Originally published as:

Wilharm, G., Piesker, J., Laue, M., Skiebe, E.
DNA Uptake by the nosocomial pathogen acinetobacter baumannii occurs during movement along wet surfaces
(2013) Journal of Bacteriology, 195 (18), pp. 4146-4153.

DOI: 10.1128/JB.00754-13

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

Gottfried Wilharm[1,*], Janett Piesker[2], Michael Laue[2], and Evelyn Skiebe[1]

[1] Robert Koch-Institute, Wernigerode Branch, Burgstr. 37, D-38855 Wernigerode, Germany

[2] Robert Koch-Institute, Nordufer 20, D-13353 Berlin, Germany

*Address correspondence to:

Gottfried Wilharm, Robert Koch-Institut, Bereich Wernigerode, Burgstr. 37,

D-38855 Wernigerode, Germany

Phone: +49 3943 679 282; Fax: +49 3943 679 207

e-mail: wilharmg@rki.de

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* paralogue *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. baylyi* 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 OD_{600nm} 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'-GCCTAGAACGTCATAGGAAGCG-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

153 0.85 ml H_2O plus 0.05 ml of 1M HEPES pH7.2) and the cells subsequently fixed by addition of
154 0.1 ml of paraformaldehyde (20%). The agarose layer was then removed from the Petri dishes
155 and the bacteria sticking to the polystyrene Petri dishes ("interphase") were gently resuspended
156 in HEPES buffer and fixed as above. Due to the poor growth of the *pilT* and *comEC* mutants at
157 the interphase, these strains were stab-inoculated ten times on each plate and material obtained
158 from three plates was pooled in 1 ml of buffer to yield enough bacteria. Actually, these mutants
159 exhibited no spread at the interphase but formed colonies at the sites of stab-inoculation.
160 Negative staining electron microscopy was conducted as described by Laue and Bannert
161 (40). Briefly, suspensions of fixed bacteria were applied onto sample supports (drop-on-grid
162 procedure) that have been pre-treated with alcian blue or by glow discharge. After brief washes
163 on distilled water, adsorbed bacteria were stained with uranyl acetate (0.5% in water). Samples
164 were inspected with a transmission electron microscope (Tecnai12, FEI Corp.) operated at 120
165 kV. Images were taken using a 1k slow-scan CCD-camera (MegaviewIII, Olympus Soft
166 Imaging Solutions). Measurements at high resolution were calibrated by using a precise
167 calibration standard (Magical, Technoorg-Linda Ltd.).
168
169 ***Galleria mellonella* infection model.** Infection of waxmoth larvae was performed as
170 described recently (26).
171

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 $3x10^{-3}$ to $6x10^{-8}$ 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

198 (chromosomal DNA of ATCC 17978 transposon mutants or plasmid pWH1266::Km) to 3 ml
199 of logarithmic LB cultures (cultures with OD_{600nm} of 0.5, 1 or 2 were tested) followed by 1 hour
200 of incubation at 37°C before plating on selective agar did not yield a single transformant.
201 Further, addition of transforming DNA (3-5 µg) to pellicle forming cultures (3 ml incubated at
202 20 and 37°C) produced no transformants. Collectively, the ten naturally competent isolates
203 described here appeared transformable only while moving on semi-solid media.

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

218 **Impact of *comEC* inactivation on motility and natural competence.** Next, to further
219 characterize the mechanistic coupling of motility and DNA uptake, we studied the impact of
220 *comEC* inactivation on motility and transformation properties. Orthologues of *comEC* are
221 required for DNA uptake in different bacteria (12, 42). A *comEC::Km* transposon mutant
222 derivative of *A. baumannii* ATCC 17978 was recently identified in a screen for mutations
223 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

276 *Enterobacteriaceae* and *Pseudomonas* (1). The apparent formation of so-called genetic
277 exchange communities (47) is further illustrated by the recent finding that a potent resistance
278 determinant, the New Delhi metallo-β-lactamase 1 (NDM-1) first discovered in *Klebsiella*
279 *pneumoniae* and *Escherichia coli* (48), probably originated in *Acinetobacter* (49) and can be
280 transferred among *A. baumannii* isolates via natural transformation competence (50).
281 Mechanistically, conjugative transfer can only partially explain the multitude of HGT events in
282 *Acinetobacter*, given that *tra* and *mob* genes required for conjugative transfer are missing on
283 most sequenced *Acinetobacter* plasmids (38). Recently, another possible HGT pathway was
284 identified in *A. baumannii* showing that outer membrane vesicles can mediate transfer of
285 resistance genes (51). Hitherto, only a single isolate of *A. baumannii* was known to be
286 competent for DNA uptake (13). Here, we add to the understanding of HGT in *A. baumannii*,
287 demonstrating natural competence in 10 out of 28 (36%) antibiotic-sensitive clinical isolates.
288 Next, we will investigate if natural competence is prevalent among multi-drug resistant
289 isolates, as this may indicate it contributes to the acquisition of novel resistance genes. Owing
290 to their multi-drug resistance, it is technically difficult and problematic from an ethical point of
291 view to transform these isolates with other resistance genes. Therefore we need to develop
292 alternative methods for the phenotypic display of transformation events.

293 So far, the only representatives of the genus *Acinetobacter* known to be naturally
294 competent were *A. baylyi* ADP1 (BD4) (7-12) and *A. baumannii* A118 (13). Both are
295 transformable when grown in liquid cultures with ADP1 known to reach highest competence
296 during early logarithmic growth (41). However, we failed to transform any of our competent
297 isolates under comparable conditions suggesting significant regulatory and/or mechanistic
298 differences. Interestingly, the ComA DNA uptake channel known to be involved in competence
299 of *A. baylyi* ADP1 (36) is only about 50% identical to ComEC of *A. baumannii*. It remains to
300 be determined whether *A. baumannii* A118 harbours an uptake channel of the ComA or the
301 ComEC type to further estimate whether different uptake channels could contribute to the

302 mechanistic differences. Another significant difference in the endowment with competence
303 genes between *A. baylyi* and *A. baumannii* as figured out by Smith et al. (37) refers to *A. baylyi*
304 *comP* which encodes a pilin-like protein involved in DNA uptake but obviously not involved in
305 pilus formation (45).

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

313 Mechanistically, our data suggest that in *A. baumannii* T4P are required for motility at
314 the interphase as this form of motility was abolished upon *pilT* inactivation. Thus, as already
315 suggested by others (17, 23, 25) this form of motility can be well termed twitching motility
316 now. The finding that *pilT* inactivation can interfere with but not abolish surface motility as
317 demonstrated here and as described by Clemmer *et al.* (24) suggests that T4P are expressed
318 under these conditions as has been demonstrated here but are not the (only) driving force of
319 surface motility. Our finding that T4P are expressed both at the surface and the interphase is
320 further compatible with our observation that transformants could be obtained by flushing off
321 bacteria from only the surface or the interphase. To control whether transformation rates were
322 different at the surface and at the interphase we mixed the DNA with the agarose medium prior
323 to casting the plates to produce a medium with a constant DNA concentration. After stab-
324 inoculation, the bacteria were then separately recovered from surface and interphase and no
325 significant difference in the transformation rates at both sites could be observed (data not
326 shown). Collectively, transformation occurs at both sites of motility and correlates with the
327 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*[9] 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 ~10^6 for isolates 07-095 and DSM 30011 as well as their mutant derivatives, and ~5x10^5 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.

REFERENCES

1. **Fournier PE, Vallenet D, Barbe V, Audic S, Ogata H, Poirel L, Richet H, Robert C, Mangenot S, Abergel C, Nordmann P, Weissenbach J, Raoult D, Claverie JM.** 2006. Comparative genomics of multidrug resistance in Acinetobacter baumannii. PLoS genetics **2:**e7.
2. **Mussi MA, Limansky AS, Relling V, Ravasi P, Arakaki A, Actis LA, Viale AM.** 2011. Horizontal gene transfer and assortative recombination within the Acinetobacter baumannii clinical population provide genetic diversity at the single carO gene, encoding a major outer membrane protein channel. Journal of bacteriology **193:**4736-4748.
3. **Sahl JW, Johnson JK, Harris AD, Phillippy AM, Hsiao WW, Thom KA, Rasko DA.** 2011. Genomic comparison of multi-drug resistant invasive and colonizing Acinetobacter baumannii isolated from diverse human body sites reveals genomic plasticity. BMC genomics **12:**291.
4. **Snitkin ES, Zelazny AM, Montero CI, Stock F, Mijares L, Program NCS, Murray PR, Segre JA.** 2011. Genome-wide recombination drives diversification of epidemic strains of Acinetobacter baumannii. Proceedings of the National Academy of Sciences of the United States of America **108:**13758-13763.
5. **Peleg AY, Seifert H, Paterson DL.** 2008. Acinetobacter baumannii: emergence of a successful pathogen. Clinical microbiology reviews **21:**538-582.
6. **Forsberg KJ, Reyes A, Wang B, Selleck EM, Sommer MO, Dantas G.** 2012. The shared antibiotic resistome of soil bacteria and human pathogens. Science **337:**1107-1111.
7. **Juni E, Janik A.** 1969. Transformation of Acinetobacter calco-aceticus (Bacterium anitratum). Journal of bacteriology **98:**281-288.

8. **Palmen R, Vosman B, Buijsman P, Breek CK, Hellingwerf KJ.** 1993. Physiological characterization of natural transformation in Acinetobacter calcoaceticus. Journal of general microbiology **139:**295-305.
9. **Lorenz MG, Wackernagel W.** 1994. Bacterial gene transfer by natural genetic transformation in the environment. Microbiological reviews **58:**563-602.
10. **Metzgar D, Bacher JM, Pezo V, Reader J, Doring V, Schimmel P, Marliere P, de Crecy-Lagard V.** 2004. Acinetobacter sp. ADP1: an ideal model organism for genetic analysis and genome engineering. Nucleic acids research **32:**5780-5790.
11. **Elliott KT, Neidle EL.** 2011. Acinetobacter baylyi ADP1: transforming the choice of model organism. IUBMB life **63:**1075-1080.
12. **Averhoff B, Friedrich A.** 2003. Type IV pili-related natural transformation systems: DNA transport in mesophilic and thermophilic bacteria. Archives of microbiology **180:**385-393.
13. **Ramirez MS, Don M, Merkier AK, Bistue AJ, Zorreguieta A, Centron D, Tolmasky ME.** 2010. Naturally competent Acinetobacter baumannii clinical isolate as a convenient model for genetic studies. Journal of clinical microbiology **48:**1488-1490.
14. **LAUTROP H.** 1961. Bacterium anitratum transferred to the genus Cytophaga. International Bulletin of Bacteriological Nomenclature and Taxonomy **11:**107-108.
15. **Lautrop H.** 1965. Gliding motility in bacteria as a taxonomic criterion. Publ. Fac. Sci. Univ. JE Purkyne, ser K **35:**322-327.
16. **Henrichsen J.** 1972. Bacterial surface translocation: a survey and a classification. Bacteriological Reviews **36:**478.
17. **Henrichsen J, Blom J.** 1975. Correlation between twitching motility and possession of polar fimbriae in Acinetobacter calcoaceticus. Acta pathologica et microbiologica Scandinavica. Section B, Microbiology **83:**103-115.
18. **Merz AJ, So M, Sheetz MP.** 2000. Pilus retraction powers bacterial twitching motility. Nature **407:**98-102.
19. **Pelicic V.** 2008. Type IV pili: e pluribus unum? Molecular microbiology **68:**827-837.
20. **Burrows LL.** 2005. Weapons of mass retraction. Molecular microbiology **57:**878-888.
21. **Mattick JS.** 2002. Type IV pili and twitching motility. Annual review of microbiology **56:**289-314.
22. **Henrichsen J.** 1983. Twitching motility. Annual review of microbiology **37:**81-93.
23. **Antunes LC, Imperi F, Carattoli A, Visca P.** 2011. Deciphering the multifactorial nature of Acinetobacter baumannii pathogenicity. PloS one **6:**e22674.
24. **Clemmer KM, Bonomo RA, Rather PN.** 2011. Genetic analysis of surface motility in Acinetobacter baumannii. Microbiology **157:**2534-2544.
25. **Eijkelkamp BA, Stroeher UH, Hassan KA, Papadimitrious MS, Paulsen IT, Brown MH.** 2011. Adherence and motility characteristics of clinical Acinetobacter baumannii isolates. FEMS microbiology letters **323:**44-51.
26. **Skiebe E, de Berardinis V, Morczinek P, Kerrinnes T, Faber F, Lepka D, Hammer B, Zimmermann O, Ziesing S, Wichelhaus TA, Hunfeld KP, Borgmann S, Grobner S, Higgins PG, Seifert H, Busse HJ, Witte W, Pfeifer Y, Wilharm G.** 2012. Surface-associated motility, a common trait of clinical isolates of Acinetobacter baumannii, depends on 1,3-diaminopropane. International journal of medical microbiology : IJMM **302:**117-128.
27. **Barker J, Maxted H.** 1975. Observations on the growth and movement of Acinetobacter on semi-solid media. Journal of medical microbiology **8:**443-446.
28. **Mussi MA, Gaddy JA, Cabruja M, Arivett BA, Viale AM, Rasia R, Actis LA.** 2010. The opportunistic human pathogen Acinetobacter baumannii senses and responds to light. Journal of bacteriology **192:**6336-6345.

29. **Stacy DM, Welsh MA, Rather PN, Blackwell HE.** 2012. Attenuation of quorum sensing in the pathogen Acinetobacter baumannii using non-native N-Acyl homoserine lactones. ACS Chem Biol **7:**1719-1728.
30. **Eijkelkamp BA, Hassan KA, Paulsen IT, Brown MH.** 2011. Investigation of the human pathogen Acinetobacter baumannii under iron limiting conditions. BMC genomics **12:**126.
31. **McQueary CN, Kirkup BC, Si Y, Barlow M, Actis LA, Craft DW, Zurawski DV.** 2012. Extracellular stress and lipopolysaccharide modulate Acinetobacter baumannii surface-associated motility. J Microbiol **50:**434-443.
32. **Wolfgang M, Lauer P, Park HS, Brossay L, Hebert J, Koomey M.** 1998. PilT mutations lead to simultaneous defects in competence for natural transformation and twitching motility in piliated Neisseria gonorrhoeae. Molecular microbiology **29:**321-330.
33. **Krüger N-J, Stingl K.** 2011. Two steps away from novelty – principles of bacterial DNA uptake. Molecular microbiology **80:**860-867.
34. **Assalkhou R, Balasingham S, Collins RF, Frye SA, Davidsen T, Benam AV, Bjoras M, Derrick JP, Tonjum T.** 2007. The outer membrane secretin PilQ from Neisseria meningitidis binds DNA. Microbiology (Reading, England) **153:**1593-1603.
35. **Claverys JP, Martin B.** 2003. Bacterial "competence" genes: signatures of active transformation, or only remnants? Trends Microbiol **11:**161-165.
36. **Friedrich A, Hartsch T, Averhoff B.** 2001. Natural transformation in mesophilic and thermophilic bacteria: identification and characterization of novel, closely related competence genes in Acinetobacter sp. strain BD413 and Thermus thermophilus HB27. Appl Environ Microbiol **67:**3140-3148.
37. **Smith MG, Gianoulis TA, Pukatzki S, Mekalanos JJ, Ornston LN, Gerstein M, Snyder M.** 2007. New insights into Acinetobacter baumannii pathogenesis revealed by high-density pyrosequencing and transposon mutagenesis. Genes & development **21:**601-614.
38. **Fondi M, Bacci G, Brilli M, Papaleo MC, Mengoni A, Vaneechoutte M, Dijkshoorn L, Fani R.** 2010. Exploring the evolutionary dynamics of plasmids: the Acinetobacter pan-plasmidome. BMC evolutionary biology **10:**59.
39. **Hunger M, Schmucker R, Kishan V, Hillen W.** 1990. Analysis and nucleotide sequence of an origin of DNA replication in Acinetobacter calcoaceticus and its use for Escherichia coli shuttle plasmids. Gene **87:**45-51.
40. **Laue M, Bannert N.** 2010. Detection limit of negative staining electron microscopy for the diagnosis of bioterrorism-related micro-organisms. J Appl Microbiol **109:**1159-1168.
41. **Palmen R, Vosman B, Kok R, van der Zee JR, Hellingwerf KJ.** 1992. Characterization of transformation-deficient mutants of Acinetobacter calcoaceticus. Molecular microbiology **6:**1747-1754.
42. **Kruger NJ, Stingl K.** 2011. Two steps away from novelty--principles of bacterial DNA uptake. Molecular microbiology **80:**860-867.
43. **Gohl O, Friedrich A, Hoppert M, Averhoff B.** 2006. The thin pili of Acinetobacter sp. strain BD413 mediate adhesion to biotic and abiotic surfaces. Appl Environ Microbiol **72:**1394-1401.
44. **Grzanka A, Gospodarek E, Domaniewski J.** 1996. A comparison of three staining methods in estimation of structures on the cell wall surface of Acinetobacter junii by using electron microscope. Acta Microbiol Pol **45:**233-239.
45. **Porstendorfer D, Gohl O, Mayer F, Averhoff B.** 2000. ComP, a pilin-like protein essential for natural competence in Acinetobacter sp. Strain BD413: regulation, modification, and cellular localization. Journal of bacteriology **182:**3673-3680.

46. **Peleg AY, Jara S, Monga D, Eliopoulos GM, Moellering RC, Jr., Mylonakis E.** 2009. Galleria mellonella as a model system to study Acinetobacter baumannii pathogenesis and therapeutics. Antimicrobial agents and chemotherapy **53:**2605-2609.
47. **Skippington E, Ragan MA.** 2011. Lateral genetic transfer and the construction of genetic exchange communities. FEMS microbiology reviews **35:**707-735.
48. **Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, Walsh TR.** 2009. Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrobial agents and chemotherapy **53:**5046-5054.
49. **Toleman MA, Spencer J, Jones L, Walsh TR.** 2012. blaNDM-1 is a chimera likely constructed in Acinetobacter baumannii. Antimicrobial agents and chemotherapy **56:**2773-2776.
50. **Pfeifer Y, Wilharm G, Zander E, Wichelhaus TA, Gottig S, Hunfeld KP, Seifert H, Witte W, Higgins PG.** 2011. Molecular characterization of blaNDM-1 in an Acinetobacter baumannii strain isolated in Germany in 2007. The Journal of antimicrobial chemotherapy **66:**1998-2001.
51. **Rumbo C, Fernandez-Moreira E, Merino M, Poza M, Mendez JA, Soares NC, Mosquera A, Chaves F, Bou G.** 2011. Horizontal transfer of the OXA-24 carbapenemase gene via outer membrane vesicles: a new mechanism of dissemination of carbapenem resistance genes in Acinetobacter baumannii. Antimicrobial agents and chemotherapy **55:**3084-3090.
52. **Wu SS, Wu J, Kaiser D.** 1997. The Myxococcus xanthus pilT locus is required for social gliding motility although pili are still produced. Molecular microbiology **23:**109-121.
53. **Bradley DE.** 1974. The adsorption of Pseudomonas aeruginosa pilus-dependent bacteriophages to a host mutant with nonretractile pili. Virology **58:**149-163.
54. **Whitchurch CB, Hobbs M, Livingston SP, Krishnapillai V, Mattick JS.** 1991. Characterisation of a Pseudomonas aeruginosa twitching motility gene and evidence for a specialised protein export system widespread in eubacteria. Gene **101:**33-44.
55. **Bhaya D, Bianco NR, Bryant D, Grossman A.** 2000. Type IV pilus biogenesis and motility in the cyanobacterium Synechocystis sp. PCC6803. Molecular microbiology **37:**941-951.
56. **Sinha S, Redfield RJ.** 2012. Natural DNA uptake by Escherichia coli. PloS one **7:**e35620.
57. **van Schaik EJ, Giltner CL, Audette GF, Keizer DW, Bautista DL, Slupsky CM, Sykes BD, Irvin RT.** 2005. DNA binding: a novel function of Pseudomonas aeruginosa type IV pili. Journal of bacteriology **187:**1455-1464.
58. **Xicohtencatl-Cortes J, Monteiro-Neto V, Saldana Z, Ledesma MA, Puente JL, Giron JA.** 2009. The type 4 pili of enterohemorrhagic Escherichia coli O157:H7 are multipurpose structures with pathogenic attributes. Journal of bacteriology **191:**411-421.

587 **Table 1**

Strain	Mutant 27 DNA Mean transformation rate (SD)[1]	Mutant 179 DNA Mean transformation rate (SD)[2]	Plasmid pWH1266::Km Mean transformation rate (SD)[3]
07-028	5,85E-08 (6,78E-08)	1,29E-06 (8,27E-07)	0 (0)
07-099	0 (0)	6,44E-08 (1,04E-07)	0 (0)
07-095	4,09E-06 (1,06E-06)	1,13E-04 (2,56E-05)	4,53E-06 (1,61E-06)
07-101	4,34E-07(2,56E-07)	5,39E-05 (3,51E-05)	3,91E-08 (3,91E-08)
07-102	5,82E-05 (5,25E-06)	2,62E-03 (7,12E-04)	0 (0)
07-105	5,90E-08 (4,82E-08)	2,41E-06 (1,17E-06)	5,16E-08 (5,16E-08)
07-111	1,87E-07 (8,50E-09)	6,37E-06 (4,09E-06)	0 (0)
10-096	7,75E-06 (3,21E-06)	5,72E-04 (2,76E-04)	9,28E-07 (1,03E-07)
DSM 30011	2,59E-06 (8,98E-07)	1,99E-04 (1,26E-04)	0 (0)
BMBF 320	2,94E-06 (1,50E-06)	1,07E-05 (3,09E-06)	1,36E-06 (5,22E-07)

588
589
590 [1] Three independent experiments
591 [2] Four independent experiments
592 [3] Two independent experiments
593
594

595 **Legend to Table 1:**

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

601

602 **Table 2**
603
604

Strain/sample	7 nm pili	rel. frequency of 7 nm pili
07-095 surface	rarely, but regularly (~1 pilus per 50 cells) length: ≤ 2 µm	+
07-095 *pilT::Km* surface	> 1 per cell length: 2-6 µm	++
07-095 *comEC::Km* surface	~1 per cell length: ≥ 2 µm	++
07-102 surface	rarely, but regularly (~1 pilus per 25-50 cells) length: ≤ 2 µm	+
07-102 *pilT::Km* surface	≥ 1 per cell length: 2-6 µm	++
07-102 *comEC::Km* surface	a single sporadic pilus detected	-
07-095 interphase	sporadic	+
07-095 *pilT::Km* interphase	>3 per cell on average length: short and long (≥ 2 µm)	+++
07-095 *comEC::Km* interphase	~1 per cell length: ≥ 2 µm	++
102/07 interphase	sporadic amount and length not determinable	+
102/07 *pilT::Km* interphase	>5 per cell on average length: short and long (≥ 2 µm)	+++
102/07 *comEC::Km* interphase	no pili detected	-

605
606

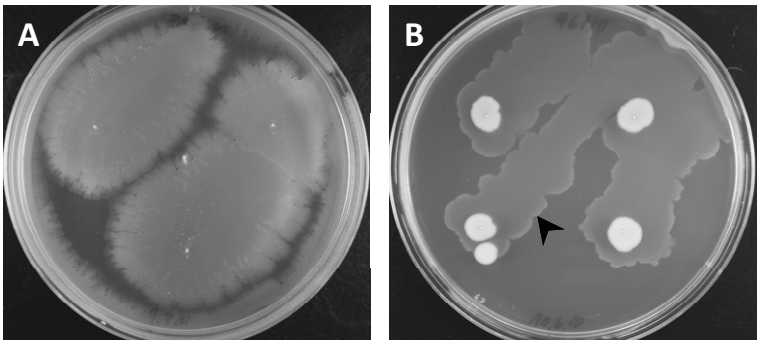
607 **Legend to Table 2:**

608 **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

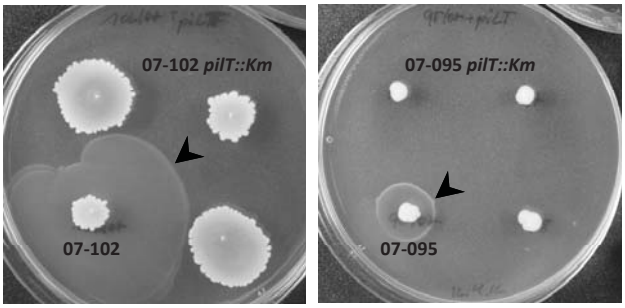
609 25-50 cells,; ++ = ~1 pilus per cell; +++ = ≥ 3 pili per cell on average; unbiased estimation of the pili distribution on the cells was not possible, because bacteria

610 formed cluster on the sample supports

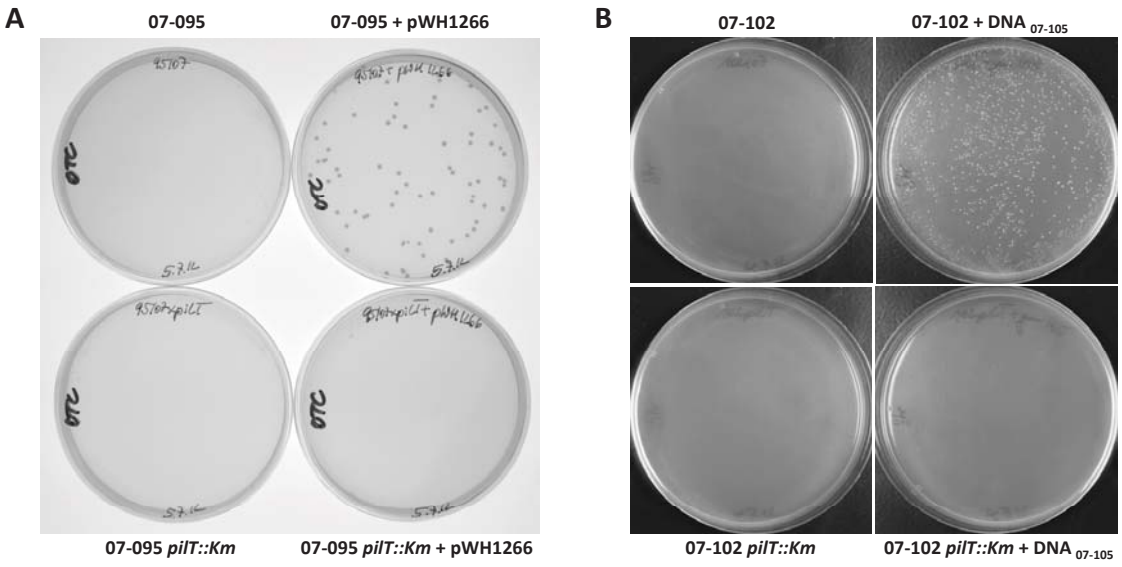
Wilharm et al.
Figure 1



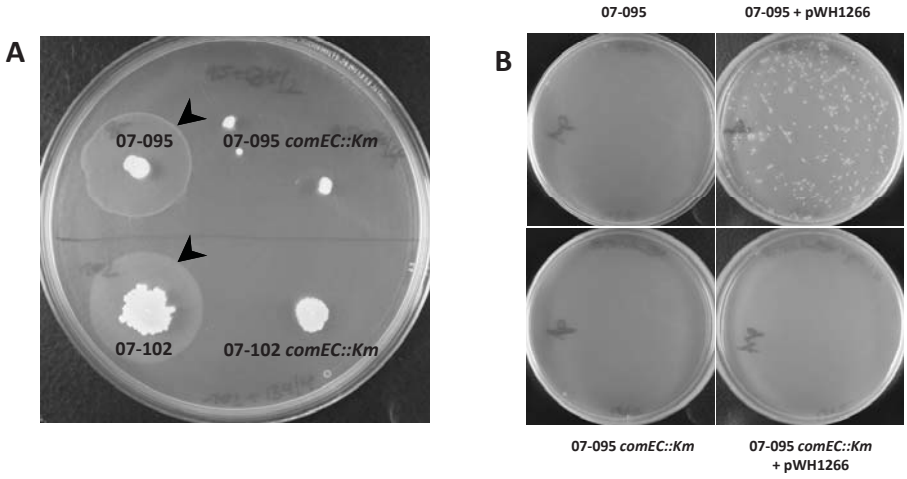
Wilharm et al.
Figure 2



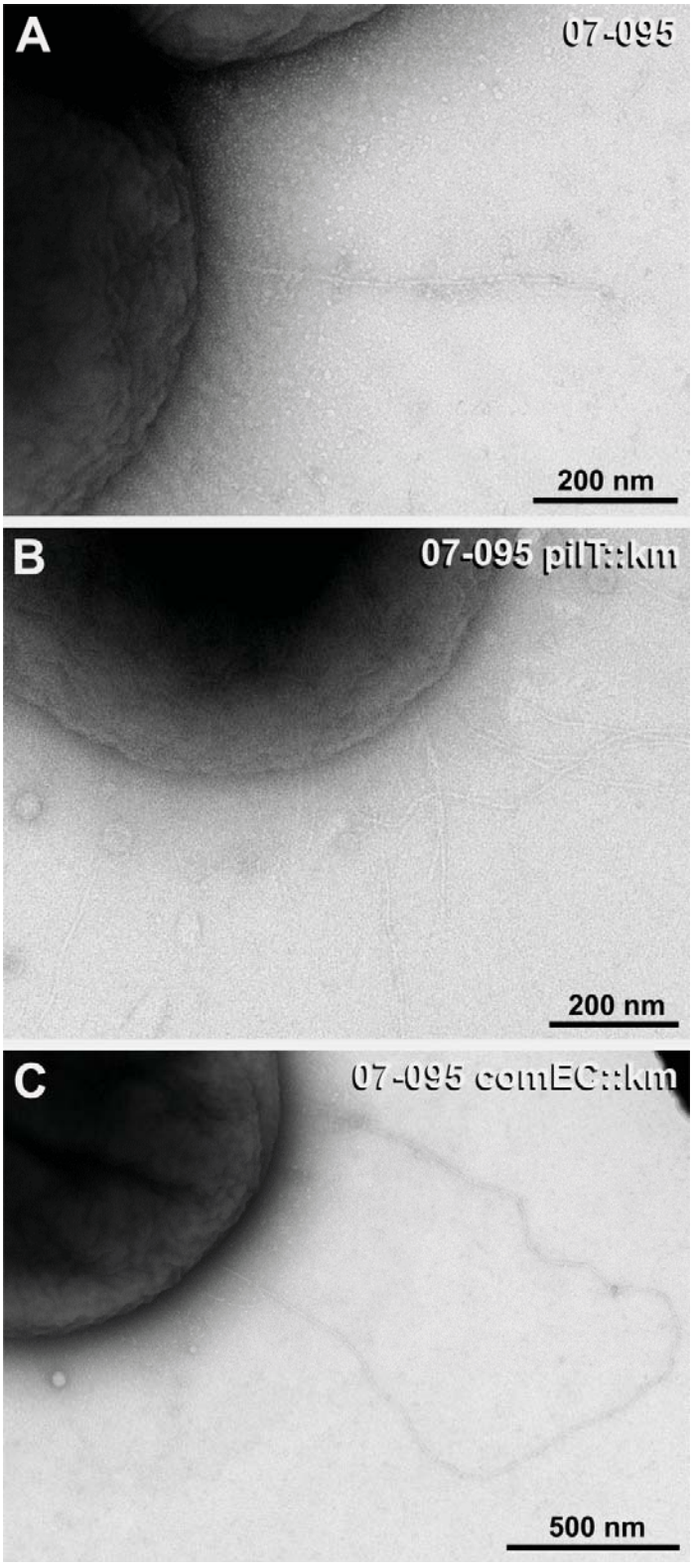
Wilharm et al.
Figure 3



Wilharm et al.
Figure 4



Wilharm et al.
Figure 5



Wilharm et al.
Figure 6