetobacter baumannii* and its association to the existence of the virulence-associated genes *pilA* and *algW*

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Abstract

Acinetobacter (*A.*) *baumannii* is one of the major nosocomial pathogens worldwide. It is associated with bloodstream infection, pneumonia, meningitis, urinary tract, soft tissue, and wound infections. Several factors contribute to its survival and spread as a nosocomial pathogen, and motility is often associated with the virulence, fitness, and tenacity of *A. baumannii* on surfaces. In the present study, the correlation between the presence of genes encoding for fimbrial protein PilA and periplasmic protease AlgW and motility was investigated in 87 clinical and non-clinical *A. baumannii* isolates from Germany. *A. baumannii* exhibited robust swimming, swarming, and twitching movement based on the percentage of agar in the medium, as well as the time and temperature of incubation. The swarm motility medium utilizing 2% agar with tetrazolium salts provided an efficient assay for the phenotypic characterization of *A. baumannii* and it was more efficient than the classical motility assays in terms of time, visibility, and biosafety. The presence of the *pilA* gene increased motility of *A. baumannii* but was not required for motility. The *algW* gene was found in 18 strains obtained from milk, all of them with proven phenotypic motility. The rapid detection of motility is essential to evaluate the virulence and fitness of *A. baumannii*. Further studies on the level of genome, transcriptome and proteome are needed to investigate the secrets behind different movement paths in each strain.

Keywords: *Acinetobacter baumannii*, Motility, Tetrazolium salts, Swimming, Swarming, and Twitching

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Introduction

Acinetobacter (*A.*) *baumannii* is an emerging pathogen widely distributed in hospital settings globally. It is a Gram-negative opportunistic bacterial pathogen associated with high morbidity and mortality rates among hospitalized patients (Kumar et al., 2021). A high degree of intrinsic and acquired resistance to different antibiotics, endurance against desiccation, motility, and biofilm formation contribute to its survival in the hospital environment (Gaddy and Actis, 2009; Lee et al., 2017). Although the genus *Acinetobacter* lacks flagella and is described as a non-motile bacterium (Baumann et al., 1968), several studies have shown various types of motility in isolates of the *A. baumannii* complex (Clemmer et al., 2011; Eijkelkamp et al., 2011; Skiebe et al., 2012).

Motility and biofilm formation have been observed to be strongly associated and linked to increased virulence in various bacteria (Josenhans and Suerbaum, 2002; Verstraeten et al., 2008). However, the role of motility and biofilm formation in promoting the virulence of *A. baumannii* is still a matter of debate. Hyper-motility of *A. baumannii* enhances adherence to human pneumocytes and promotes virulence in a nematode infection model (Eijkelkamp et al., 2013). Contrariwise, mutants defective in motility show impaired virulence (Pérez-Varela et al., 2017). A study on clinical isolates of *A. baumannii* revealed that motility might confer a fitness advantage at different predilection sites of the body, and severity and type of infection depend on phenotypic variations of *A. baumannii* (Vijayakumar et al., 2016).

However, several studies have mentioned that *A. baumannii* possesses both twitching and surface-associated motility (Clemmer et al., 2011; Corral et al., 2021), investigation of the mechanisms of biofilm formation and motility in*A. baumannii* isolates is poorly understood. Almost all studies used Luria-Bertani (LB) or fresh-agarose medium plates to investigate the twitching and surface-associated motility in *A.*

baumannii (Skiebe et al., 2012; Corral et al., 2021). Indeed, the bacterial movement appeared as a faint densely branched halo and required extra staining for better visualization. To visualize bacterial movement path that is sticking to the surface, different staining materials such as Coomassie and Crystal violet have been used after removal of the semi-solid medium. However, this step consumes time, destroys the morphotype of moving colonies, and the risk of contamination of the workplace is high.

Therefore, this report aimed to assess a rapid and sensitive method for the efficient detection of motility in *A. baumannii*, utilizing a motility medium containing tetrazolium salts and varying agar concentrations. Additionally, we analyzed the correlation of motility and existence of the *pilA* gene encoding type IV pilus fimbrial protein PilA and the *algW* gene, the regulator of flagellin abundance the periplasmic protease AlgW in *A. baumannii* isolates from human and non-human origin.

Material and Methods

Acinetobacter baumannii isolates

A total of 87 well-characterized *A. baumannii* strains originating from Germany 14 clinical isolates from human origin, 71 non-clinical isolates from milk powder, and two DSMZ control strains (DSM30007 and DSM105126), were used in the current study. The isolates were identified using a combination of *bla*OXA-51-like carbapenemase gene PCR (Turton et al., 2006), together with the MALDI-TOF, and were sequenced using Illumina MiSeq sequencer (Illumina, USA) as previously mentioned (Wareth et al., 2021b). The whole-genome sequences of these strains were analyzed for the presence of the type IV pilus gene *pilA* and the regulator of flagellin abundance *algW* (Wareth et al., 2021a). All isolates either harbored or devoid of *pilA* and *algW* genes were subjected to motility testing using the swarm medium.

Preparation of motility medium

The swarm medium is a semi-solid nutrient medium for the mobility testing of different bacterial species. It is prepared from Brain Heart Infusion (BHI) broth containing 37g basic nutrient brain-heart-broth (Mast Diagnostica Labortechnik, Schleswig-Holstein, Germany) dissolved in 1000 ml sterile H_2O_2. Agar (1-3%) was added to the broth and then heated until wholly dissolved and thoroughly mixed. The pH7 was adjusted and the content was autoclaved for 15 min at 121°C and then cooled down to 45°C. Then 0.05g (0.5% w/v) triphenyl tetrazolium chloride (TTC) colorless crystals (Merck KGaA, Darmstadt, Germany) were added to be as an indicator for bacterial growth. The TTC or simply tetrazolium chloride with the formula (2,3,5-Triphenyltetrazolium chloride) is a redox indicator commonly used in biochemical experiments to indicate cellular respiration (Villegas-Mendoza et al., 2019). The final content of the agar medium is clear and yellowish. Around 6 ml of medium were poured in 15 ml tubes, and 25 ml were poured in Petri dishes (100x15 mm) to give a double layer of medium and then cooled and kept at 2-10°C until use.

Testing of movement

A single fresh colony of each *A. baumannii* strain was picked from blood agar using the needle tip of a plastic loop and stabbed into swarm agar [BHI (3.7%, w/v), agar (1-3%; w/v), pH 7 containing (0.5%; w/v) TTC]. To examine surface motility, a single colony was inoculated between the media and the bottom of a polystyrene Petri dish or between the wall and the media in a polystyrene test tube. To examine the ability of *A. baumannii* to grow at different temperatures, all inoculated tubes and dishes were incubated in triplicate at 4°C, room temperature, and 37°C. The inoculated tubes and dishes were monitored, photographed every 12 hrs, and changes were documented. In parallel, the motility was tested by motility assays using LB media and crystal violet staining as described previously (Shamim et al., 2014; Biswas et al., 2019). Each experiment was conducted three times, and each strain was tested in duplicate.

Results and discussion

Although *A. baumannii* is described historically as a non-motile bacterium due to lack of flagella, several clinical isolates showed different patterns of movement (Eijkelkamp et al., 2011; Corral et al., 2021). In the current study, the swarm medium containing tetrazolium salts was used to investigate the motility in well-characterized clinical (n=14) and non-clinical (n=71), and two control isolates. The appearance of insoluble red formazan crystals in both tubes and Petri dishes was considered proof of motility. No growth or motility was seen in tubes incubated at 4°C for up to 7 days. Colour development was observed after 72 hrs in test tubes and Petri dishes incubated at room temperature and after 24 hrs in those incubated at 37°C.

Acinetobacter spp. are able to grow at temperatures between 20–44°C and clinical isolates grow readily at 37°C (Wisplinghoff, 2017). It has been shown that twitching motility in *A. baumannii* is thermoregulated, and its surface-associated motility has been reduced significantly at 28°C compared to strains grown at 37°C (De Silva et al., 2018). In the current study, some super and weak motile *A. baumannii* strains at 37°C showed slower motility and non-motility at room temperature, respectively (Table 1). It is worth mentioning that tubes and dishes kept at room temperature were facing light. Previously, motility and biofilm formation were improved in *A. baumannii* when bacterial cells were incubated in darkness (Mussi et al., 2010).

In Petri dishes and tubes containing 1% agar, some *A. baumannii* strains with the *pilA* gene showed robust swimming motility within 15 hrs, while motility became visible after 24 hrs in some strains devoid of *pilA*. However, the 1% swarm agar medium was semi-liquid, and the dishes were difficult to handle. The swimming movement is the most general type of motility and is defined as the diffuse movement of bacteria in an unorganized pattern inside the liquid or low viscosity medium (Shamim et al., 2014).

Table 1: The description of the results regarding the presence of *pilA* and *algW* genes, and different temperatures with association with the motility results.

Isolates	*pilA*	*algW*	*pilA*+*algW*	Temperature [1]		
				37°C	RT	4°C
Milk powder (n=33/71)	-	-	13	12 motile - 1 weak motile	12 motile - 1 non-motile	13 non-motile
	15	-	-	15 motile	14 motile - 1 non-motile	15 non-motile
	-	5	-	5 motile	5 motile	5 non-motile
Humans (n=1/14)	1	-	-	Weak motile	Non-motile	Non-motile
DSMZ-1[2]	1	-	-	Motile	Motile	Non-motile
DSMZ-2[3]	1	-	-	Weak motile	Non-motile	Non-motile
Total (n=36/87)	18	5	13			

[1]Room temperature at 20-25°C for three days; In the fridge at 4°C for one week.

[2]DSMZ-1: DSM105126.

[2]DSMZ-2: DSM30007.

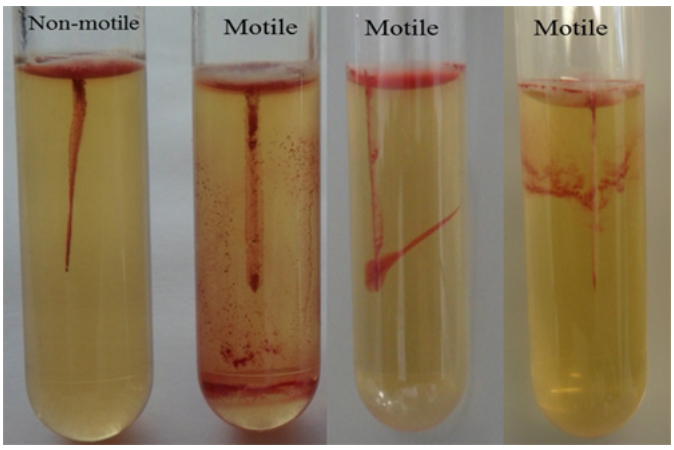

Figure 1: Motility of *A. baumannii* in tubes containing 2% swarm agar medium after 48 hrs at 37°C. The strains of *A. baumannii* harbor *pilA* gene showed a different pattern of movement and a strain lack of the *pilA* gene showed no motility.

In Petri dishes and tubes containing 2% agar, the red formazan crystals required 24 hrs to be detected and became clearly visible after 48 hrs. Strains containing the *pilA* gene did grow faster. The red colour was difficult to be detected in Petri dishes and tubes containing 3% agar before 48 hrs (Table 2). When a single colony was inoculated between the wall of the tubes and the medium, *A. baumannii* exhibited evident surface-associated motility faster than inside the medium. Each strain was tested in duplicate and each experiment was conducted three times. Each strain of *A. baumannii* showed a different pattern of movement (Figure 1).

Detection of the motility was more prominent and easier to be seen in Petri dishes than in tubes. Movement of *A. baumannii* was easy to be seen in Petri dishes containing 2% agar within 16 hr in case of super motile with *pilA* gene and became clear and prominent after 24 hr. The movement was detected in Petri dishes containing 3% agar after three days post-incubation. The swarming (coordinated and organized motility), as well as the twitching (bacterial translocation on a solid surface in a less organized manner than swarming), were clear and the distinct motility path was easy to distinguish in 2% agar medium kept at 37°C for 16 hrs and became prominent after 24 hrs (Figure 2). When a 3% swarm agar medium was used, movement on the plates was not seen before 48 hrs of growth.

The use of LB medium to monitor motility in *A. baumannii* has restrictions in the evaluation of the result as the uniform faded pale appearance must be stained to be evaluated. The path of movement and shape of motile colonies was difficult to identify and require either a high-resolution camera with specific light facilities or extra staining with crystal violet (Figure 3A). It is worth mentioning that staining of motility path for one hour and several washing steps (3x5 minutes) by phosphate buffer saline (PBS) is time-consuming and may result in contamination of the environment. Moreover, these procedures destroy the movement path (Figure 3B).

Additionally, crystal violet is a triphenylmethane dye extensively used as a biological stain, mutagenic and bacteriostatic agent in human and veterinary medicine. However, it acts as a mitotic poison, a potent carcinogen, and poses toxic effects on the environment (Mani and Bharagava, 2016). Compared to the classical motility assay, the current method utilizing swarm agar with tetrazolium salts reduced possible contamination and further spread of bacteria consider-

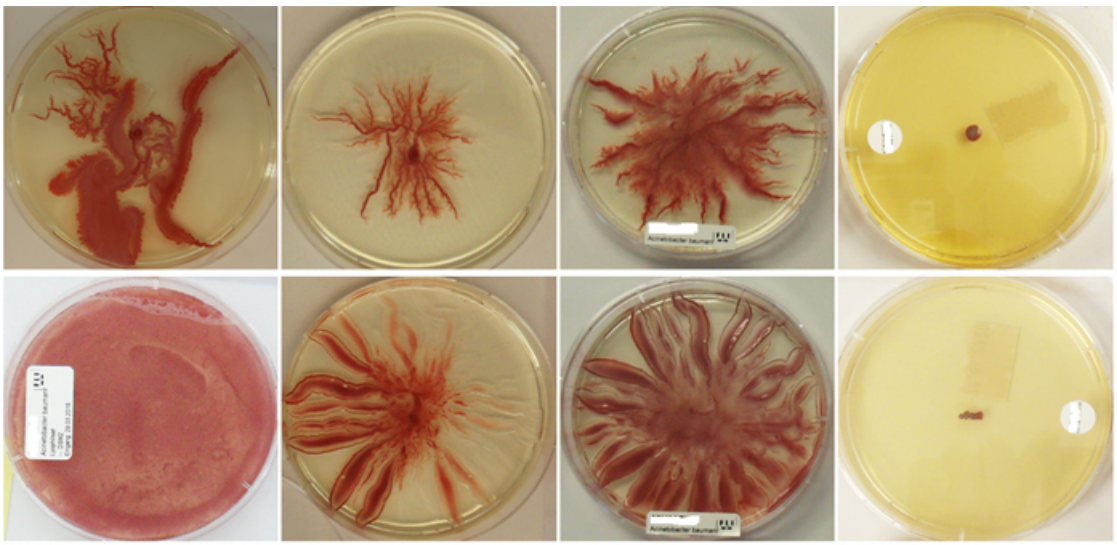

Figure 2: Two super motile *A. baumannii* strains harbored *pilA* gene showing the random swimming pattern of motility in 1% swarm agar medium (A), and swarming and twitching surface movement in 2% swarm agar medium after 15 hrs (B) and 24 hrs (C), and absence of movement in 3% swarm agar medium after 24 hrs (D). The upper strain was a nonclinical strain isolated from milk powder, and the lower is the DSMZ strain (DMS105126).

Table 2: The proportionality between the percentage of agar in swarm media and the movement of *A. baumannii* strains in association with the existence and absence of the *pilA* gene.

Agar concentration	***pilA* in genome**	**Time required to be detected (hour)**	**Assessment**[1]
1%	+	12	- Swimming motility.
	-	24	- Semi-liquid medium and dishes were difficult to be handled.
2%	+	24	- Swarming and twitching movement.
	-	48	- Semi-solid medium and was easy to be handled.
3%	+	<72	- Movement difficult to be detected within first 48 hrs, semi-hard solid.
	-	<72	

[1]Swimming: diffuse unorganized movement; Swarming: coordinated and organized motility; Twitching: bacterial translocation on a solid surface in a less organized manner than swarming.

ably. It also reduced the hands-on time as no staining and washing are required. An additional advantage is that the different distinct shapes of the motility path can easily be recognized with the naked eye (Figure 3C).

The BHI has been used as a culture medium since it is a highly nutritious medium (http://www.usbio.net/item/B2701-07). It is recommended to cultivate fastidious pathogenic microorganisms and is also used to prepare the inoculate for antimicrobial susceptibility testing. Tetrazolium salts were firstly described in 1894. However, since the early 1950s, they have been used in broad applications in microbiology due to their reducing function (Pegram, 1972). The TTC is a colorless, water-soluble non-specific substrate for microorganisms. It can form water-insoluble red triphenylformazan crystals after reduction and can be seen easily with the naked eye. Thus, it has been used as a colorimetric dye to detect the presence of bacteria. The formation of red formazan can be considered a qualitative indication of activity. The TTC has a toxic effect only on Gram-positive bacteria leading to impaired growth (Pegram, 1969; Junillon et al., 2012). However, no adverse effect has been reported on the growth of the Gram-negative bacteria, making it a suitable indicator for the growth of *A. baumannii*.

Different kinds of motility were seen in either tubes or on plates, and the phenotype of *A. baumannii* was easy to define by the naked eye without further staining. The motility allows bacteria to search for nutrients and survive under adverse environmental conditions. The swimming pattern was best observed using a 1% agar medium in Petri dishes, while swarming and twitching surface movement was more pronounced in the 2% agar medium. The bacteria could twitch and swarm on swarm agar better than on LB media, the classical medium used.

Eighty-seven isolates of clinical and non-clinical *A.*

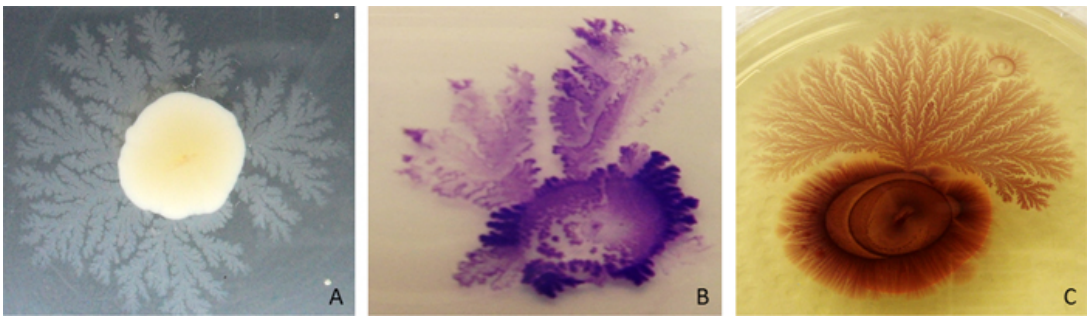

Figure 3: A super-motile *A. baumannii* nonclinical strain isolated from milk powder and harbored *pilA* gene showing twitching motility on 1% LB media (37°C, 72 hrs) without tetrazolium salt represented by faint pale shape colonies (A), stained with crystal violet (1 hr and washed 3x5 min by PBS) (B), and on 3% swarm agar medium contain tetrazolium salt (37°C, >72 hrs) (C).

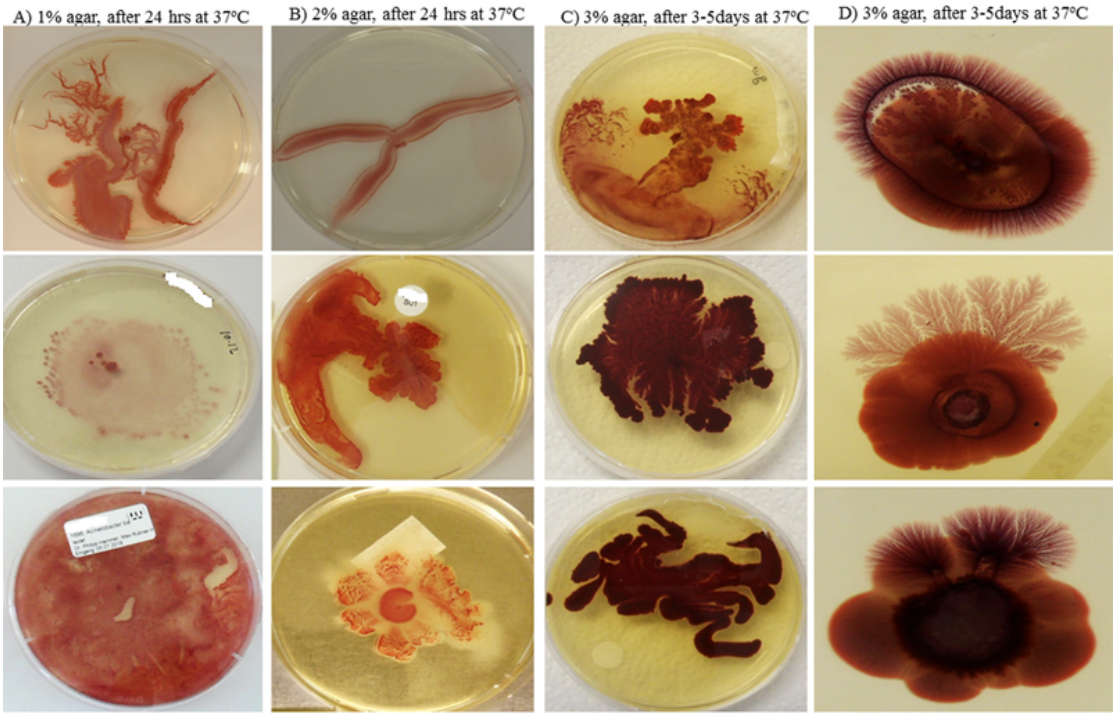

Figure 4: Three different motile *A. baumannii* strains harbored *pilA* gene showed different patterns of motility on 1-3% swarm agar medium at 37°C: (A) 1% after 24 hrs; (B) 2% after 24 hrs; (C-D) 3% after 3-5 days.

baumannii were tested. Among them, eleven strains were non-motile and were missing the *pilA*. Seventy-six strains showed different patterns of motility, among them, only 31 isolates harbored the *pilA* gene and 45 were missing the *pilA*. Only one clinical isolate, the two control strains, and 28 isolates from milk powder were demonstrated to harbor the *pilA* gene, while the *algW* gene was present in 18 isolates from milk powder. Interestingly, each isolate showed a different style of motility, and the movement path of each isolate was unique (Figure 4).

The movement was detected in strains harboring and not harboring the *pilA* gene. Those thirty-one isolates harboring *pilA* genes were faster in movement than strains lacking *pilA*. Therefore, they are considered super motile strains. Type IV pili PilA is composed of protein that is essential for twitching motility, biofilm and microcolony formation, cell adhesion, virulence, and natural competence (Richter et al., 2017; Ronish et al., 2019). It is also required for the phototactic movement of some bacteria (Bhaya et al., 1999). The genes encoding type IV pili are found universally in non-flagellated, Gram-negative pathogenic *A. baumannii*, but there is considerable variation in the *pilA* genes, both in amino acid sequence and in glycosylation patterns (Ronish et al., 2019). The mechanism and role of type IV pili in the movement of *A. baumannii* is still a subject of discussion. The presence of the *pilA* gene improves the movement of *A. baumannii*, which was associated with hypermotility patterns. However, its existence was not necessary for motility, suggesting additional, still unknown other factors contributing to the motility in *A. baumannii*.

The periplasmic protease encoded *algW* gene was found in 18 *A. baumannii* isolates obtained from powdered milk and all were motile. The AlgW protein reg-

ulates multiple virulence mechanisms in bacteria such as *Pseudomonas syringae* (Schreiber and Desveaux, 2011). It is considered a key negative regulator of flagellin abundance, while its association with bacterial movement is not completely understood. Rapid detection of motility of *A. baumannii* is essential in epidemiological studies, as well as in designing and performing experiments to evaluate the virulence and fitness of this pathogenic bacterium. The swarm agar medium containing tetrazolium salt provides an efficient method to detect motility in *A. baumannii* and may be used to assess biofilm formation. Further investigations are required to fully understand the mechanisms of motility, adherence, and biofilm formation in *A. baumannii* and their roles in promoting virulency.

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