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DNA uptake by the nosocomial pathogen *Acinetobacter baumannii* occurs while moving along wet surfaces

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**RUNNING TITLE**

Natural competence of *Acinetobacter baumannii*

**KEYWORDS**

*Acinetobacter baumannii* – natural competence – DNA uptake – twitching motility – type IV pili – antibiotic resistance – nosocomial pathogen
ABSTRACT

The emergence of *Acinetobacter baumannii* as an increasingly multidrug-resistant nosocomial pathogen largely relies on acquisition of resistance genes via horizontal gene transfer. Here, we demonstrate that many clinical isolates of *A. baumannii* take up DNA while they move along wet surfaces. We show that both motility and DNA uptake is abolished after inactivation of *pilT*, putatively encoding the type 4 pilus (T4P) retraction ATPase, and *comEC*, putatively encoding the DNA uptake channel, respectively. Inactivation of *pilT* correlates with an increase in the number and length of pili with an average diameter of 7.2 nm. In the *Galleria mellonella* infection model the *comEC* mutant is significantly attenuated whereas the *pilT* mutant is not, dissecting biologically distinct roles of T4P and the DNA uptake channel. Collectively, these findings promote our understanding of the mechanisms of DNA uptake and resistance development in *A. baumannii* which may also apply to other important pathogens.

INTRODUCTION

The capability of *A. baumannii* to undergo horizontal gene transfer (HGT) events considerably contributes to the alarming resistance development of this emerging pathogen (1-6). However, while the low-pathogenic relative *A. baylyi* ADP1 (BD4) is a model organism to study DNA uptake from the environment (7-12), to date only a single isolate of *A. baumannii* has been shown to be naturally competent for transformation (13).

It is long known that members of the genus *Acinetobacter*, though lacking flagella, can move along wet surfaces in an intermittent and jerky way termed twitching motility (14-16). Henrichsen & Blom were the first to propose that *Acinetobacter* twitching motility was related to the expression of polar fimbriae (16, 17). Since that time, twitching motility has been
intensively studied in many genera including *Myxococcus, Neisseria, Pseudomonas* and *Haemophilus* firmly establishing that this form of movement is powered by depolymerization of type 4 pili (T4P) (18-21). Only very recently *Acinetobacter* motility was further elucidated, providing evidence that at least in part it is driven by means of T4P in *A. baumannii* (22-26). Specifically, inactivation of *pilT*, putatively encoding an ATPase responsible for T4P retraction, reduced surface motility by roughly 50% (24). Residual activity observed with this *pilT* mutant could be due to the *pilT* parologue *pilU* known to be present in many representatives of *A. baumannii* (23). Alternatively, another mode of surface-associated motility could be active under the same experimental conditions. Actually, forms of motility seemingly different from twitching have been described for *Acinetobacter*. Barker and Maxted (27) found that when *Acinetobacter* strains were stab-inoculated into semi-solid media some showed surface motility called “swarming” while others exhibited spreading at the bottom of the Petri dish beneath the medium or both forms in parallel. In addition, spreading at the surface sometimes was found to be accompanied by the formation of ditches in the agar surface and no signs of jerking movements were found by these authors under the conditions tested (27). Even though phenotypically distinct, all forms of motility described for *A. baumannii* have been shown to depend on synthesis of the polyamine 1,3-diaminopropane (26). Surface motility of *A. baumannii* was further shown to be controlled by blue light sensing (28), quorum sensing (24, 29) and depending on iron availability (30, 31).

Besides conveying twitching motility, T4P also permit DNA uptake in a number of Gram-negative bacteria (19) and in *Neisseria gonorrhoeae*, for example, T4P are involved in both motility and DNA uptake (32). The mechanistic role that T4P play in DNA uptake is not clearly defined but requires the secretin PilQ for DNA passage through the outer membrane (33, 34). Transport via the inner membrane is mediated by a ComA/ComEC membrane channel (12, 33, 35). While *A. bayyi* harbours a *comA*-like transporter gene that has been shown to be required for natural transformation (36), *A. baumannii* harbours a *comEC*-like gene (37) for
which no functional characterization is published to date and which exhibits only about 50% identity to *A. baylyi* ComA on the protein level.

Sequencing of *A. baumannii* genomes is steadily revealing that members of this species are in extensive genetic exchange with related species and also across the genus, family and order barrier suggesting that natural competence could contribute to this continuous DNA uptake (1, 4, 37, 38). Although an apparently complete set of genes required for natural transformation competence seems to be present in *A. baumannii* (23, 25, 37) to date only a single isolate has been described to be naturally competent (13). Given the potential role of T4P in surface-associated motility of *A. baumannii* (17, 23-26) and their established contribution to DNA uptake in various species (12, 19, 35) we speculated that *A. baumannii* might develop competence for DNA uptake while moving along wet surfaces in a T4P-dependent manner.

**MATERIALS AND METHODS**

**Motility and transformation.** Motility plates were composed of 0.5% agarose, 5 g/l tryptone and 2.5 g/l NaCl as described (26). The inoculum was stabbed into the semi-solid medium to enable spread of bacteria at both the surface of the medium and the interphase between the bottom of the Petri dish and the medium. Two alternative transformation procedures were performed. The transforming DNA (30 μg per plate) can be either added to the molten medium immediately before pouring the plates. The plates were then inoculated by stabbing with a pipette tip. A single colony from a blood agar plate stored in the fridge for no longer than two weeks was touched with the pipette tip which was then stabbed into the DNA-doped motility plate seven times. Alternatively, the DNA can be mixed with the inoculum of bacteria and can then be stabbed into the motility medium (seven times, pipetting 2 μl of the mixture with each stabbing). To this end a suspension of bacteria (generated from a single
colony resuspended in 20 μl of sterile PBS) is produced and mixed with equal volumes of the transforming DNA (400 ng/μL). The precise OD of the bacterial suspension had no significant effect on the transformation efficiency. This latter procedure yielded higher transformation rates compared to the standard procedure where the transforming DNA (30 μg per plate) was mixed into the medium prior to pouring into Petri dishes albeit at the expense of somewhat increased variance. The method using mixtures of bacteria and DNA was also used to determine the transformation rates given in Table 1. After inoculation, the transformation plates were sealed with parafilm to prevent desiccation which proved detrimental to both motility and transformation efficiency. The plates were incubated for 18 hours at 37°C. The bacteria were then flushed off the motility medium with 1 ml of sterile PBS. The suspension was adjusted to 10 optical densities (so that the tenfold dilution yielded an OD₆₀₀nm of 1.0) and 100 μl was plated on the appropriate selective agar (typically 30 μg/ml of kanamycin). Tenfold dilution series were performed from the OD-adjusted PBS suspension to determine the number of colony forming units (CFU) for calculation of transformation rates (number of transformants divided by total CFU). Chromosomal DNA for transformation experiments was purified with the MasterPure DNA Purification Kit (Epicentre Biotechnologies). Sterility of transforming DNA was controlled by plating. Effective transformation with DNA from ATCC 17978 mutants 27 and 179, respectively, was confirmed by PCR on selected colonies after sub-culturing of these colonies. Direct colony-PCR from the selection plate is not recommended since the background of transforming DNA as well as the background of DNA from killed bacteria can lead to ambiguous results. Subsequently, DNA sequencing was performed to confirm homologous recombination events. Phenotypic features such as motility morphotypes were used as additional controls. DNase I treatment of the mixture of transforming DNA and bacterial inoculum significantly reduced the transformation rates while treatment of the bacteria flushed off the motility plates with DNase I did not interfere with the transformation rates.
Plasmid transformation was studied with a derivative of pWH1266 (39), designated pWH1266::Km, which was isolated from *E. coli* DH5α. Plasmid pWH1266 confers resistance to ampicillin and tetracycline. Since all ten naturally competent isolates are sensitive to kanamycin but not all are sensitive to either ampicillin or tetracycline, we mutagenized the plasmid with transposon EZ-Tn5 <KAN-2> (Epicentre Biotechnologies) to obtain pWH1266::Km. Transposon insertion after nucleotide position 207 (39) as verified by DNA sequencing did not interfere with plasmid stability or copy number. Effective transformation with plasmid pWH1266::Km was confirmed by isolation of the plasmid from a number of colonies and detection of the Km\(^R\) cassette in the pWH1266 background by PCR. To this end forward primer FP3 5’-GAGTTGAAGGATCAGATCACGC-3’ binding inside EZ-Tn5 <KAN-2> and reverse primer pWH1266-rev1 5’-GCCTAGAACGTACATAGGAAGCG-3’ binding inside pWH1266 were combined resulting in a PCR product of approx. 1250 bp.

*A. baumannii* mutants. Transforming DNA was obtained from transposon mutant derivatives of *A. baumannii* ATCC 17978 mutagenized with the EZ-Tn5 <KAN-2> transposon (Epicentre Biotechnologies). Screening of a transposon mutant library of *A. baumannii* ATCC 17978 for motility phenotypes revealed a motility-deficient mutant with a transposon insertion in A1S_2610, encoding a homologue of the ComEC competence protein family. Since ATCC 17978 is unable to move at the interphase between the medium and the bottom of the Petri dish (26), we used the chromosomal DNA of this *comEC::Km* mutant to transform naturally competent isolates 07-095, 07-102 and DSM 30011 exhibiting motility at the interphase. Chromosomal DNA of *A. baumannii* M2 pilT::Km (24) was obtained from Philip N. Rather.

Electron microscopy studies. Appropriate strains were stab-inoculated seven times into motility agarose (0.5% agarose) and incubated at 37°C for approximately 18 h. Colonies formed on the agarose surface were gently resuspended in 0.9 ml of HEPES buffer (mixture of
0.85 ml H₂O plus 0.05 ml of 1M HEPES pH7.2) and the cells subsequently fixed by addition of 0.1 ml of paraformaldehyde (20%). The agarose layer was then removed from the Petri dishes and the bacteria sticking to the polystyrene Petri dishes (“interphase”) were gently resuspended in HEPES buffer and fixed as above. Due to the poor growth of the pilT and comEC mutants at the interphase, these strains were stab-inoculated ten times on each plate and material obtained from three plates was pooled in 1 ml of buffer to yield enough bacteria. Actually, these mutants exhibited no spread at the interphase but formed colonies at the sites of stab-inoculation.

Negative staining electron microscopy was conducted as described by Laue and Bannert (40). Briefly, suspensions of fixed bacteria were applied onto sample supports (drop-on-grid procedure) that have been pre-treated with alcian blue or by glow discharge. After brief washes on distilled water, adsorbed bacteria were stained with uranyl acetate (0.5% in water). Samples were inspected with a transmission electron microscope (Tecnai12, FEI Corp.) operated at 120 kV. Images were taken using a 1k slow-scan CCD-camera (MegaviewIII, Olympus Soft Imaging Solutions). Measurements at high resolution were calibrated by using a precise calibration standard (Magical, Technoorg-Linda Ltd.).

**Galleria mellonella infection model.** Infection of waxmoth larvae was performed as described recently (26).
RESULTS

Do *A. baumannii* isolates take up DNA while they move? To challenge this hypothesis, we selected 28 clinical isolates of *A. baumannii* from our collection sensitive to the antibiotic kanamycin (Km). We performed transformation experiments using chromosomal DNA of Km-resistant transposon mutant derivatives of *A. baumannii* strain ATCC 17978. We doped a semi-solid medium facilitating surface-associated motility with the transforming DNA and subsequently inoculated *A. baumannii* isolates to allow them to move along the wet surface. Fig. 1 illustrates the morphotypic variance among the isolates under these conditions. After 18 hours, the bacteria were rinsed off and plated on kanamycin plates to select for transformants (Materials & Methods, Table 1). We identified 10 out of 28 isolates (36%) that were competent for the uptake of the naked DNA. Transformation rates varied depending on isolates and on the locus of homologous recombination with rates ranging from $3 \times 10^{-3}$ to $6 \times 10^{-8}$ for the most efficiently transforming DNA (Table 1). Only 5 of the 10 naturally competent isolates could be transformed with the plasmid tested, a derivative of pWH1266 (39) harbouring an insertion of transposon EZ-Tn5 Kan2 (Table 1). The transformation competence and efficiency appeared unpredictable from the motility phenotypes and did not correlate with the velocity of motility.

In contrast to *A. baylyi* BD413 (9) and *A. baumannii* A118 (13) planktonic cells of our isolates were not naturally competent. While competence of *A. baylyi* BD413 depends on the growth phase and reaches its maximum during early logarithmic growth (41) we could not observe transformation of *A. baumannii* isolates under any condition other than in association with motility. In effect, when we spread the bacteria on DNA-doped solid medium which did not permit movement of the bacteria and which differed only in the concentration of agarose (1.5% instead of 0.5%) from transformation-permissive conditions, not a single transformation event was detectable with any of our strains. Also, addition of 3-5 μg of transforming DNA...
(chromosomal DNA of ATCC 17978 transposon mutants or plasmid pWH1266::Km) to 3 ml of logarithmic LB cultures (cultures with OD$_{600nm}$ of 0.5, 1 or 2 were tested) followed by 1 hour of incubation at 37°C before plating on selective agar did not yield a single transformant. Further, addition of transforming DNA (3-5 μg) to pellicle forming cultures (3 ml incubated at 20 and 37°C) produced no transformants. Collectively, the ten naturally competent isolates described here appeared transformable only while moving on semi-solid media.

**Impact of pilT inactivation on motility and natural competence.** Our discovery of a direct coupling of motility and DNA uptake suggests the involvement of T4P and competence proteins mediating DNA import. To challenge this hypothesis, we first made use of a recently described pilT mutant of *A. baumannii* M2 (24). Also illustrating the methodological impact of our finding, we used chromosomal DNA of this mutant to generate pilT mutant derivatives of our naturally competent isolates 07-095 and 07-102 (Fig. 2). The pilT disruption abolished spread of the mutant bacteria at the boundary between the semi-solid medium and the bottom of the Petri dish (“interphase” motility) but had comparably little influence on motility along the air-medium boundary (“surface”). Surface motility of mutant 07-102 pilT::Km was slightly elevated compared to its parental strain (Fig. 2A) while surface motility of 07-095 pilT::Km was unaffected (Fig. 2B). Taken together, this may suggest that motility at the interphase is indeed driven by T4P and therefore may represent twitching motility as recently claimed by others (23, 25). Moreover, we could demonstrate that pilT inactivation annihilated natural transformation competence in both isolates (Fig. 3).

**Impact of comEC inactivation on motility and natural competence.** Next, to further characterize the mechanistic coupling of motility and DNA uptake, we studied the impact of comEC inactivation on motility and transformation properties. Orthologues of comEC are required for DNA uptake in different bacteria (12, 42). A comEC::Km transposon mutant derivative of *A. baumannii* ATCC 17978 was recently identified in a screen for mutations affecting motility (unpublished results). Since ATCC 17978 was not naturally competent in our
hands we used the chromosomal DNA of the ATCC 17978 comEC::Km mutant to transform naturally competent isolates 07-095 and 07-102. We found that inactivation of comEC abolished both twitching motility at the interphase and natural transformation competence (Fig. 4). Motility at the surface was also significantly reduced in line with the identification of the ATCC 17978 comEC::Km mutant in the course of a screening for motility defects.

**Electron microscopy studies reveal a hyperpiliation phenotype of pilT mutants.**

We then applied transmission electron microscopy (TEM) to identify T4P in *A. baumannii* and to determine the influence of pilT and comEC inactivation on the piliation state. To this end, naturally competent isolates 07-095 and 07-102 and their pilT and comEC mutant derivatives were stab-inoculated into motility-agarose and the bacteria collected from the surface and the interphase. In accordance with published work on *Acinetobacter* pili (17, 43-45) thin (~ 4 nm wide) and thick (~ 7 nm wide) pili could be observed. In both parental strains the thick pili were only rarely found in surface-grown bacteria (approx. 1 pilus per 25-50 cells with a typical length of up to 2 μm) and even more sporadic in the interphase-derived preparations (Table 2). By contrast, with both pilT mutants in average more than one thick pilus was found per cell in surface-derived preparations and the length of the pili was significantly increased compared to the parental strains (typically between 2 and 6 μm) (Fig. 5A and B). Even more pili were found in the pilT mutant preparations derived from the interphase (more than 3-5 pili per cell). With regard to the comEC mutant phenotypes the strains differed. While the comEC mutant of 07-095 was similar to the pilT mutant (Fig. 5C), thick pili were only sporadically found in 07-102 comEC::Km. Taken together, our data demonstrate a hyperpiliation phenotype of the pilT mutants regarding the thick pili suggesting that these represent indeed T4P. These supposable T4P have an average diameter of 7.2 nm (standard deviation ±1 nm) as determined from n = 109 individual measurements on 20 pili.

**Dissection of independent functions of pilT and comEC in the Galleria mellonella infection model.** Finally, we additionally generated pilT and comEC mutants of naturally
competent strains DSM 30011 and 10-096 to study the applicability of natural competence for rapid generation of mutants and to compare the mutants in the *Galleria mellonella* caterpillar infection model (46). While we were able to introduce *pilT::Km* into strain 10-096 by natural transformation with chromosomal DNA derived from *A. baumannii* M2 *pilT::Km* (24), we were unsuccessful in generating 10-096 *comEC::Km* using donor DNA from ATCC 17978 *comEC::Km*, 07-095 *comEC::Km*, and 07-102 *comEC::Km* although we had confirmed the presence of the *comEC* locus in strain 10-096. Conversely, we successfully generated DSM 30011 *comEC::Km* using ATCC 17978 *comEC::Km* donor DNA while we failed to generate a *pilT* mutant despite confirmed presence of the *pilT* gene in DSM 30011. Detailed sequence analyses of donor and acceptor sites may pave the way to identification of determinants that restrict uptake and recombination events in these strains.

The *pilT* and *comEC* derivatives of the naturally competent isolates 07-095, 07-102, 10-096 and DSM 30011 were then characterized in the *Galleria mellonella* infection model in comparison to their parental strains (Fig. 6). Consistently, these experiments revealed a significant attenuation of the *comEC* mutants in all strains tested (Fig. 6A, 6B, 6D) whereas *pilT* mutants were not significantly attenuated (Fig. 6B, 6C) compared to their parental strains or was only marginally attenuated in the case of 07-102 *comEC::Km* (Fig. 6A; compare parental strain and mutant 48 hours and 72 hours post infection). Collectively, these data demonstrate that *comEC* fulfils an important function during infection and that PilT-driven T4P retraction is dispensable under these conditions.

**DISCUSSION**

*A. baumannii* genomes are significantly formed by HGT events (1-4). This is particularly true with respect to genetic determinants conferring antibiotic resistance which have been presumably acquired in part from distinctly related species belonging to the
Enterobacteriaceae and Pseudomonas (1). The apparent formation of so-called genetic exchange communities (47) is further illustrated by the recent finding that a potent resistance determinant, the New Delhi metallo-\(\beta\)-lactamase 1 (NDM-1) first discovered in Klebsiella pneumoniae and Escherichia coli (48), probably originated in Acinetobacter (49) and can be transferred among A. baumannii isolates via natural transformation competence (50). Mechanistically, conjugative transfer can only partially explain the multitude of HGT events in Acinetobacter, given that tra and mob genes required for conjugative transfer are missing on most sequenced Acinetobacter plasmids (38). Recently, another possible HGT pathway was identified in A. baumannii showing that outer membrane vesicles can mediate transfer of resistance genes (51). Hitherto, only a single isolate of A. baumannii was known to be competent for DNA uptake (13). Here, we add to the understanding of HGT in A. baumannii, demonstrating natural competence in 10 out of 28 (36%) antibiotic-sensitive clinical isolates. Next, we will investigate if natural competence is prevalent among multi-drug resistant isolates, as this may indicate it contributes to the acquisition of novel resistance genes. Owing to their multi-drug resistance, it is technically difficult and problematic from an ethical point of view to transform these isolates with other resistance genes. Therefore we need to develop alternative methods for the phenotypic display of transformation events.

So far, the only representatives of the genus Acinetobacter known to be naturally competent were A. baylyi ADP1 (BD4) (7-12) and A. baumannii A118 (13). Both are transformable when grown in liquid cultures with ADP1 known to reach highest competence during early logarithmic growth (41). However, we failed to transform any of our competent isolates under comparable conditions suggesting significant regulatory and/or mechanistic differences. Interestingly, the ComA DNA uptake channel known to be involved in competence of A. baylyi ADP1 (36) is only about 50% identical to ComEC of A. baumannii. It remains to be determined whether A. baumannii A118 harbours an uptake channel of the ComA or the ComEC type to further estimate whether different uptake channels could contribute to the
mechanistic differences. Another significant difference in the endowment with competence genes between *A. baylyi* and *A.baumannii* as figured out by Smith et al. (37) refers to *A. baylyi* *comP* which encodes a pilin-like protein involved in DNA uptake but obviously not involved in pilus formation (45).

Inactivation of *pilT* has been studied in a number of bacteria exhibiting twitching motility. In *Neisseria gonorrhoeae* inactivation of *pilT* abolished both natural transformation and twitching motility even though the amount and length of T4P was found unaffected (32). Similarly, T4P-driven motility was abolished in the *pilT* mutant of *Myxococcus xanthus* while piliation was apparently unaffected (52). By contrast and similar to our observations, *pilT* inactivation in *Pseudomonas aeruginosa* resulted in a hyperpiliation phenotype (53, 54) and the same was also found in *Synechocystis* sp. PCC6803 (55).

Mechanistically, our data suggest that in *A. baumannii* T4P are required for motility at the interphase as this form of motility was abolished upon *pilT* inactivation. Thus, as already suggested by others (17, 23, 25) this form of motility can be well termed twitching motility now. The finding that *pilT* inactivation can interfere with but not abolish surface motility as demonstrated here and as described by Clemmer *et al.* (24) suggests that T4P are expressed under these conditions as has been demonstrated here but are not the (only) driving force of surface motility. Our finding that T4P are expressed both at the surface and the interphase is further compatible with our observation that transformants could be obtained by flushing off bacteria from only the surface or the interphase. To control whether transformation rates were different at the surface and at the interphase we mixed the DNA with the agarose medium prior to casting the plates to produce a medium with a constant DNA concentration. After stab-inoculation, the bacteria were then separately recovered from surface and interphase and no significant difference in the transformation rates at both sites could be observed (data not shown). Collectively, transformation occurs at both sites of motility and correlates with the presence of T4P.
It will be interesting to learn whether the unprecedented direct mechanistic coupling of motility and DNA uptake applies to other bacteria. A number of pathogens harboring T4P including *Pseudomonas aeruginosa* and enterohemorrhagic *E. coli* (EHEC) are highly suspicious of being competent given the excessive HGT documented in their genomes, but to date have not been shown to undergo transformation naturally (56-58).

Our finding that the *comEC* mutants are attenuated in the *Galleria mellonella* infection model while the *pilT* mutants are not is unexpected. To our knowledge, this is the first time that DNA uptake channels of the *comA/comEC* type have been implicated in virulence. This could point to a role of the channel independent of DNA uptake and T4P-dependent motility. Alternatively, it is tempting to speculate that DNA uptake could become important during infection as a way to open up DNA as a nutrient source. However, the fact that *pilT* inactivation abolished DNA uptake on motility plates but had little to no effect on virulence argues against this speculation. The contribution of DNA uptake channels to virulence should now be tested in other pathogens and other infection models. Targeting DNA uptake systems might become an interesting strategy to suppress virulence and resistance development of pathogens in the hospital environment.

**AUTHORS’ CONTRIBUTIONS**

GW conceived of the study. GW, JP, ML and ES performed experiments, analysed and interpreted the data. GW wrote the manuscript. All authors read and approved the final manuscript.

**ACKNOWLEDGEMENTS**
We would like to thank Philip N. Rather for providing chromosomal DNA of *A. baumannii* M2 *pilT::Km* and Paul G. Higgins and Christine Heider for critical reading of a previous version of this manuscript.

**LEGENDS TO FIGURES**

**Fig. 1: Transformation of *A. baumannii* on motility medium.** Semi-solid medium facilitating surface motility (26) was doped with transforming DNA and inoculated with *A. baumannii* (the medium was stabbed four times with A: DSM 30011; B: 10-096). The plates were incubated overnight at 37°C and the bacteria floated off the medium the next day and plated on selective medium. The arrowhead indicates the frontline of growth at the “interphase” (between medium and bottom of Petri dish).

**Fig. 2: Inactivation of *pilT* in *A. baumannii* abolishes twitching-like motility.** *A. baumannii* isolates 07-095 and 07-102 were transformed on motility plates as described using chromosomal DNA derived from *A. baumannii* M2 *pilT::Km* to generate *pilT* mutants 07-095 *pilT::Km* and 07-102 *pilT::Km*, respectively. Of the mutants, three independent colonies were inoculated each on a motility plate together with the respective parental strain. The photos shown were taken after incubation for 18 hours at 37°C and subsequent incubation for 24 hours at 20°C. The latter incubation was solely to intensify the biofilm formed at the interphase (arrowheads) to facilitate photography.

**Fig. 3: Inactivation of *pilT* annihilates natural transformation competence of *A. baumannii*.** Mutant strain 07-095 *pilT::Km* and its parental strain were incubated on motility plates with or without plasmid pWH1266 (39) conferring resistance to ampicillin and tetracycline. The bacteria were then floated off the motility plates and after adjustment of
optical densities the bacteria were plated on selective LB agar plates containing 20 μg/ml of oxytetracycline to select for transformants (A). While the parental strain 07-095 was transformed, its 07-095 pilT::Km mutant derivative was not. (B) Isolate 07-102, which is unable to take up plasmid pWH1266 by natural competence (see Table 1), and its mutant 07-102 pilT::Km were incubated on motility plates doped with or without chromosomal DNA derived from the streptomycin-resistant isolate 07-105 and subsequently plated on selective LB agar with streptomycin (20 μg/ml) (B). The 07-102 pilT::Km mutant was not transformable in contrast to its parental strain.

Fig. 4: Inactivation of comEC in A. baumannii abolishes twitching-like motility and natural transformation competence. A. baumannii isolates 07-095 and 07-102 were transformed on motility plates as described using chromosomal DNA derived from A. baumannii ATCC 17978 comEC::Km to generate comEC mutants 07-095 comEC::Km and 07-102 comEC::Km, respectively. (A) Subsequently, both mutants and their respective parentals were inoculated into motility medium as described. Motility at the interphase was observed with the parental strains (arrowheads) but not with the mutant derivatives. (B) To prove an involvement of comEC in natural competence, 07-095 comEC::Km and its parental strain were incubated on motility plates with or without plasmid pWH1266 conferring resistance to ampicillin and tetracycline. The bacteria were then floated off the motility plates and after adjustment of optical densities the bacteria were plated on LB agar plates containing 100 μg/ml of ampicillin to select for transformants. While strain 07-095 was readily transformable, its comEC-inactivated derivative was not.

Fig. 5: Transmission electron microscopy (TEM) reveals a hyperpiliation phenotype of pilT mutants. Images show representative cells of naturally competent A. baumannii 07-095 (A) and its pilT (B) and comEC (C) mutant derivatives. In the pilT::Km (B) and comEC::Km
(C) mutants number of pili and length are increased in comparison to the wild type (A) (compare also Table 2).

Fig. 6: The comEC locus is important for virulence in the Galleria mellonella infection model while pilT is not. Galleria mellonella caterpillars were infected with A. baumannii strains as indicated or mock infected with PBS. The number of bacteria used for infection (determined as colony forming units; CFU) was \( \sim 10^6 \) for isolates 07-095 and DSM 30011 as well as their mutant derivatives, and \( \sim 5 \times 10^5 \) for isolates 07-102 and 10-096 and respective derivatives. The average of three independent replicates (groups of 16 larvae each) is plotted with error bars representing +/- one standard deviation.

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Table 1

<table>
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<tr>
<th>Strain</th>
<th>Mutant 27 DNA (Mean transformation rate (SD))</th>
<th>Mutant 179 DNA (Mean transformation rate (SD))</th>
<th>Plasmid pWH1266::Km (Mean transformation rate (SD))</th>
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</tr>
<tr>
<td>10-096</td>
<td>7.75E-06 (3.21E-06)</td>
<td>5.72E-04 (2.76E-04)</td>
<td>9.28E-07 (1.03E-07)</td>
</tr>
<tr>
<td>DSM 30011</td>
<td>2.59E-06 (8.98E-07)</td>
<td>1.09E-04 (1.26E-04)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>BMBF 320</td>
<td>2.94E-06 (1.50E-06)</td>
<td>1.07E-05 (3.09E-06)</td>
<td>1.36E-06 (5.22E-07)</td>
</tr>
</tbody>
</table>

1 Three independent experiments
2 Four independent experiments
3 Two independent experiments

Legend to Table 1:

Transformation rates of ten naturally competent *A. baumannii* isolates. To obtain chromosomal DNA for transformation experiments *Acinetobacter baumannii* ATCC 17978 was mutagenized with transposon EZ-Tn5 <KAN-2> (Epicentre Biotechnologies) as previously described (26). From resulting mutants 27 and 179 harboring transposon insertions in genes A1S_2167 (encoding cytochrome o ubiquinol oxidase subunit I) and A1S_2846 (encoding sulfite reductase), respectively, chromosomal DNA was purified. Plasmid transformation was studied with a derivative of pWH1266 (39), designated pWH1266::Km. Transformation experiments were performed as described in the Materials and Methods section.
### Table 2

<table>
<thead>
<tr>
<th>Strain/sample</th>
<th>7 nm pili</th>
<th>rel. frequency of 7 nm pili</th>
</tr>
</thead>
<tbody>
<tr>
<td>07-095</td>
<td>rarely, but regularly (~1 pilus per 50 cells)</td>
<td>+</td>
</tr>
<tr>
<td>07-095 pilT::Km surface</td>
<td>&gt; 1 per cell</td>
<td>++</td>
</tr>
<tr>
<td>07-095 comEC::Km surface</td>
<td>~1 per cell</td>
<td>++</td>
</tr>
<tr>
<td>07-102</td>
<td>rarely, but regularly (~1 pilus per 25-50 cells)</td>
<td>+</td>
</tr>
<tr>
<td>07-102 pilT::Km surface</td>
<td>≥ 1 per cell</td>
<td>++</td>
</tr>
<tr>
<td>07-102 comEC::Km surface</td>
<td>a single sporadic pilus detected</td>
<td>*</td>
</tr>
<tr>
<td>07-095 interphase</td>
<td>sporadic</td>
<td>+</td>
</tr>
<tr>
<td>07-095 pilT::Km interphase</td>
<td>&gt;3 per cell on average</td>
<td>+++</td>
</tr>
<tr>
<td>07-095 comEC::Km interphase</td>
<td>~1 per cell</td>
<td>++</td>
</tr>
<tr>
<td>102/07 pilT::Km interphase</td>
<td>&gt;5 per cell on average</td>
<td>+++</td>
</tr>
<tr>
<td>102/07 comEC::Km interphase</td>
<td>no pilus detected</td>
<td>*</td>
</tr>
</tbody>
</table>

**Legend to Table 2:**

Evaluation of TEM negative staining of *A. baumannii* obtained from motility plates. 7 nm pili: - = no pili or single detection; + = sporadic or up to 1 pilus per 25-50 cells; ++ = ~1 pilus per cell; +++ = >3 pilus per cell on average; unbiased estimation of the pili distribution on the cells was not possible, because bacteria formed cluster on the sample supports.
Wilharm et al.
Figure 1
Wilharm et al.
Figure 2
Wilharm et al.
Figure 3

A

07-095 07-095 + pWH1266

07-095 pili\textsuperscript{\text{::}Km} 07-095 pili\textsuperscript{\text{::}Km} + pWH1266

B

07-102 07-102 + DNA\textsubscript{07-105}

07-102 pili\textsuperscript{\text{::}Km} 07-102 pili\textsuperscript{\text{::}Km} + DNA\textsubscript{07-105}
Figure 4

A

07-095

07-095 comEC::Km

07-102

07-102 comEC::Km

B

07-095

07-095 + pWH1266

07-095 comEC::Km

07-095 comEC::Km

+ pWH1266
Wilharm et al.
Figure 6

A

\[ \text{no. of surviving caterpillars} \]
\[ \text{time [h]} \]

PBS
- 07-102
- 07-102 comEC::Km
- 07-102 pilT::Km

B

\[ \text{no. of surviving caterpillars} \]
\[ \text{time [h]} \]

PBS
- 07-095
- 07-095 comEC::Km
- 07-095 pilT::Km

C

\[ \text{no. of surviving caterpillars} \]
\[ \text{time [h]} \]

PBS
- 10-096
- 10-096 pilT::Km

D

\[ \text{no. of surviving caterpillars} \]
\[ \text{time [h]} \]

PBS
- DSM 30011
- DSM 30011 comEC::Km