Originally published as:

Skiebe, E., de Berardinis, V., Morczinek, P., Kerrinnes, T., Faber, F., Lepka, D., Hammer, B., Zimmermann, O., Ziesing, S., Wichelhaus, T.A., Hunfeld, K.-P., Borgmann, S., Gröbner, S., Higgins, P.G., Seifert, H., Busse, H.-J., Witte, W., Pfeifer, Y., Wilharm, G.
Surface-associated motility, a common trait of clinical isolates of Acinetobacter baumannii, depends on 1,3-diaminopropane
(2012) International Journal of Medical Microbiology, 302 (3), pp. 117-128.

DOI: 10.1016/j.ijmm.2012.03.003

Surface-associated motility, a common trait of clinical isolates of *Acinetobacter baumannii*, depends on 1,3-diaminopropane

Evelyn Skiebe[1], Véronique de Berardinis[2], Peter Morczinek[1], Tobias Kerrinnes[1], Franziska Faber[1], Daniela Lepka[1], Bettina Hammer[3], Ortrud Zimmermann[4], Stefan Ziesing[5], Thomas A. Wichelhaus[6], Klaus-Peter Hunfeld[7], Stefan Borgmann[8], Sabine Gröbner[9], Paul G. Higgins[10], Harald Seifert[10], Hans-Jürgen Busse[11], Wolfgang Witte[1], Yvonne Pfeifer[1], and Gottfried Wilharm[1,*]

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

[2]CEA, DSV, IG, Genoscope, 2 rue Gaston Crémieux, 91057 Evry, France

[3]MVZ Labor Dr. Switkowski, Wagner, Dr. Bauermann, D-10711 Berlin, Germany

[4]Institute for Medical Microbiology, Georg-August-University, D-37075 Göttingen, Germany

[5]Institute for Medical Microbiology and Hospital Epidemiology, Hannover Medical School, D-30625 Hannover, Germany

[6]Institute of Medical Microbiology and Infection Control, Hospital of Goethe-University, D-60596 Frankfurt/Main, Germany

[7]Institute for Laboratory Medicine, North West Medical Centre, D-60488 Frankfurt/Main, Germany

[8]Synlab Medical Care Service, Medical Care Centre Weiden, D-92637 Weiden, Germany

[9]Institute of Medical Microbiology and Hygiene, University of Tübingen, D-72076 Tübingen, Germany

[10]Institute for Medical Microbiology, Immunology and Hygiene, University of Cologne, D-50935 Cologne, Germany

[11]Institute of Bacteriology, Mycology and Hygiene, University of Veterinary Medicine, A-1210 Vienna, Austria

*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: *Acinetobacter baumannii* motility

Abstract

While flagella-independent motility has long been described in representatives of the genus *Acinetobacter*, the mechanism of motility remains ambiguous. *Acinetobacter baumannii*, a nosocomial pathogen appearing increasingly multidrug-resistant, may profit from motility during infection or while persisting in the hospital environment. However, data on the frequency of motility skills among clinical *A. baumannii* isolates is scarce. We have screened a collection of 83 clinical *A. baumannii* isolates of different origin and found that, with the exception of one isolate, all were motile on wet surfaces albeit to varying degrees and exhibiting differing morphologies. Screening a collection of transposon mutants of strain ATCC 17978 for motility defects, we identified two akinetic mutants carrying transposon insertions in the *dat* and *ddc* gene, respectively. These neighbouring genes contribute to synthesis of 1,3-diaminopropane (DAP), a polyamine ubiquitously produced in *Acinetobacter*. Supplementing semi-solid media with DAP cured the motility defect of both mutants. HPLC analyses confirmed that DAP synthesis was abolished in *ddc* and *dat* mutants of different *A. baumannii* isolates and was re-established after genetic complementation. Both, the *dat* and *ddc* mutant of ATCC 17978 were attenuated in the *Galleria mellonella* caterpillar infection model. Taken together, surface-associated motility is a common trait of clinical *A. baumannii* isolates that requires DAP and may play a role in its virulence.

Keywords

Acinetobacter baumanniii – motility – 1,3-diaminopropane – *dat* – *ddc* – *A. baylyi* ADP1 – *Galleria mellonella*

Introduction

Acinetobacter baumannii is an emerging nosocomial pathogen. Of major concern is the increase of multi-drug resistance within this species (Munoz-Price and Weinstein, 2008; Peleg et al., 2008; Alsan and Klompas, 2010; Gordon and Wareham, 2010). It is believed that its ability to withstand desiccation, to form biofilms and to acquire antibiotic resistance contribute to its survival in the hospital environment (Jawad et al., 1998; Gaddy and Actis, 2009). Besides the few virulence determinants identified, in particular OmpA (Choi et al., 2005) and phospholipase D (Jacobs et al., 2010), biofilm formation is also considered to contribute to virulence (de Breij et al., 2010). Biofilm formation and motility are intimately connected processes in many organisms (Verstraeten et al., 2008) and motility contributes to virulence in many pathogens (Josenhans and Suerbaum, 2002). However, it is not known whether motility contributes to persistence and virulence of *A. baumannii*.

The genus name *Acinetobacter* was coined to indicate that representatives were akinetic due to the lack of flagella (Brisou and Prevot, 1954; Peleg et al., 2008). However, fifty years ago, certain isolates belonging to the genus have been reported to jerkily move on wet surfaces, a phenomenon called 'twitching motility' (Lautrop, 1961; Lautrop, 1965; Henrichsen, 1972; Henrichsen, 1983). Henrichsen and Blom (1975) later suggested that pili could be involved in *Acinetobacter* twitching motility. While twitching motility has since been studied intensively in other genera like *Neisseria*, *Myxococcus* and *Pseudomonas*, and found to rely on type IV pilus retraction (Merz et al., 2000; Skerker and Berg, 2001; Mattick, 2002; Burrows, 2005; Bulyha et al., 2009), the genetic and molecular basis of *Acinetobacter* motility is still ambiguous (McBride, 2010). Interestingly, twitching motility was found to be essential for virulence in *Dichelobacter nodosus*, the causative agent of ovine footrot (Han et al., 2008). Furthermore, a role of twitching motility in *Pseudomonas aeruginosa* virulence has been recently demonstrated in a murine model of keratitis (Alarcon et al., 2009).

Henrichsen and Blom (1975) distinguished another form of motility, called sliding, among *Acinetobacter* strains. Sliding was defined as "… surface translocation produced by the expansive forces in a growing culture in combination with special surface properties of the cells resulting in reduced friction …" (Henrichsen, 1972). However, this form of motility has not been substantiated mechanistically in *Acinetobacter*. Studied in some more detail, *P. aeruginosa* sliding motility along wet surfaces has been demonstrated to be independent of type IV pili and flagella, but facilitated by rhamnolipid surfactant production (Murray and Kazmierczak, 2008).

Barker and Maxted (1975) characterized the growth of 29 *Acinetobacter* isolates of clinical origin on various semi-solid media and found that 19 out of 29 strains showed a surface motility they called "swarming". Inoculating the media by stabbing, they found that 11 of their strains moved beneath the agar and 14 strains formed what they called "ditches" (Barker and Maxted, 1975). Of further interest, in contrast to what Lautrop (1961) reported, Barker and Maxted (1975) could not observe the jerking movement of cells, leaving open the question of whether they had observed "twitching" motility. Later, attempts to link the surface motility of the environmental strain *Acinetobacter baylyi* BD413 to specific pili failed (Herzberg et al., 2000; Gohl et al., 2006). Very recently, Mussi et al. (2010) reported that surface-associated motility of *A. baumannii* is controlled by sensing of blue light and Clemmer et al. (2011) demonstrated that *A. baumannii* motility requires autoinducer synthesis. In addition, motility was found to be partially reduced by inactivation of *pilT*, encoding an ATPase involved in pilus retraction. Further, Eijkelkamp et al. (2011a) found an inhibition of surface motility of *A. baumannii* under iron-limiting conditions and an accompanying down-regulation of type IV pilus genes. A recent study involving 50 clinical *A. baumannii* strains describes "twitching" motility to be a common trait of international clone I strains while "swarming" motility was only observed with some isolates not classified within the clonal lineages (Eijkelkamp et al., 2011b).

Considering the ability to move to be of potential use for *A. baumannii* both to persist in the hospital environment and to colonize the host, we have investigated motility skills of a collection of 83 clinical *A. baumannii* isolates and found only one isolate to be completely immotile under the various conditions tested. Generating and screening transposon mutants we identified the genes *ddc* and *dat* involved in synthesis of 1,3-diaminopropane as being essential for *A. baumannii* motility and for virulence in the *Galleria mellonella* infection model.

Materials and methods

Bacterial strains. *A. baumannii* strains ATCC 17978 and ATCC 19606^T were purchased from the American Type Culture Collection. All other clinical isolates are listed in Supplemental Table 1 indicating their source countries. API 20NE tests were performed on all isolates for presumptive identification as *A. baumannii*. Further, presence of $bla_{\text{OXA-51}}$-like genes was tested by PCR to confirm identification as *A. baumannii* (Turton et al., 2006). Isolates 44-83 (Supplemental Table 1) were speciated by *gyrB* multiplex PCR (Higgins et al., 2007). Typing of selected isolates was performed by pulsed-field gel electrophoresis (PFGE) using restriction enzyme ApaI (Seifert et al., 2005). PFGE patterns were interpreted according to previously described criteria (Tenover et al., 1995). Typing of isolates by rep-PCR to identify global lineages was performed as recently described (Higgins et al., 2010).

Motility assays. Unless otherwise stated, motility was analysed on 0.5% agarose plates supplemented with 5 g/l tryptone and 2.5 g/l NaCl after incubation for 16 hours at 37°C. For inoculation, the agarose layer was punctured to enable growth not only at the surface but also at the boundary between the bottom of the agar layer and the polystyrene Petri-dish ("interphase"). Where appropriate, formation of a biofilm at the interphase of the plate was visualized by Coomassie-staining of bacterial mass sticking to the Petri-dish after

removal of the agarose layer (Volkmann et al., 2010). As a crucial point of the motility assays, the plates were sealed with parafilm to prevent drying and in order to obtain reproducible results, incubators were not run under strong circulating air flow conditions. Reproducibility was optimum when strains were plated on LB agar from glycerol stock, and single colonies used for inoculation of motility plates were taken within a week after plating. To prepare thread agar, agar threads purchased from Robert KIND GmbH, Lichtenfels, Germany (cat. no. 950230) were ground and used instead of powdered agar. Where appropriate, 1,3-diaminopropane was supplemented to motility media at 0.001%.

Bacterial transformation. Electro-competent cells were prepared essentially as described (Choi et al., 2006). Efficiency of this published protocol was further improved by preparing cells at 4°C instead of room temperature and by using cells from the late logarithmic phase (optical density of approx. 1 OD_{600nm}). Alternatively, plasmids were transformed applying the recently described nanopiercing method (Wilharm et al., 2010). Transformation of naturally competent *A. baumannii* strains will be described elsewhere (Skiebe & Wilharm, manuscript in preparation).

Transposon mutagenesis. Electro-competent ATCC 17978 was transformed with EZ-Tn5 <KAN-2> transposome complexes (Epicentre Biotechnologies, Madison, Wisconsin) and transformants were selected on LB agar plates containing 6 µg/ml kanamycin. Selection with higher concentrations of kanamycin reduced the yield of transposon mutants considerably. Subsequently, transformants were inoculated into 0.5% agarose plates as described above to screen for motility phenotypes. Motility mutants (with phenotypes reproduced at least three times) were subjected to single-primer PCR to identify the transposon insertion sites (see below).

Determination of transposon insertion sites by single-primer PCR. Single primer PCR (Hermann et al., 2000) was conducted using chromosomal DNA as a template on a gradient PCR machine as follows. Chromosomal DNA was isolated using the DNA isolation

158 reagent for genomic DNA (AppliChem, Germany) according to the manufacturer's
159 recommendations. Following 30 cycles under stringent conditions (1 min at 95°C, 30 s at
160 58°C, 1.5 min at 72°C), a single cycle was conducted under low stringency conditions (1 min
161 at 95°C, 30 s at 40°C +/-10°C, 3 min at 72°C) to enable unspecific priming of the second
162 strand. Subsequently, 30 cycles were performed under stringent conditions (1 min at 95°C, 30
163 s at 58°C, 1.5 min at 72°C). As the sole primer either the forward primer 5'-
164 GAGTTGAAGGATCAGATCACGC-3' (binding to nucleotides 1043-1064 of transposon
165 EZ-Tn5 <KAN-2>) or the reverse primer 5'-CGCGGCCTCGAGCAAGACG-3' (binding to
166 nucleotides 160-178 of transposon EZ-Tn5 <KAN-2>) was used. Following agarose gel
167 electrophoresis, unique bands were cut out from agarose gels, eluted and subjected to Sanger
168 sequencing using an appropriate nested primer (nested forward primer 5'-
169 CTTCCCGACAACGCAGACCG-3', binding to nucleotides 1067-1086 of transposon EZ-
170 Tn5 <KAN-2>; nested reverse primer 5'-CCCTTGTATTACTGTTTATGTAAGC-3',
171 binding to nucleotides 108-132). Transposon insertions were subsequently confirmed by PCR
172 combining transposon primers as above with appropriate target site primers and subsequent
173 re-sequencing of the PCR products.

174 **Construction of *dat-ddc* complementation plasmid.** A 3815 bp fragment
175 encompassing genes A1S-2453 (*ddc*) and A1S-2454 (*dat*) as well as the putative promoter
176 and terminator regions (analysed with the softberry package available online:
177 http://linux1.softberry.com/berry.phtml) was amplified by PCR (see Fig. 4) using primers 5'-
178 ATTAGGATCCGAGTGCTTGGTTCATTAACTGG-3' (BamHI-2454-for) and 5'-
179 ATTAGGATCCGTGCCGTATTGATATATCAATGCG-3' (BamHI-2453-rev) thereby
180 introducing flanking BamHI restriction sites (underlined). The BamHI-cleaved fragment was
181 ligated into BamHI-digested plasmid pWH1266 (Hunger et al., 1990) resulting in plasmid
182 p*dat-ddc*. *A. baumannii* ATCC 17978 transformants were selected with 100 µg/ml ampicillin.

183 **Determination of polyamines.** Polyamines were determined by high performance
184 liquid chromatography (HPLC) from lyophilized bacterial pellets and culture supernatants as
185 described (Busse & Auling, 1988). The HPLC apparatus was equipped as described by Stolz
186 et al. (2006). Bacteria were cultured overnight at 37°C in broth containing 5 g/l tryptone and
187 2.5 g/l NaCl which corresponds to the nutritional conditions on motility plates. Overnight
188 cultures were diluted 1:20 into 30 ml of fresh broth as above and cultured until late
189 logarithmic state (6 hours, OD_{600nm} of 2) at 37°C. Bacteria were harvested by centrifugation
190 and pellets were frozen and lyophilized. 25 ml of each culture supernatant were collected,
191 frozen and lyophilized. For comparison, ATCC 17978 was cultured on a motility plate
192 overnight and the bacterial mass covering the surface was floated off with broth and the
193 bacteria were harvested by centrifugation and were frozen and lyophilized.
194 ***Galleria mellonella* caterpillar infections.** *G. mellonella* caterpillar infections were
195 essentially performed as described by Peleg et al. (2009). Bacteria were grown in LB broth
196 overnight at 37°C, diluted 1:50 in LB broth and cultured for another 3 hours at 37°C. Bacteria
197 were then washed and resuspended in sterile phosphate-buffered saline (PBS) and adjusted to
198 an optical density (OD_{600nm}) of 0.2. 5 µl-aliquots corresponding to $3x10^5$ CFU were injected
199 into the last left proleg of *G. mellonella* caterpillars (200-300 mg in weight) purchased from
200 Reptilienkosmos.de, Niederkrüchten, Germany. CFUs were determined by serial dilutions that
201 were plated on Mueller-Hinton agar. Groups of 16 caterpillars were incubated at 37°C for 5
202 days in Petri dishes and daily checked for vitality by touching. Two control groups were
203 included; either injected with 5 µl-aliquots of sterile PBS or were untreated. Experiments with
204 more than two dead caterpillars within 5 days in any control group were not considered valid.
205 Survival was plotted according to Kaplan-Meier using Origin 8 software and statistical
206 significance was tested by a two-tailed, unpaired t test pooling data from three independent
207 experiments; *P* values below 0.05 were considered to be statistically significant.
208

Results

Conditions and forms of Acinetobacter motility. Hypothesizing that motility could be an important trait of *A. baumannii* isolates for colonizing biotic or abiotic surfaces and thus for persistence in the hospital environment, we were interested to know how widely such traits were distributed among clinical isolates. Given that several putative different forms of motility have been described in *Acinetobacter*, termed twitching, swarming, sliding and ditching motility and analysed under various conditions (Barker and Maxted, 1975; Henrichsen and Blom, 1975), we tested and varied several published motility assays irrespective of their original dedication to analysis of swimming, swarming, twitching or other forms of motility (Barker and Maxted, 1975; Semmler et al., 1999; Rashid and Kornberg, 2000).

We found that the morphotype of moving colonies of an individual isolate could differ significantly depending on the media composition. As an example, motility of *A. baumannii* ATCC 17978 is illustrated on 0.5% agarose supplemented with either 5 g/l or 2 g/l tryptone (compare Fig. 1A and B) and on 0.8% thread agar supplemented with either 10 g/l or 5 g/l tryptone (compare Fig. 1C and D). The colony morphotypes observed on thread agar resemble the "ditching" described by Barker and Maxted (1975) using 0.3% "Davis Agar" and 1% peptone. The bacteria grow in rifts filled with fluid developing on the agar surface. Very recently, Clemmer et al. (2011) described similar "ditching" phenomena when *A. baumannii* M2 was grown on 0.25% LB-Difco Bacto agar.

Barker and Maxted (1975) further described the growth of some isolates at the bottom of the petri dish beneath the semi-solid medium (designated "interphase" here) when inoculating plates by stabbing into the medium. We could reproduce this phenomenon as illustrated in Supplementary Fig. 1. Coomassie-staining was used to visualize bacteria

234 sticking to the polystyrene surface after removal of the semi-solid medium (0.5% agarose as
235 matrix).

236 ***Strain-specificity of motility morphotypes.*** In accordance with Barker and Maxted
237 (1975), we found that the morphotypes of individual isolates differed significantly (Fig. 2A-
238 F). While some strains moved along the agarose surface and the interphase between agarose
239 and petri dish (see Fig. 2A-B), others preferentially or exclusively moved on the agarose
240 surface exhibiting different forms (see Fig. 2C-F). Also, the rate of progression of the
241 spreading zone differed significantly among strains and was in the range of 0.2-4 mm per
242 hour. We found that using 0.5% agarose as the matrix of semi-solid media was superior to
243 agar (both from agar powder and threads) with respect to the discriminatory power and
244 reproducibility of the morphotypes (data not shown). Our attempts to correlate motility
245 morphotypes to relationship of the strains according to repetitive sequence-based PCR
246 clustering (rep-PCR; DiversiLab) (Higgins et al., 2010) failed. We suppose that variability of
247 surface properties such as determined by LPS and outer membrane proteins (OMPs) critically
248 influence motility morphotypes. In support of an influence of LPS composition and OMPs on
249 motility of *A. baumannii*, Clemmer et al. (2011) identified motility-deficient mutants affected
250 in biosynthesis of dTDP-4-dehydro-6-deoxy-D-glucose, a precursor of the O-antigen sugar
251 dTDP-L-rhamnose, and the outer membrane protein OmpA, respectively.

252 ***Surface-associated motility is a common trait of clinical A. baumannii isolates.*** We
253 screened a collection of 83 clinical *A. baumannii* isolates, confirmed by the presence of the
254 $bla_{\text{OXA-51}}$-like genes indicative of *A. baumannii* (Turton et al., 2006), for their capability to
255 move at the surface and interphase of 0.5% agarose plates and on 0.8% thread agar plates.
256 Altogether, on 0.5% agarose plates we found that all isolates except one showed clear signs of
257 motility at the surface of 0.5% agarose. By contrast, only 41% of the isolates (34 out of 83
258 isolates) exhibited significant motility at the interphase (see Supplementary Table 1).
259 Approximately 66% of the isolates (55 out of 83 isolates) formed ditches on thread agar. A

group of four isolates to which *A. baumannii* ATCC 19606^T belonged showed a slimy morphology and slime and/or release of water from the agarose matrix seemed to promote some sliding-like motility. However, upon prolonged incubation at room temperature for seven days all these isolates showed clear signs of motility on 0.5% agarose (data not shown). Lastly, one slowly growing isolate (07-116) exhibited no significant motility. We only observed the distribution of colony-forming bacteria at the interphase, likely due to physical dispersion of bacteria along a liquid flow occurring at the interphase (data not shown). Comparing growth rates of slowly moving isolates to growth rates of fast moving ones we ruled out a correlation between growth rates in broth and the velocity of motility (data not shown). Taken together, all isolates except one proved motile on 0.5% agarose; a subpopulation of all isolates exhibited motility at the interphase between agarose and petri dish and/or formed ditches on thread agar.

The spreading zone is preceded by a translucent film. Upon microscopic examination of motility plates we observed that the spreading zone of bacteria was preceded by a translucent film on the agarose surface. This phenomenon is illustrated in Fig. 3. In Fig. 3C, black triangles mark the frontline of the spreading bacteria and white triangles indicate the frontline of the translucent film that is free of bacteria. This phenomenon is only observed at the frontline where bacteria spread, and not at the sides of these branches, suggesting that the actively moving bacteria manipulate the surface, e.g. by secreting surfactant or by causing the release of liquid from the semi-solid medium. While this translucent film has dimensions of width of less than 1 mm in different *A. baumannii* isolates, we found that the film can expand to several mm with the rapidly moving soil isolate *A. baylyi* ADP1 (data not shown).

Identification of motility mutants affected in biosynthesis of 1,3-diaminopropane. Next, we used the EZ-Tn5 <KAN-2> transposome system to generate insertional mutants of *A. baumannii* ATCC 17978 which were screened for motility defects on 0.5% agarose plates. The transposon insertion site of motility mutants showing reproducible and stable phenotypes

(at least three independent motility assays) was determined by single-primer PCR and subsequent DNA sequencing and was then confirmed by PCR. In doing so, we identified the motility deficient mutants 129 and 277 which harboured transposon insertions in the neighbouring genes A1S-2453 (*ddc*) and A1S-2454 (*dat*), respectively. These have been previously characterized as genes encoding L-2,4-diaminobutyrate decarboxylase (DABA DC) and L-2,4-diaminobutyrate:2-ketoglutarate 4-aminotransferase (DABA AT) that are involved in biosynthesis of 1,3-diaminopropane (Yamamoto et al., 1992; Ikai and Yamamoto, 1997). 1,3-diaminopropane (DAP) is the major polyamine produced by *Acinetobacter* (Auling et al., 1991; Kämpfer et al., 1991; Hamana and Matsuzaki, 1992), but no function has hitherto been attributed to this compound. Figure 4 illustrates the genome arrangement of *ddc* and *dat* and indicates the transposon insertion sites of mutants 129 and 277.

1,3-diaminopropane (DAP) restores the motility defect of dat and ddc mutants. To test whether the supposed lack of 1,3-diaminopropane (DAP) in the mutants could be compensated by its supplementation, 0.5% agarose plates were supplemented with 0.001% DAP (120 μM) and inoculated with the mutant strains 129 (*ddc*) and 277 (*dat*). While both mutants were immotile on the surface of 0.5% agarose and also at the interphase (only physical dispersion of some bacteria could be observed similar to the phenotype of the immotile isolate 07-116), supplementation with DAP promoted strong motility of both mutants (Fig. 5). This result suggests that the motility defect of mutants 129 and 277 is due to a lack of DAP synthesis mediated by *dat* and *ddc* and, consequently, that DAP is required for efficient spreading of *A. baumannii* ATCC 17978.

Complementation of dat and ddc mutants. Since sequence analyses of transposon insertion sites revealed some mutants harbouring at least two transposons at different sites, we had to ensure that the phenotypes of mutants 129 and 277 were exclusively due to insertional inactivation of the *ddc* and *dat* genes, respectively. To this end, a plasmid for complementation was constructed. Previous work by Ikai and Yamamoto (1997) had

suggested that *ddc* and *dat* are organized in a bi-cistronic operon, therefore we amplified by PCR a 3815 bp region encompassing both open reading frames as well as the putative promoter and ρ-independent terminator of the supposed bi-cistronic operon (see Fig. 4). This PCR product was inserted into the shuttle vector pWH1266 (Hunger et al., 1990) to yield plasmid p*dat-ddc* for complementation experiments. Transformation of mutants 129 and 277 with p*dat-ddc* reconstituted motility of both mutants (Fig. 6). This corroborates our conclusion that inactivation of either *ddc* or *dat,* respectively, determines a motility defect.

Transformation of clinical isolates with mutant DNA. Further, we tested whether the motility defects associated with transposon insertions in *dat* and *ddc* could be reconstituted in clinical *A. baumannii* isolates by transformation with DNA isolated from mutants 129 and 277. We made use of recently identified naturally competent clinical *A. baumannii* isolates (Skiebe and Wilharm, manuscript in preparation) and selected two of these competent isolates, designated 07-095 and 07-102 (see Supplementary Table 1), with distinguishable motility phenotypes for transformation experiments. While isolate 07-102 exhibits a unique morphotype on the surface of 0.5% agarose, isolate 07-095 preferentially moves at the interphase between agarose and petri dish. Both isolates were successfully transformed with DNA from both mutants (129/*ddc* and 277/*dat*), as was confirmed by PCR demonstrating recombination of the transposon into the *dat* and *ddc* gene, respectively (data not shown). Transformation of isolates 07-095 and 07-102 with DNA from mutants 129/*ddc* and 277/*dat* resulted in motility defects in all cases. As with mutants 129/*ddc* and 277/*dat* of ATCC 17978, motility of the corresponding mutants of isolates 07-095 and 07-102 could be restored by supplementation with DAP (Figures 7 and 8). Collectively, these results demonstrate that DAP is required for motility in different *A. baumannii* isolates and that motility at the surface as well as at the interphase depends on DAP.

Ditching depends on DAP. We were further interested to learn whether formation of ditches as first described by Barker and Maxted (1975) and illustrated above also requires

DAP. Supplementary Fig. 2 illustrates that mutants 129/*ddc* and 277/*dat* were unable to form ditches on thread agar and that supplementation with DAP restored the ability to form ditches in both mutants.

Contribution of DAP synthesis to motility of Acinetobacter baylyi ADP1. Next, we made use of a complete collection of single-gene deletion mutants available for *Acinetobacter baylyi* ADP1 DSM 24193 (de Berardinis et al., 2008) to analyse the influence of the homologues of *dat* and *ddc*, ACIAD1210 and ACIAD1211, respectively, on motility. Deletion of ACIAD1210 or ACIAD1211 in *A. baylyi* ADP1 affected motility to some extent, however, effects were significantly less pronounced compared to mutations in *dat* and *ddc* of *A. baumannii* strains (see Supplementary Fig. 3). Reproducibly, the motility defect caused by deletion of ACIAD1210 (*dat*) was more pronounced compared to deletion of ACIAD1211 (*ddc*). The motility defects of both mutants could be partially restored by supplementing DAP or by trans-complementation of the mutant strains with plasmid p*dat-ddc* harbouring the homologous genes of *A. baumannii* ATCC 17978 (Supplementary Fig. 3). In control experiments, transformation of *A. baylyi* ADP1 with the pWH1266 backbone plasmid did not interfere with motility (data not shown). The extent of deficiency differed depending on the motility media tested and was more pronounced on 0.5% agar plates (Supplementary Fig. 3) compared to 0.5% agarose plates (data not shown). Similar results were obtained with other semi-solid media including thread agar. Taken together, while ACIAD1210 (*dat*) and ACIAD1211 (*ddc*) contribute to motility of *A. baylyi* ADP1 in a DAP-related manner, we could not observe a dependency as strong as that observed in *A. baumannii*.

DAP synthesis is abolished in *ddc* and *dat* mutants of *A. baumannii*. To confirm the contribution of *ddc* and *dat* to synthesis of DAP in *A. baumannii*, we determined the DAP content of bacteria by HPLC (see Supplementary Table 2). Bacteria were cultivated in broth with a nutrient content corresponding to our standard motility medium (5 g/l tryptone, 2.5 g/l NaCl). We found a DAP content of approximately 70-80 µmol per g of dry bacterial mass for

364 *A. baumannii* strains ATCC 17978, 07-095 and 07-102 and a comparable content in ATCC
365 17978 harvested from motility plates. By contrast, the *ddc* and *dat* mutants of all three *A.*
366 *baumannii* isolates exhibited a low DAP content of 0.5-5 µmol/g dry mass thus confirming
367 the essential role of *dat* and *ddc* in biosynthesis of DAP in *A. baumannii*. Further,
368 complementation of ATCC 17978 mutants 129/*ddc* and 277/*dat* with plasmid p*dat-ddc*
369 restored DAP synthesis to wild-type levels. Collectively, these findings are in full agreement
370 with the requirement of DAP for motility of *A. baumannii*.

371 Interestingly, however, the situation is different with *A. baylyi* ADP1. As outlined
372 above, motility defects of the *A. baylyi* mutants deleted in ACIAD1210 (corresponding to *dat*)
373 and ACIAD1211 (corresponding to *ddc*), respectively, were less pronounced compared to
374 *A. baumannii* mutants. The DAP content of mutant ΔACIAD1210, which was more affected
375 in motility compared to ACIAD1211 (see Suppl. Fig. 3), was reduced to one third of the wild-
376 type level but was at least one order of magnitude higher than in any *A. baumannii* mutant.
377 The DAP content of mutant ΔACIAD1211, being only slightly affected in motility, was even
378 higher than in the wild-type strain. Taken together, these findings suggest that in *A. baylyi*
379 ADP1 an alternative pathway for the biosynthesis of DAP synthesis exists besides the one
380 mediated by ACIAD1210 and ACIAD1211. The identification of this alternative pathway is
381 required to fully assess the impact of DAP on motility of *A. baylyi* ADP1.

382 **The *ddc* and *dat* mutants of *A. baumannii* ATCC 17978 are attenuated in the**
383 ***Galleria mellonella* caterpillar infection model.** Finally, we have tested the impact of *ddc*
384 and *dat* in the *Galleria mellonella* caterpillar infection model recently established for
385 *A. baumannii* (Peleg et al., 2009). The larvae were infected with $3x10^5$ bacteria that were
386 injected into the hemocoel and incubated for five days at 37°C. In line with previous results
387 reported by Peleg et al. (2009), more than 80% of the larvae were killed by *A. baumannii*
388 ATCC 17978 at this infection dose within 24 hours. By contrast, killing by the mutants
389 129/*ddc* and 277/*dat* was markedly reduced and delayed ($p < 0.05$; see Fig. 9). Melanisation

390 reactions caused by the mutants were accordingly delayed compared to the wild-type (see
391 Suppl. Fig. 4). In summary, these data support an impact of DAP synthesis during infection
392 by *A. baumannii*.
393
394

Discussion

Previous work from different groups reported various manifestations of surface-associated motility of *Acinetobacter* isolates (Lautrop, 1961; Barker and Maxted, 1975; Henrichsen and Blom, 1975; Mussi et al., 2010; Clemmer et al., 2011; Eijkelkamp et al., 2011b; Antunes et al., 2011). However, none of these phenomena has been mechanistically clarified. Therefore it is unclear whether all the phenomena have the same mechanistic basis or whether different forms of motility exist in *Acinetobacter*. Despite this, there are some indications that the jerky movement of *Acinetobacter* cells reported previously relies on pilus retraction and is therefore correctly termed "twitching motility" (Lautrop, 1961; Henrichsen and Blom, 1975). Applying phase-contrast microscopy on *A. baumannii* isolates spreading at the interphase, we could clearly observe the jerky movement of single cells (Suppl. Fig. 5) suggesting a twitching-like mechanism in some isolates. It has to be noted, however, that twitching motility in prototypic organisms typically appears in rafts of cells rather than in individual cells. Thus, we refrain from using any such terminology before the underlying mechanisms are more disclosed. To clarify this, genetic and electron microscopy studies are underway. Very recently, Clemmer et al. (2011) showed that inactivation of *A. baumannii pilT*, encoding an ATPase required for type IV pilus retraction, resulted in approximately 50 % reduced motility. The significant residual motility, however, is ambiguous leaving open the question whether type IV pili are fundamental in *A. baumannii* motility. Furthermore, Clemmer et al. (2011) identified several genes whose inactivation significantly reduced motility. These genes were unrelated to type IV pili suggesting a more complex or even a type IV pili-independent mechanism.

Evidently, however, *Acinetobacter* motility in its various forms involves manipulation of wet surfaces and requires synthesis of 1,3-diaminopropane (DAP). The role of DAP in motility of any organism, to our knowledge, is unprecedented. Despite DAP being the

dominant polyamine produced by *Acinetobacter* (Auling et al., 1991; Kämpfer et al., 1991; Hamana and Matsuzaki, 1992), its role is unclear. More generally, polyamines such as spermidine and putrescine are involved in various cellular processes as they can modulate the functions of nucleic acids and proteins (Igarashi and Kashiwagi, 2010). Therefore it seems reasonable to speculate that DAP takes over at least some of these functions in *Acinetobacter*. Of specific interest, evidence accumulates that polyamines play a crucial role in maintaining fitness of pathogens during infection (Shah and Swiatlo, 2008). In accordance, we have shown here that virulence of *A. baumannii* is attenuated if DAP synthesis is abolished. Furthermore, polyamines have been implicated in tolerance to desiccation in plants (Cona et al., 2006) and our unpublished preliminary data suggest that DAP synthesis contributes to desiccation tolerance (data not shown). Considering that the well-known ability of *A. baumannii* to withstand desiccation (Jawad et al., 1998) might be crucial for dissemination in the hospital environment, studying the functions of DAP in more detail could prove fruitful in understanding the basic principles of persistence. It is worthwhile to state again that the polyamine production profile of *Acinetobacter* spp. is very characteristic in that DAP, being the predominate polyamine, can serve as a valuable taxonomic tool (Auling et al., 1991; Kämpfer et al., 1991; Hamana and Matsuzaki, 1992). Targeting DAP biosynthesis in *A. baumannii* may thus become an interesting option to treat *Acinetobacter* infections specifically. Thus, future work should aim at evaluating the role of DAP during infection and as a persistence factor contributing to withstand desiccation and antibiotic stress.

Our studies do not answer the question in which way DAP is involved in motility. Stimulated by a recent report of Kurihara et al. (2011), describing the role of the polyamine putrescine on type 1-pili dependent motility in *E. coli* K-12 and in particular the sensing of extracellular putrescine to determine the cell density and coordinate motility, we hypothesised that secreted DAP could act as quorum sensing-like molecule to coordinate motility in *A. baumannii*. However, when analysing the DAP content of *A. baumannii* culture

447 supernatant, no indication of DAP secretion augmenting the intrinsic background of the broth
448 (approx. 0.2 μM) was found (data not shown). Since the concentration of supplemented DAP
449 required to fully restore motility in the *ddc* and *dat* mutants (120 μM) is more than two orders
450 of magnitude above the background level of the broth, the mode of action of DAP in motility
451 does not likely include DAP secretion and extracellular signalling.

452 We and others (Clemmer et al., 2011) observed a translucent film preceding the
453 spreading zone of moving *A. baumannii* thus suggesting surface manipulation to support
454 motility. However, a role of DAP as a lubricant promoting sliding across wet surfaces seems
455 unlikely given the lack of evidence of DAP secretion into the culture supernatant. Moreover,
456 recent attempts of Clemmer et al. (2011) to identify a surfactant in *A. baumannii* failed.
457 Hence, we favour a mechanism involving the release of liquid from the medium to explain the
458 translucent film.

459 Very recently, Eijkelkamp et al. (2011b) published a study on *Acinetobacter* motility
460 including 50 *A. baumannii* strains. However, in contrast to our claim that motility is a
461 common trait of *A. baumannii*, they only found motility in less than 40% of their strains.
462 Also, Clemmer et al. (2011), comparing 12 isolates, identified motility in only 75% of the
463 isolates. We ascribe this to the conditions tested by these authors that differ considerably from
464 ours both with respect to the matrix (agar versus agarose) and the nutrient content (Mueller-
465 Hinton and LB, respectively, versus 5 g/l tryptone). Eijkelkamp et al. (2011b) further claim
466 that what they called "swarming" motility, that is motility at the surface of LB medium
467 containing 0.25% agar, is observed only with isolates not classified within the international
468 clonal lineages. However, we have found that on the surface of our motility plates, with the
469 exception of a single isolate, all isolates of our collection, representing eight globally spread
470 lineages (which include international clonal lineages I-III) (Higgins et al., 2010; see
471 Supplementary Table 1), exhibited motility. Figure 2 illustrates that strains belonging to
472 lineages I and II can move along wet surfaces efficiently (strain BMBF 49 representing

lineage I (Fig. 2C) and strain BMBF 509 representing lineage II (Fig. 2D)). We thus conclude that our conditions are more favourable to demonstrate the different forms of motility.

Collectively, we have shown here that surface-associated motility is a common trait of *A. baumannii* that requires 1,3-diaminopropane. This capability could be important for colonisation of medical devices and tissue as well as wet areas within the hospital environment. Additionally, DAP mutants showed decreased virulence in a caterpillar infection model. Thus, continued investigation of *A. baumannii* motility may yield novel therapeutic avenues and targets. Possibly, inhibition of the processes of synthesis and functioning of 1,3-diaminopropane can contribute to therapy and control of the spread of this pathogen.

Acknowledgements

We thank members of the sequencing core facility at the Robert Koch-Institute for DNA sequencing and Rolf Reissbrodt for valuable advice on semi-solid media. PGH and HS were supported by a grant from Bundesministerium für Bildung und Forschung (BMBF), Germany, Klinische Forschergruppe Infektiologie (grant number 01 KI 0771).

References

Alarcon, I., Evans, D.J., Fleiszig, S.M., 2009. The role of twitching motility in *Pseudomonas aeruginosa* exit from and translocation of corneal epithelial cells. Invest. Ophthalmol. Vis. Sci. 50, 2237-2244.

Alsan, M., Klompas, M., 2010. *Acinetobacter baumannii*: An emerging and important pathogen. J. Clin. Outcomes Manage. 17, 27-35.

498 Antunes, L.C., Imperi, F., Carattoli, A., Visca, P., 2011. Deciphering the multifactorial nature
499 of *Acinetobacter baumannii* pathogenicity. PLoS One 6:e22674.
500 Auling, G., Pilz, F., Busse, H.-J., Karrasch, S., Streichan, M., Schön, G., 1991. Analysis of the
501 polyphosphate accumulating microflora in phosphorus eliminating anaerobic-aerobic
502 activated sludge systems using diaminopropane (DAP) as a bio marker for rapid
503 estimation of *Acinetobacter*. Appl. Environ. Microbiol. 57, 3585-3592.
504 Barker, J., Maxted, H., 1975. Observations on the growth and movement of *Acinetobacter* on
505 semi-solid media. J. Med. Microbiol. 8, 443-446.
506 Brisou, J., Prevot, A.R., 1954. Studies on bacterial taxonomy. X. The revision of species
507 under *Acromobacter* group. Ann. Inst. Pasteur (Paris) 86, 722-728.
508 Bulyha, I., Schmidt, C., Lenz, P., Jakovljevic, V., Höne, A., Maier, B., Hoppert, M., Søgaard-
509 Andersen, L., 2009. Regulation of the type IV pili molecular machine by dynamic
510 localization of two motor proteins. Mol. Microbiol. 74, 691-706.
511 Burrows, L.L., 2005. Weapons of mass retraction. Mol. Microbiol. 57, 878-888.
512 Busse, J., Auling, G., 1988. Polyamine pattern as a chemotaxonomic marker within the
513 Proteobacteria. Syst. Appl. Microbiol. 11, 1-8.
514 Choi, C.H., Lee, E.Y., Lee, Y.C., Park, T.I., Kim, H.J., Hyun, S.H., Kim, S.A., Lee, S.K., Lee,
515 J.C., 2005. Outer membrane protein 38 of *Acinetobacter baumannii* localizes to the
516 mitochondria and induces apoptosis of epithelial cells. Cell. Microbiol. 7, 1127-1138.
517 Choi, K.H., Kumar, A., Schweizer, H.P., 2006. A 10-min method for preparation of highly
518 electrocompetent *Pseudomonas aeruginosa* cells: application for DNA fragment transfer
519 between chromosomes and plasmid transformation. J. Microbiol. Methods 64, 391-397.
520 Clemmer, K.M., Bonomo, R.A., Rather, P.N., 2011. Analysis of surface motility in
521 *Acinetobacter baumannii*. Microbiology 157, 2534-2544.
522 Cona, A., Rea, G., Angelini, R., Federico, R., Tavladoraki, P., 2006. Functions of amine
523 oxidases in plant development and defence. Trends Plant Sci. 11, 80-88.

524 de Berardinis, V., Vallenet, D., Castelli, V., Besnard, M., Pinet, A., Cruaud, C., Samair, S.,
525 Lechaplais, C., Gyapay, G., Richez, C., Durot, M., Kreimeyer, A., Le Fèvre, F.,
526 Schächter, V., Pezo, V., Döring, V., Scarpelli, C., Médigue, C., Cohen, G. N., Marlière,
527 P., Salanoubat, M., Weissenbach, J., 2008. A complete collection of single-gene deletion
528 mutants of *Acinetobacter baylyi* ADP1. Mol. Syst. Biol. 4, 174.
529 de Breij, A., Dijkshoorn, L., Lagendijk, E., van der Meer, J., Koster, A., Bloemberg, G.,
530 Wolterbeek, R., van den Broek, P., Nibbering, P., 2010. Do biofilm formation and
531 interactions with human cells explain the clinical success of *Acinetobacter baumannii*?
532 PLoS One 5, e10732.
533 Eijkelkamp, B. A., Hassan, K. A., Paulsen, I. T., Brown, M. H., 2011a. Investigation of the
534 human pathogen *Acinetobacter baumannii* under iron limiting conditions. BMC
535 Genomics 12:126.
536 Eijkelkamp, B. A., Stroeher, U. H., Hassan, K. A., Papadimitrious, M. S., Paulsen, I. T.,
537 Brown, M. H., 2011b. Adherence and motility characteristics of clinical *Acinetobacter*
538 *baumannii* isolates. FEMS Microbiol. Lett. 323, 44-51.
539 Gaddy, J.A., Actis, L.A., 2009. Regulation of *Acinetobacter baumannii* biofilm formation.
540 Future Microbiol. 4, 273-278.
541 Gohl, O., Friedrich, A., Hoppert, M., Averhoff, B., 2006. The thin pili of *Acinetobacter* sp.
542 strain BD413 mediate adhesion to biotic and abiotic surfaces. Appl. Environ. Microbiol.
543 72, 1394-1401.
544 Gordon, N.C., Wareham, D.W., 2010. Multidrug-resistant *Acinetobacter baumannii*:
545 mechanisms of virulence and resistance. Int. J. Antimicrob. Agents. 35, 219-226.
546 Hamana, K., Matsuzaki, S., 1992. Diaminopropane occurs ubiquitously in *Acinetobacter* as
547 the major polyamine. J. Gen. Appl. Microbiol. 38, 191-194.

548 Han, X., Kennan, R.M., Davies, J.K., Reddacliff, L.A., Dhungyel, O.P., Whittington, R.J.,
549 Turnbull, L., Whitchurch, C.B., Rood, J.I., 2008. Twitching motility is essential for
550 virulence in *Dichelobacter nodosus*. J. Bacteriol. 190, 3323-3335.
551 Henrichsen, J., 1972. Bacterial surface translocation: a survey and a classification. Bacteriol.
552 Rev. 36, 478-503.
553 Henrichsen, J., 1983. Twitching motility. Annu. Rev. Microbiol. 37, 81-93.
554 Henrichsen, J., Blom, J., 1975. Correlation between twitching motility and possession of polar
555 fimbriae in *Acinetobacter calcoaceticus*. Acta Pathol. Microbiol. Scand. B 83, 103-115.
556 Hermann, S.R., Miller, J.A., O'Neill, S., Tsao, T.T., Harding, R.M., Dale, J.L., 2000. Single-
557 primer amplification of flanking sequences. Biotechniques 29, 1176-1180.
558 Herzberg, C., Friedrich, A., Averhoff, B., 2000. comB, a novel competence gene required for
559 natural transformation of *Acinetobacter* sp. BD413: identification, characterization, and
560 analysis of growth-phase-dependent regulation. Arch. Microbiol. 173, 220-228.
561 Higgins, P.G., Dammhayn, C., Hackel, M., Seifert, H., 2010. Global spread of carbapenem-
562 resistant *Acinetobacter baumannii*. J. Antimicrob. Chemother. 65, 233-238.
563 Higgins, P.G., Wisplinghoff, H., Krut, O., Seifert, H., 2007. A PCR-based method to
564 differentiate between *Acinetobacter baumannii* and *Acinetobacter* genomic species
565 13TU. Clin. Microbiol. Infect. 13, 1199-1201.
566 Hunger, M., Schmucker, R., Kishan, V., Hillen, W., 1990. Analysis and nucleotide sequence
567 of an origin of DNA replication in *Acinetobacter calcoaceticus* and its use for
568 *Escherichia coli* shuttle plasmids. Gene 87, 45-51.
569 Igarashi, K., Kashiwagi, K., 2010. Characteristics of cellular polyamine transport in
570 prokaryotes and eukaryotes. Plant Physiol. Biochem. 48, 506-512.
571 Ikai, H., Yamamoto, S., 1997. Identification and analysis of a gene encoding L-2,4-
572 diaminobutyrate:2-ketoglutarate 4-aminotransferase involved in the 1,3-diaminopropane
573 production pathway in *Acinetobacter baumannii*. J. Bacteriol. 179, 5118-5125.

574 Jacobs, A.C., Hood, I., Boyd, K.L., Olson, P.D., Morrison, J.M., Carson, S., Sayood, K.,
575 Iwen, P.C., Skaar, E.P., Dunman, P.M., 2010. Inactivation of phospholipase D
576 diminishes *Acinetobacter baumannii* pathogenesis. Infect Immun. 78, 1952-1962.
577 Jawad, A., Seifert, H., Snelling, A.M., Heritage, J., Hawkey, P.M., 1998. Survival of
578 *Acinetobacter baumannii* on dry surfaces: comparison of outbreak and sporadic isolates.
579 J. Clin. Microbiol. 36, 1938-1941.
580 Josenhans, C., Suerbaum, S., 2002. The role of motility as a virulence factor in bacteria. Int. J.
581 Med. Microbiol. 291, 605-614.
582 Kämpfer, P., Bark, K., Busse, H.-J., Auling, G., Dott., W., 1991. Numerical and
583 chemotaxonomy of polyphosphate accumulating *Acinetobacter* strains with high
584 polyphosphate: AMP Phosphotransferase (PPAT) activity. Syst. Appl. Microbiol. 15,
585 409-419.
586 Kurihara, S., Suzuki, H., Oshida, M., Benno, Y., 2011. A novel putrescine importer required
587 for type 1 pili-driven surface motility induced by extracellular putrescine in *Escherichia*
588 *coli* K-12. J. Biol. Chem. 286, 10185-10192.
589 Lautrop, H., 1961. *Bacterium anitratum* transferred to the genus *Cytophaga*. Int. Bull.
590 Bacteriol. Nomencl. 11, 107-108.
591 Lautrop, H., 1965. Gliding motility in bacteria as a taxonomic criterion. Publ. Fac. Sci. Univ.
592 J. E. Purkyne, ser. K, 35, 322-327.
593 Mattick, J.S., 2002. Type IV pili and twitching motility. Annu. Rev. Microbiol. 56, 289-314.
594 McBride, M.J., 2010. Shining a light on an opportunistic pathogen. J. Bacteriol. 192, 6325-
595 6326.
596 Merz, A.J., So, M., Sheetz, M.P., 2000. Pilus retraction powers bacterial twitching motility.
597 Nature 407, 98-102.
598 Munoz-Price, L.S., Weinstein, R.A., 2008. *Acinetobacter* infection. N. Engl. J. Med. 358,
599 1271-1281.

600 Murray, T.S., Kazmierczak, B.I., 2008. *Pseudomonas aeruginosa* exhibits sliding motility in
601 the absence of type IV pili and flagella. J. Bacteriol. 190, 2700-2708.
602 Mussi, M.A., Gaddy, J.A., Cabruja, M., Arivett, B.A., Viale, A.M., Rasia, R., Actis. L.A.,
603 2010. The opportunistic human pathogen *Acinetobacter baumannii* senses and responds
604 to light. J. Bacteriol. 192, 6336-6345.
605 Peleg, A.Y., Jara, S., Monga, D., Eliopoulos, G.M., Moellering, R.C. Jr., Mylonakis, E., 2009.
606 *Galleria mellonella* as a model system to study *Acinetobacter baumannii* pathogenesis
607 and therapeutics. Antimicrob Agents Chemother. 53, 2605-2609.
608 Peleg, A.Y., Seifert, H., Paterson, D.L., 2008. *Acinetobacter baumannii*: emergence of a
609 successful pathogen. Clin. Microbiol. Rev. 21, 538-582.
610 Rashid, M.H., Kornberg, A., 2000. Inorganic polyphosphate is needed for swimming,
611 swarming, and twitching motilities of *Pseudomonas aeruginosa*. Proc. Natl. Acad. Sci.
612 U S A 97, 4885-4890.
613 Seifert, H., Dolzani, L., Bressan, R., van der Reijden, T., van Strijen, B., Stefanik, D.,
614 Heersma, H., Dijkshoorn, L., 2005. Standardization and interlaboratory reproducibility
615 assessment of pulsed-field gel electrophoresis-generated fingerprints of *Acinetobacter*
616 *baumannii*. J. Clin. Microbiol. 43, 4328-4335.
617 Semmler, A.B., Whitchurch, C.B., Mattick, J.S., 1999. A re-examination of twitching motility
618 in *Pseudomonas aeruginosa*. Microbiology 145, 2863-2873.
619 Shah, P., Swiatlo, E., 2008. A multifaceted role for polyamines in bacterial pathogens. Mol.
620 Microbiol. 68, 4-16.
621 Skerker, J.M., Berg, H.C., 2001. Direct observation of extension and retraction of type IV pili.
622 Proc. Natl. Acad. Sci. U S A 98, 6901-6904.
623 Stolz, A., Busse, H.-J., Kämpfer, P., 2007. *Pseudomonas knackmussii* sp. nov.. Int. J. Syst.
624 Evol. Microbiol. 57, 572–576.

625 Sturgill, G., Rather, P.N., 2004. Evidence that putrescine acts as an extracellular signal
626 required for swarming in *Proteus mirabilis*. Mol. Microbiol. 51, 437-446.
627 Tenover, F.C., Arbeit, R.D., Goering, R.V., Mickelsen, P.A., Murray, B.E., Persing, D.H.,
628 Swaminathan, B., 1995. Interpreting chromosomal DNA restriction patterns produced
629 by pulsed-field gel electrophoresis: criteria for bacterial strain typing. J. Clin. Microbiol.
630 33, 2233-2239.
631 Turton, J.F., Woodford, N., Glover, J., Yarde, S., Kaufmann, M.E., Pitt, T.L., 2006.
632 Identification of *Acinetobacter baumannii* by detection of the $bla_{\text{OXA-51}}$-like
633 carbapenemase gene intrinsic to this species. J. Clin. Microbiol. 44, 2974-2976.
634 Verstraeten, N., Braeken, K., Debkumari, B., Fauvart, M., Fransaer, J., Vermant, J., Michiels,
635 J., 2008. Living on a surface: swarming and biofilm formation. Trends Microbiol. 16,
636 496-506.
637 Volkmann, M., Skiebe, E., Kerrinnes, T., Faber, F., Lepka, D., Pfeifer, Y., Holland, G.,
638 Bannert, N., Wilharm, G., 2010. *Orbus hercynius* gen. nov., sp. nov., isolated from
639 faeces of wild boar, is most related to *Enterobacteriales* and *Pasteurellales*. Int. J. Syst.
640 Evol. Microbiol. 60, 2601-2605.
641 Wilharm, G., Lepka, D., Faber, F., Hofmann, J., Kerrinnes, T., Skiebe, E., 2010. A simple and
642 rapid method of bacterial transformation. J. Microbiol. Methods 80:215-216.
643 Yamamoto, S., Tsuzaki, Y., Tougou, K., Shinoda, S., 1992. Purification and characterization
644 of L-2,4-diaminobutyrate decarboxylase from *Acinetobacter calcoaceticus*. J. Gen.
645 Microbiol. 138, 1461-1465.
646

Legends to illustrations

Fig. 1: *A. baumannii* ATCC 17978: motility phenotypes depend on nutrient composition of semi-solid media. *A. baumannii* ATCC 17978 was stab-inoculated into semi-solid media and incubated for 16 h at 37°C. (A) 0.5% agarose, 5 g/l tryptone (B) 0.5% agarose, 2 g/l tryptone (C) 0.8% thread agar, 10 g/l tryptone (D) 0.8% thread agar, 5 g/l tryptone. Pictures are representative of three independent experiments performed consecutively.

Fig. 2: Variation range of *A. baumannii* motility morphotypes. Isolates BMBF 425 (A), 10-092 (B), BMBF 49 (C), (D), BMBF 509 (D), BMBF 368I (E) and BMBF 142 (F) were stab-inoculated into 0.5% agarose supplemented with 5 g/l tryptone and 2.5 g/l NaCl. Each strain was inoculated four times on a single plate and incubated for 16 h at 37°C. Biofilms formed after movement at the interphase are indicated by arrows. Plate shown in (B) was incubated at room temperature for another 24 h to improve visibility of the interphase biofilm. Pictures are representative of three independent experiments.

Fig. 3: Evidence of surface manipulation contributing to *Acinetobacter* motility. Phase-contrast microscopy examination of the moving front of an *A. baumannii* ATCC 17978 colony on 0.5% agarose supplemented with 5 g/l tryptone and 2.5 g/l NaCl. (A) Macroscopic photograph of the colony and microscopic photographs of indicated details at 40-fold (B) and 200-fold magnification (C). A liquid zone (indicated by white triangles) precedes the moving bacterial mass (indicated by black triangles).

Fig. 4: Schematic overview of the genetic organization of *A. baumannii* ATCC 17978 *ddc* and *dat* genes involved in production of 1,3-diaminopropane. Gene A1S-2453 (abbrev. 2453/*ddc*) encodes L-2,4-diaminobutyrate decarboxylase (DABA DC); gene A1S-2454

673 (abbrev. 2454/*dat*) encodes L-2,4-diaminobutyrate:2-ketoglutatrate 4-aminotransferase
674 (DABA AT); transposon insertion sites of mutants 129 and 277 are indicated by triangles;
675 primers indicated were used for amplification of the *dat-ddc* fragment to construct
676 complementation plasmid p*dat-ddc*.
677
678 **Fig. 5: The motility defect of mutants 129 (*ddc*) and 277 (*dat*) is restored by**
679 **supplementation with 1,3-diaminopropane (DAP).** (A) Phenotype of parental strain
680 *A. baumannii* ATCC 17978 and transposon insertion mutants 129/*ddc* and 277/*dat* on 0.5%
681 agarose motility plates incubated for 16 h at 37°C. Each strain was stab-inoculated four times.
682 (B) Phenotype of strains as above and as indicated on 0.5% agarose motility plates
683 supplemented with 0.001% 1,3-diaminopropane (DAP). These results were reproduced twice.
684
685 **Fig. 6: Complementation of *dat* and *ddc* mutants restores motility.** Plasmid p*dat-ddc*
686 harbouring the *dat-ddc* operon including putative promoter and terminator regions or its
687 backbone plasmid pWH1266 was transformed into motility mutants 277/*dat* and 129/*ddc*,
688 respectively. Motility phenotypes were compared on 0.5% agarose motility plates after
689 incubation for 16 h at 37°C. Four transformants/colonies were stab-inoculated on each plate.
690 The pictures shown are representative of three independent experiments.
691
692 **Fig. 7: Transformation of naturally competent *A. baumannii* isolate 07-095 with**
693 **chromosomal DNA derived from mutants 129/*ddc* and 277/*dat* reveals a motility**
694 **phenotype at the interphase.** *A. baumannii* 07-095, an isolate preferentially moving at the
695 interphase when inoculated on 0.5% agarose motility plates, was transformed with
696 chromosomal DNA prepared from mutants 129/*ddc* and 277/*dat* of *A. baumannii* ATCC
697 17978. Homologous recombination of the kanamycin cassette of EZ-Tn5 into the *ddc* and *dat*
698 gene, respectively, was confirmed by PCR. Four transformants each were stab-inoculated into

699 0.5% agarose motility plates with or without DAP supplementation (0.001%) as indicated.
700 After incubation for 16 h at 37°C, plates were incubated for another 48 h at room temperature
701 to facilitate visualization of biofilms formed at the interphase after movement of colonies. All
702 transformation experiments were reproduced at least three times.
703
704 **Fig. 8: Transformation of naturally competent *A. baumannii* isolate 07-102 with**
705 **chromosmal DNA derived from mutants 129/*ddc* and 277/*dat*.** Analogous to the procedure
706 described in legend to Fig. 7, *A. baumannii* 07-102 was transformed with chromosomal DNA
707 prepared from mutants 129/*ddc* and 277/*dat* of *A. baumannii* ATCC 17978 and analysed on
708 0.5% agarose motility plates with or without DAP supplementation (0.001%).
709
710 **Fig. 9: Mutants 129/*ddc* and 277/*dat* of ATCC 17978 are attenuated in the *Galleria***
711 ***mellonella* caterpillar infection model.** *G. mellonella* caterpillars were infected with $3x10^5$
712 CFU of bacteria as indicated and incubated at 37°C for 5 days in Petri dishes. Curves
713 represent a single experiment with 16 larvae in each group. Photographs of the larvae taken
714 24, 48 and 96 hours after infection/injection are available as Supplementary Fig. 4. $P < 0.05$
715 for comparison of ATCC 17978 and mutant 129/*ddc* and for comparison of ATCC 17978 and
716 mutant 277/*dat* including data from three independent experiments.

Figure 1

Fig. 1 Skiebe et al.

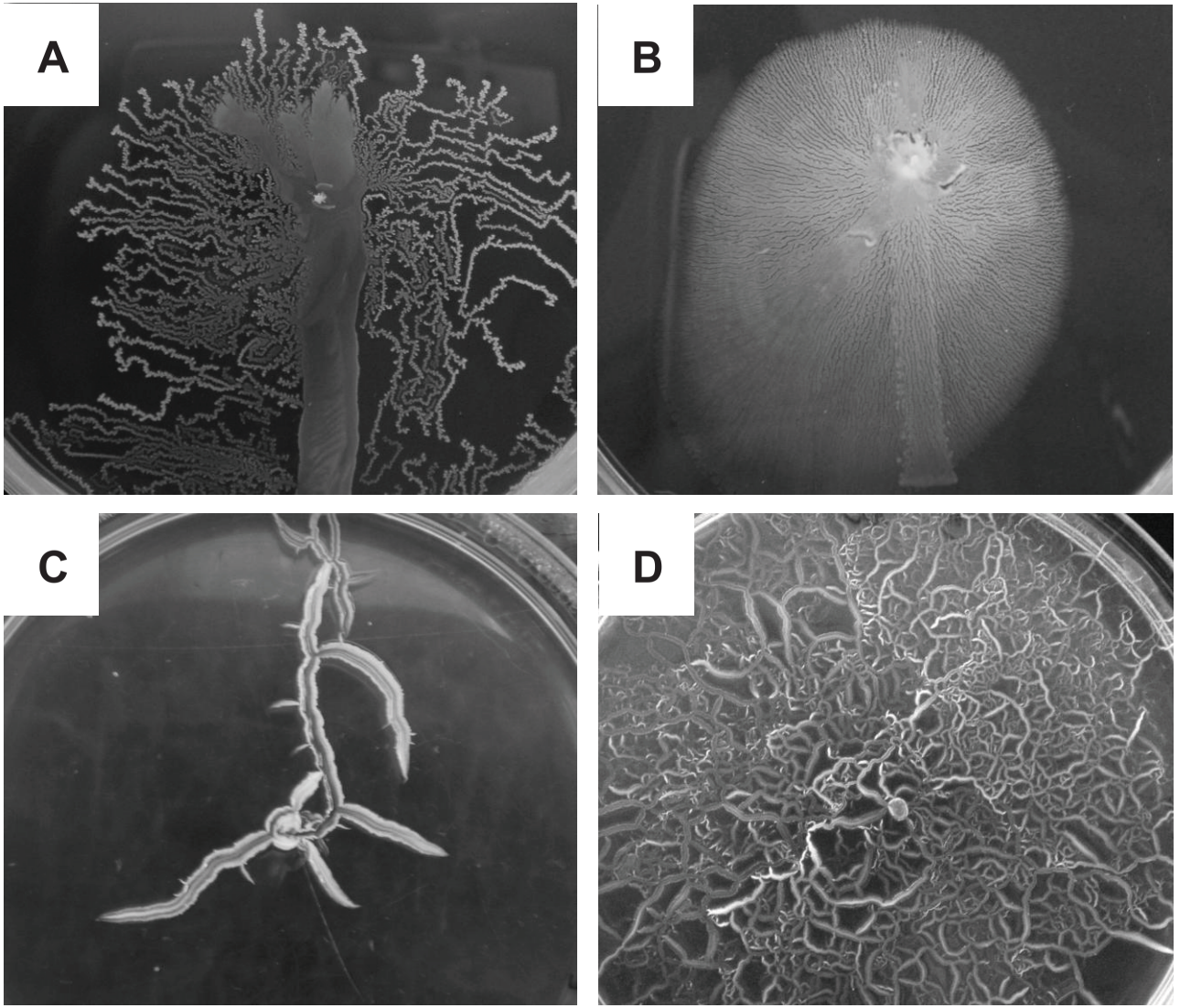


Figure 2

Fig. 2 Skiebe et al.

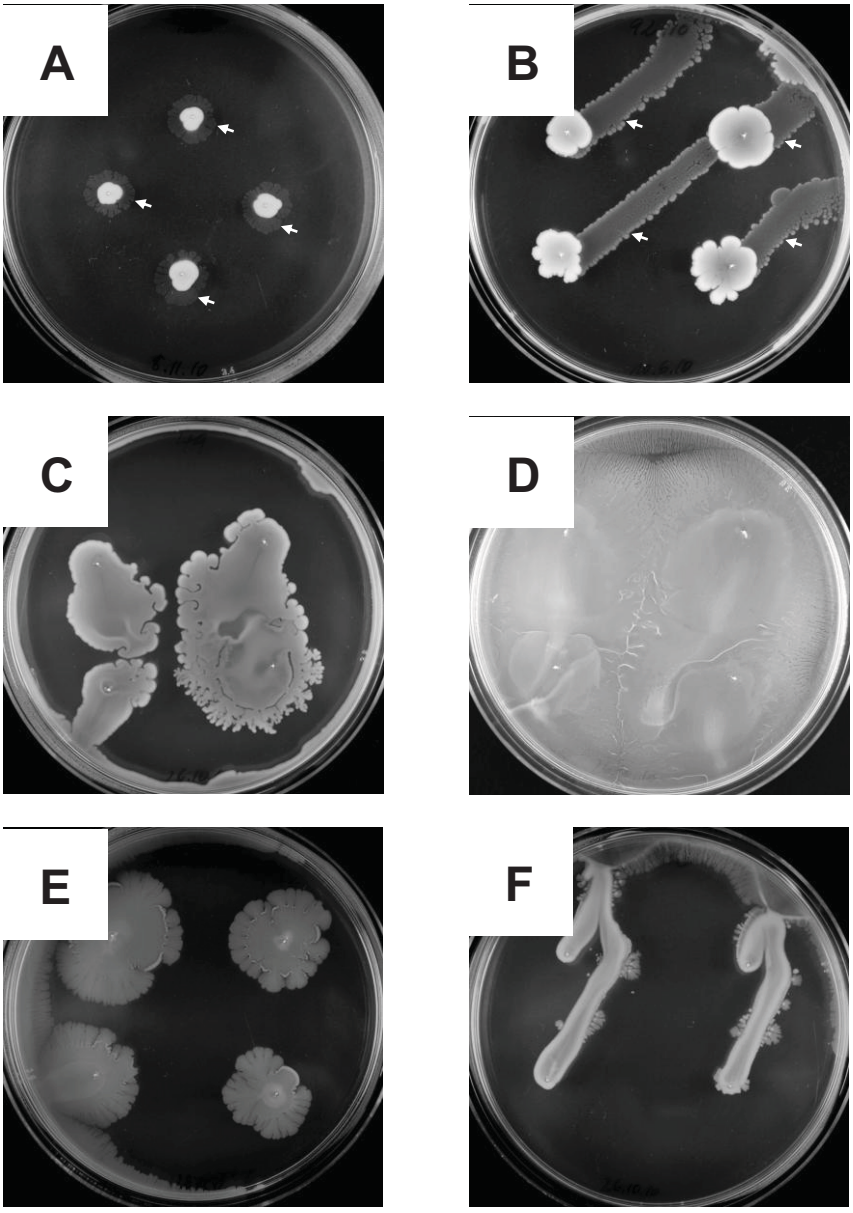


Figure 3

Fig. 3 Skiebe et al.

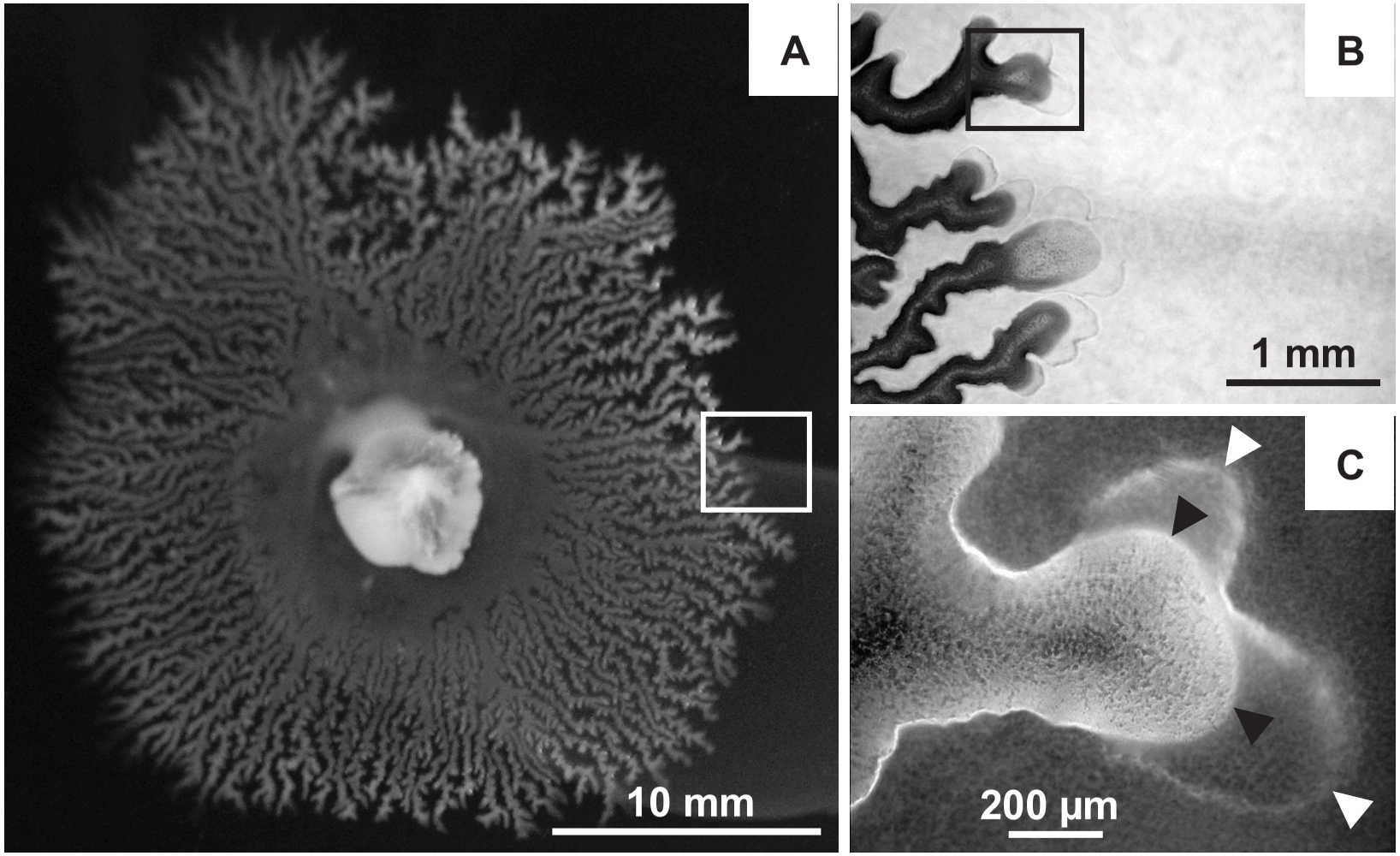


Figure 4

Fig. 4 Skiebe et al.

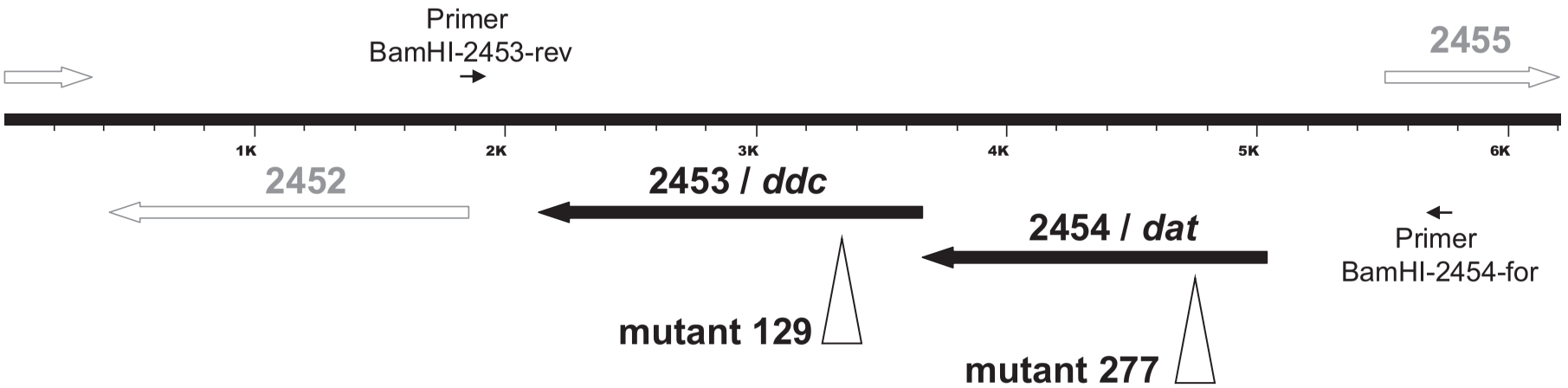


Figure 5

Fig. 5 Skiebe et al.

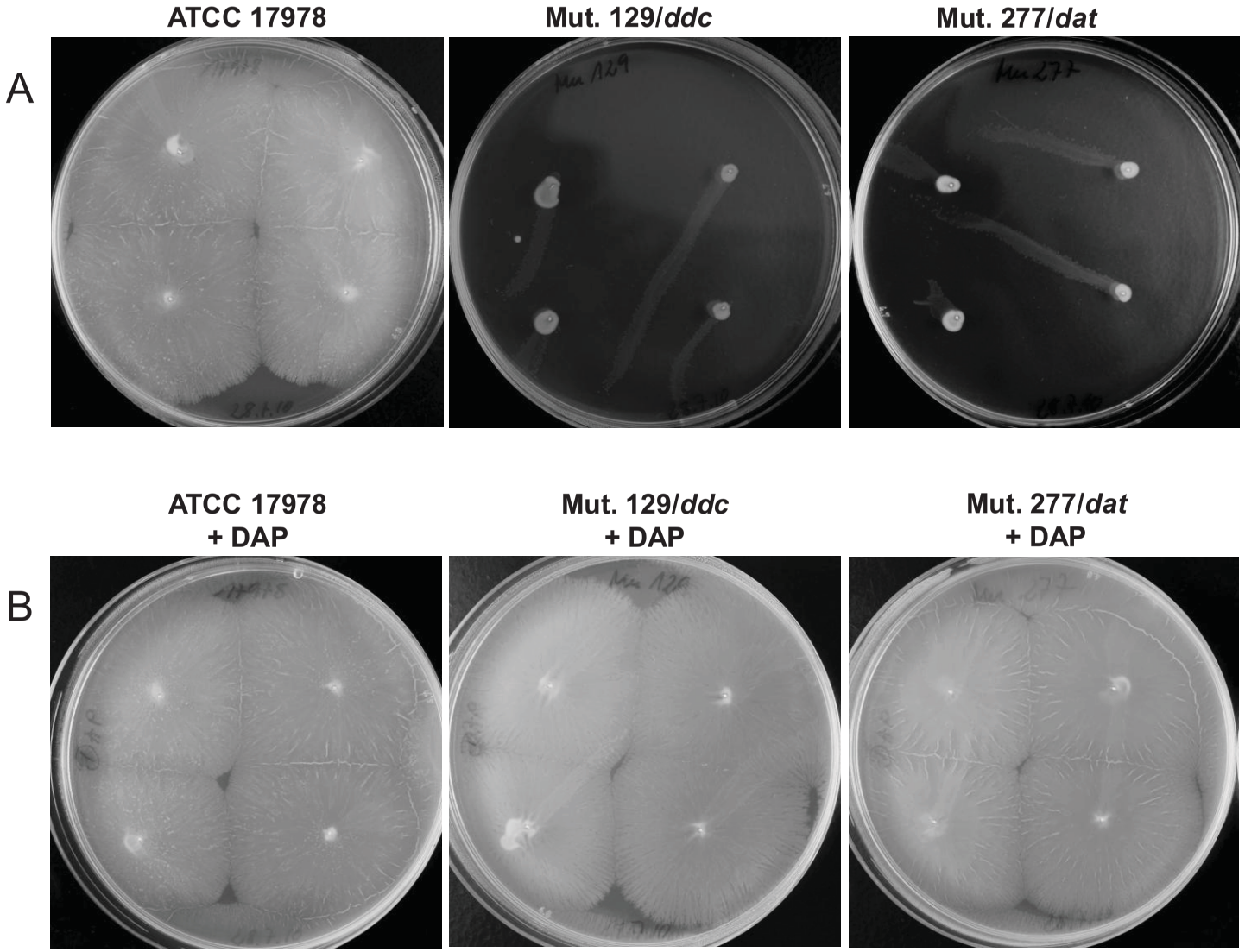


Figure 6

Fig. 6 Skiebe et al.

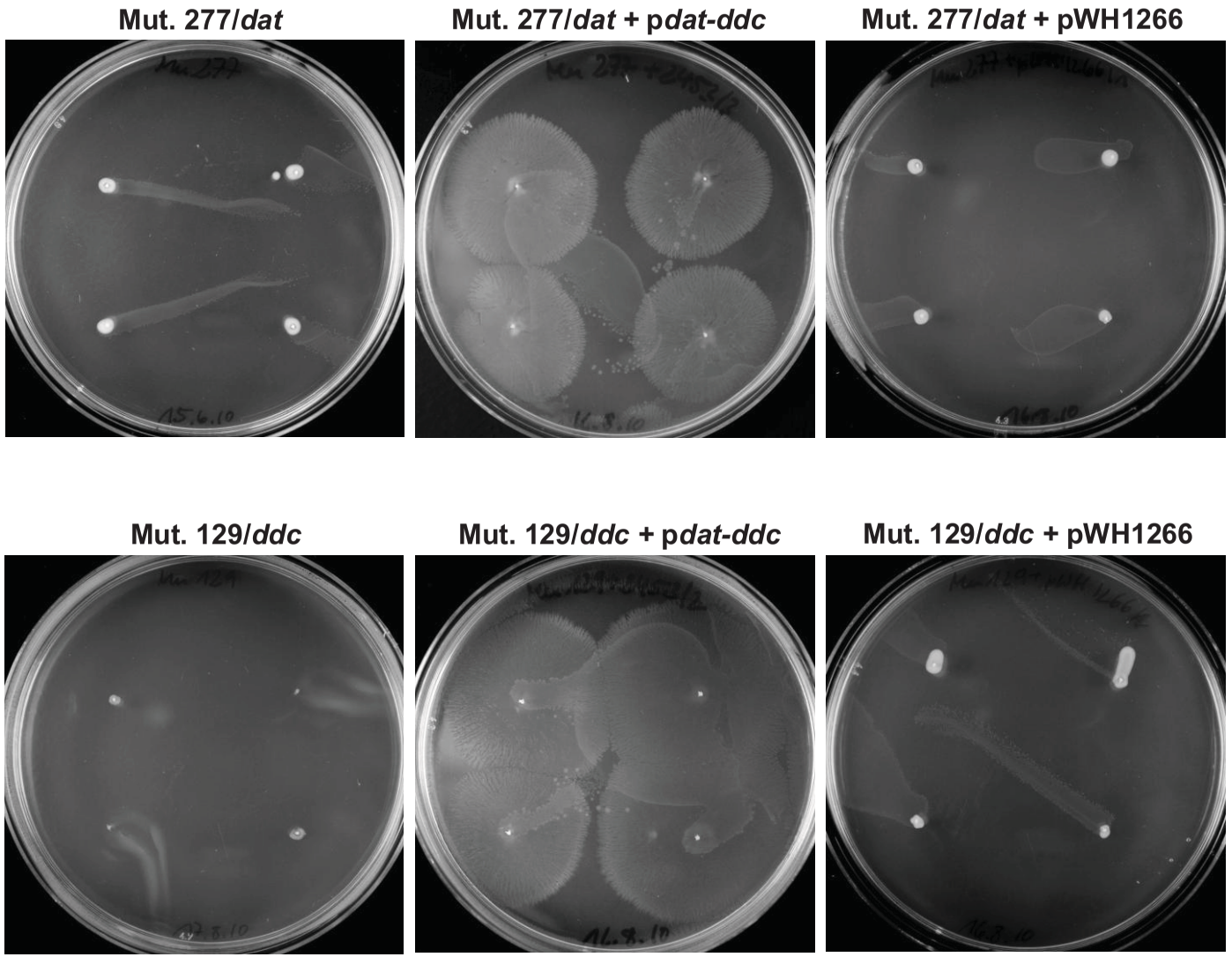


Figure 7

Fig. 7 Skiebe et al.

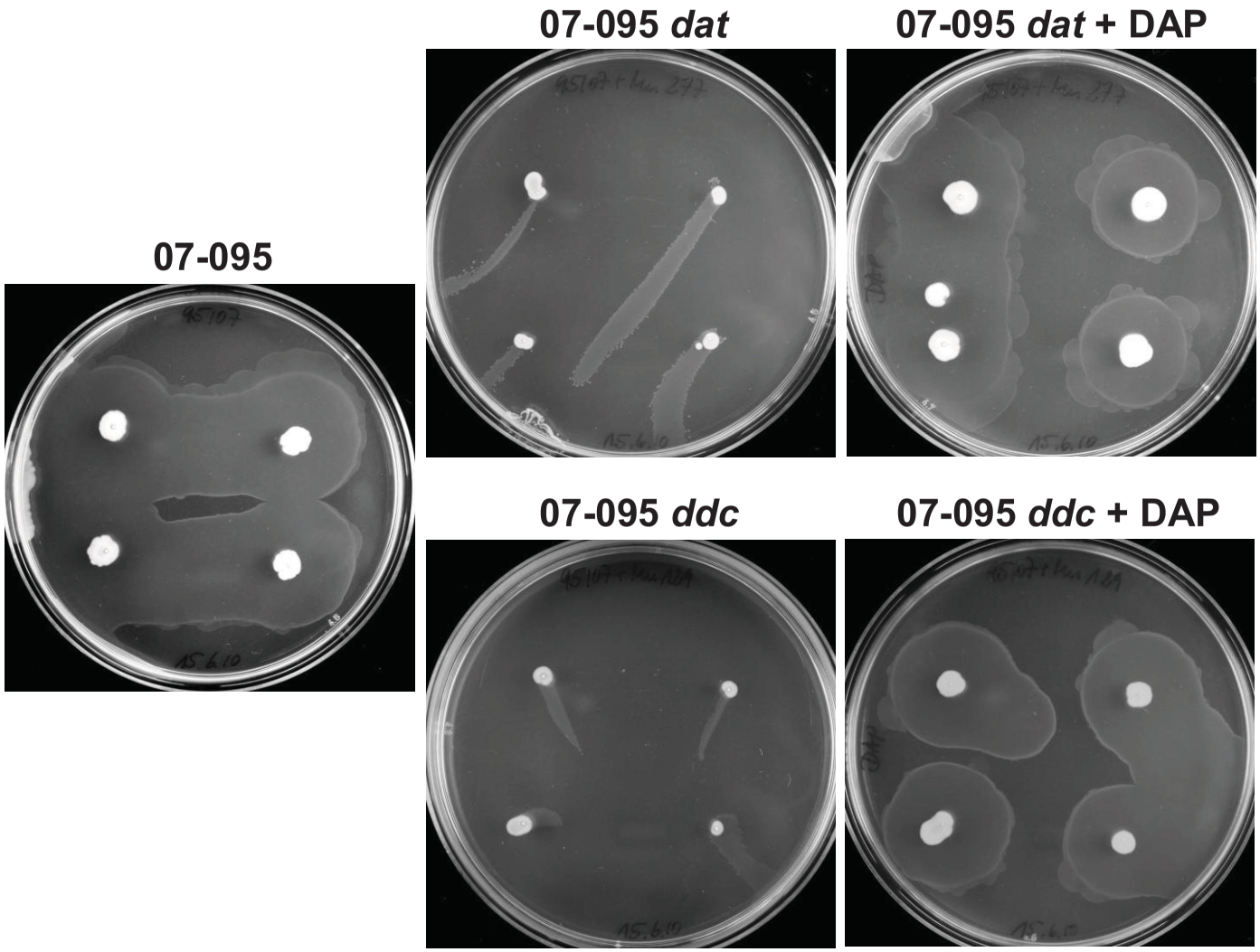


Figure 8

Fig. 8 Skiebe et al.

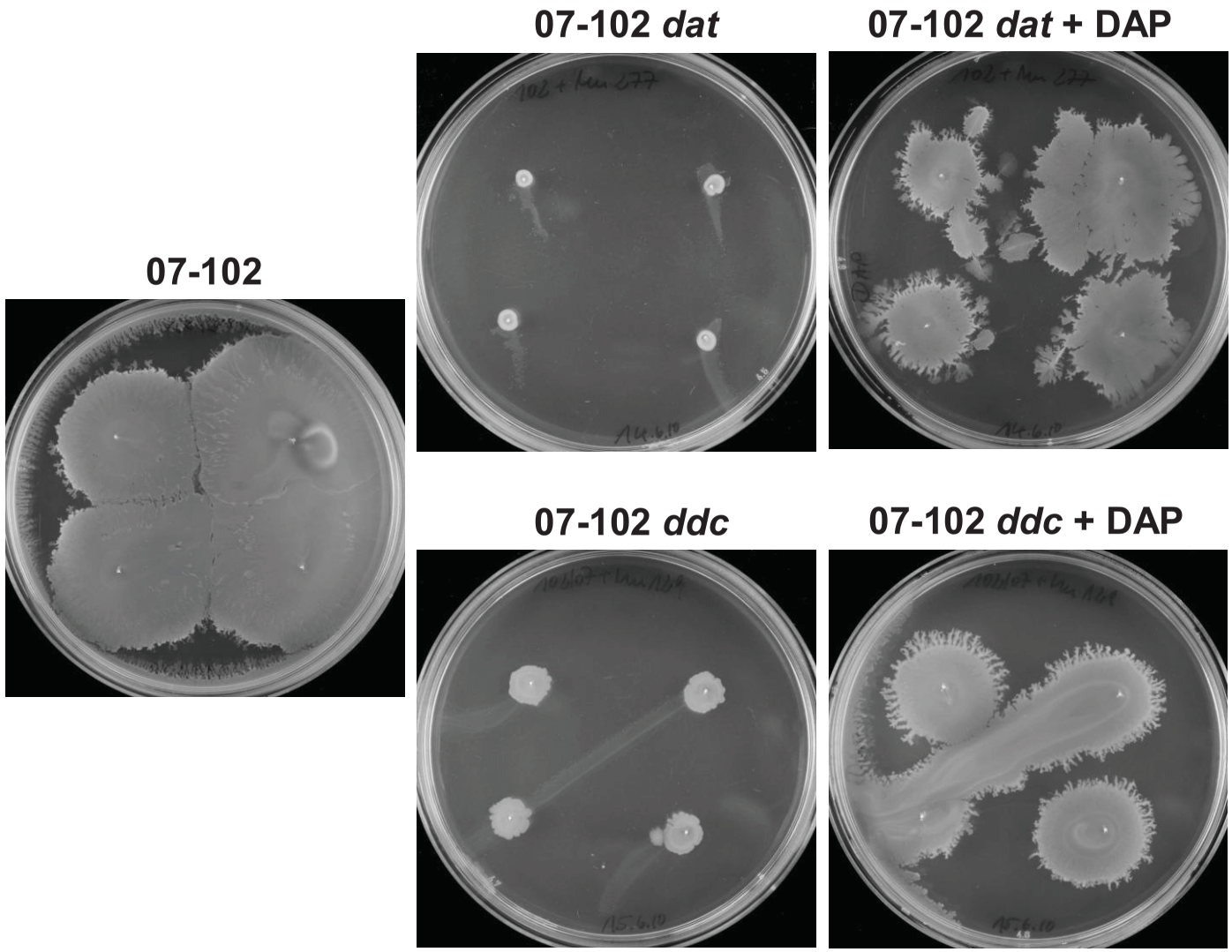
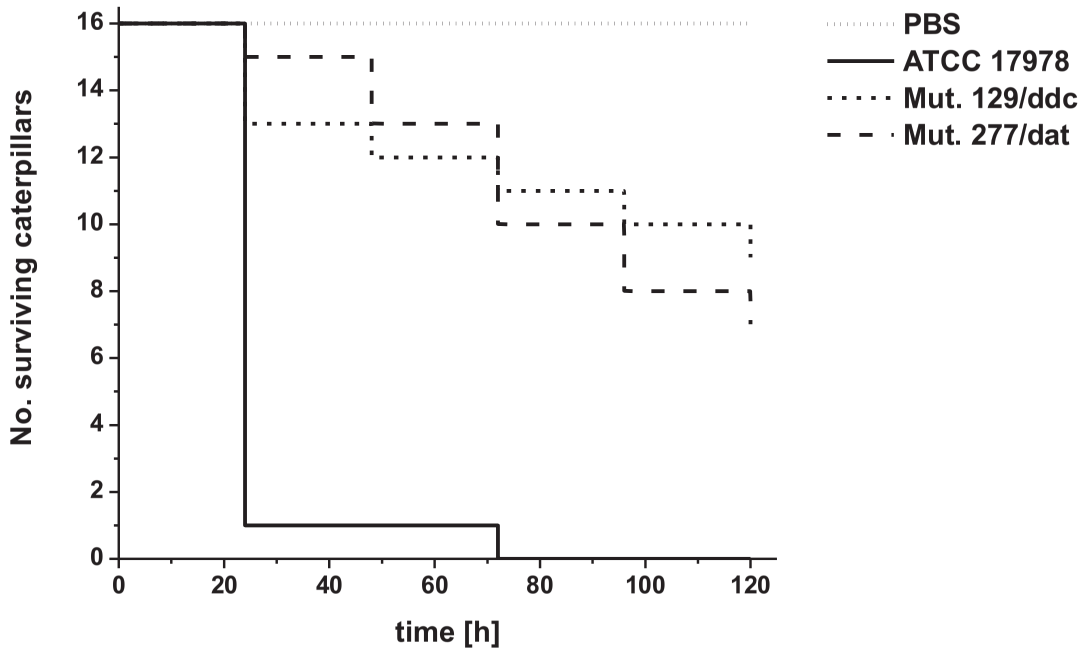


Figure 9

Fig. 9 Skiebe et al.



Table

Supplementary Table 1 Skiebe et al.

	Strain identity	Source (Country) / epidemiological group#	Motility at the surface of 0.5% agarose	Motility at the interphase (0.5% agarose)	Ditching on 0.8% thread agar
1	ATCC 17978	ATCC	+++	-	+++
2	*A. baylyi* ADP1	ATCC	+++	-	++
3	ATCC 19606^{T}	ATCC	+	-	-
4	07-016	Germany	+	-	-
5	07-028	Germany	++	-	+
6	07-029	Germany	++	+++	+++
7	07-032	Germany, probably imported from Romania	++	-	++
8	07-033	Germany	+++	-	++
9	07-034	Germany	+	-	-
10	07-037	Germany, probably imported from Croatia	++	-	+++
11	07-038	Germany, probably imported from Cameroon	+	-	+
12	07-095	Germany	+	+++	+
13	07-097	Germany	++	++	+
14	07-099	Germany	+	-	-
15	07-100	Germany	+	-	++
16	07-101	Germany	+++	-	++
17	07-102	Germany	+++	++	+++
18	07-103	Germany	+	-	-
19	07-104	Germany	+++	-	+++
20	07-105	Germany	+++	-	+
21	07-107	Germany	+	-	+
22	07-108	Germany	++	-	+
23	07-110	Germany	++	-	+
24	07-111	Germany	++	++	++
25	07-112	Germany	++	-	++
26	07-113	Germany	+	-	+

27	07-114	Germany	+	-	+
28	07-116	Germany	-	-	-
29	07-156	Germany, probably imported from Egypt	+	-	++
30	07-161	Germany, probably imported from Serbia (Göttig *et al.*, 2010)	+++	-	+++
31	09-013	Germany	+	+	-
32	09-015	Germany	+++	-	++
33	09-018	Germany	+	++	-
34	09-022	Germany	+	-	++
35	09-023	Germany	++	+	+
36	09-024	Germany	+	++	-
37	09-025	Germany	+	-	+++
38	09-039	Germany	++	+	++
39	09-046	Germany, probably imported from Egypt	+	++	-
40	10-042	Germany	+	-	++
41	10-092	Germany	++	-	+
42	10-095	Germany	++	-	+++
43	10-096	Germany	+	++	-
44	BMBF 18	USA (WW 3)	++	++	-
45	BMBF 28	Poland (WW 4)	+	-	-
46	BMBF 34	Germany (WW 1)	++	-	+++
47	BMBF 40	Latvia (WW 7)	++	+	+++
48	BMBF 49	Pakistan (WW 1)	+++	++	+++
49	BMBF 59	Spain (WW 3)	++	++	+
50	BMBF 60	Spain (WW 8)	+	-	-
51	BMBF 83	Spain (WW 1)	+	-	+
52	BMBF 93	Spain (WW 5)	+	++	-
53	BMBF 134	Turkey (WW 4)	++	-	-
54	BMBF 139	Turkey (WW 8)	++	-	-
55	BMBF 142	Turkey (WW 8)	++	-	-

56	BMBF 206	USA	(WW 3)	++	-	-
57	BMBF 232	Argentina	(WW 5)	+++	-	+++
58	BMBF 247	Argentina	(WW 4)	++	-	-
59	BMBF 258	South Africa	(WW 3)	++	++	++
60	BMBF 262	USA	(WW 5)	+	-	-
61	BMBF 320	Chile	(WW 4)	++	+++	-
62	BMBF 342	France	(WW 8)	++	-	++
63	BMBF 344	India	(WW 4)	++	++	-
64	BMBF 345	India	(WW 1)	++	+	+++
65	BMBF 355	Switzerland	(WW 7)	++	++	-
66	BMBF 384	USA	(WW 2)	+	+	+++
67	BMBF 394	Greece	(WW 1)	++	++	++
68	BMBF 399	S. Africa	(WW 2)	+++	+	+++
69	BMBF 417	Mexico	(WW 7)	+++	+	+++
70	BMBF 425	Colombia	(WW 7)	++	++	++
71	BMBF 437	Colombia	(WW 5)	++	+++	-
72	BMBF 440	Germany	(WW 5)	+	-	+
73	BMBF 441	Taiwan	(WW 2)	+	-	+++
74	BMBF 446	Honduras	(WW 6)	+	+	++
75	BMBF 448	Honduras	(WW 6)	+	++	++
76	BMBF 449	Honduras	(WW 6)	+	+	+
77	BMBF 450	Honduras	(WW 6)	++	-	++
78	BMBF 456	Australia	(WW 2)	++	+	+
79	BMBF 459	South Korea	(WW 8)	++	-	-
80	BMBF 468	Italy	(WW 6)	++	-	-
81	BMBF 483	USA	(WW 3)	++	++	-
82	BMBF 509	Spain	(WW 2)	+++	-	+++

83	BMBF 368l	Venezuela	(WW 7)	+++	++	+++

Evaluation of motility on 0.5% agarose (surface and interphase) after incubation for 16 hours at 37°C*:
Spreading zone
3-5 mm: +
6 to 15 mm: ++
>15 mm: +++
*All isolates only scoring "+" were subsequently incubated at room temperature for seven days; in all cases additional spread could be observed.

Evaluation of formation of ditches on 0.8% thread agar after incubation for 16 hours at 37°C:

Ditches < 20 mm: +
Ditches 20 to 40 mm: ++
Ditches > 40 mm: +++

All experiments were performed at least in independent triplicates and the determined values were averaged.

Epidemiological group (if available) according to Higgins et al. (2010); worldwide clusters 1-3 (WW 1-3) include representatives of European clones I-III, respectively.

References:

Göttig, S., Pfeifer, Y., Wichelhaus, T.A., Zacharowski, K., Bingold, T., Averhoff, B., Brandt, C., Kempf, V.A., 2010. Lancet Infect. Dis. 10:828-829.

Higgins, P.G., Dammhayn, C., Hackel, M., Seifert, H., 2010. J. Antimicrob. Chemother. 65, 233-238.

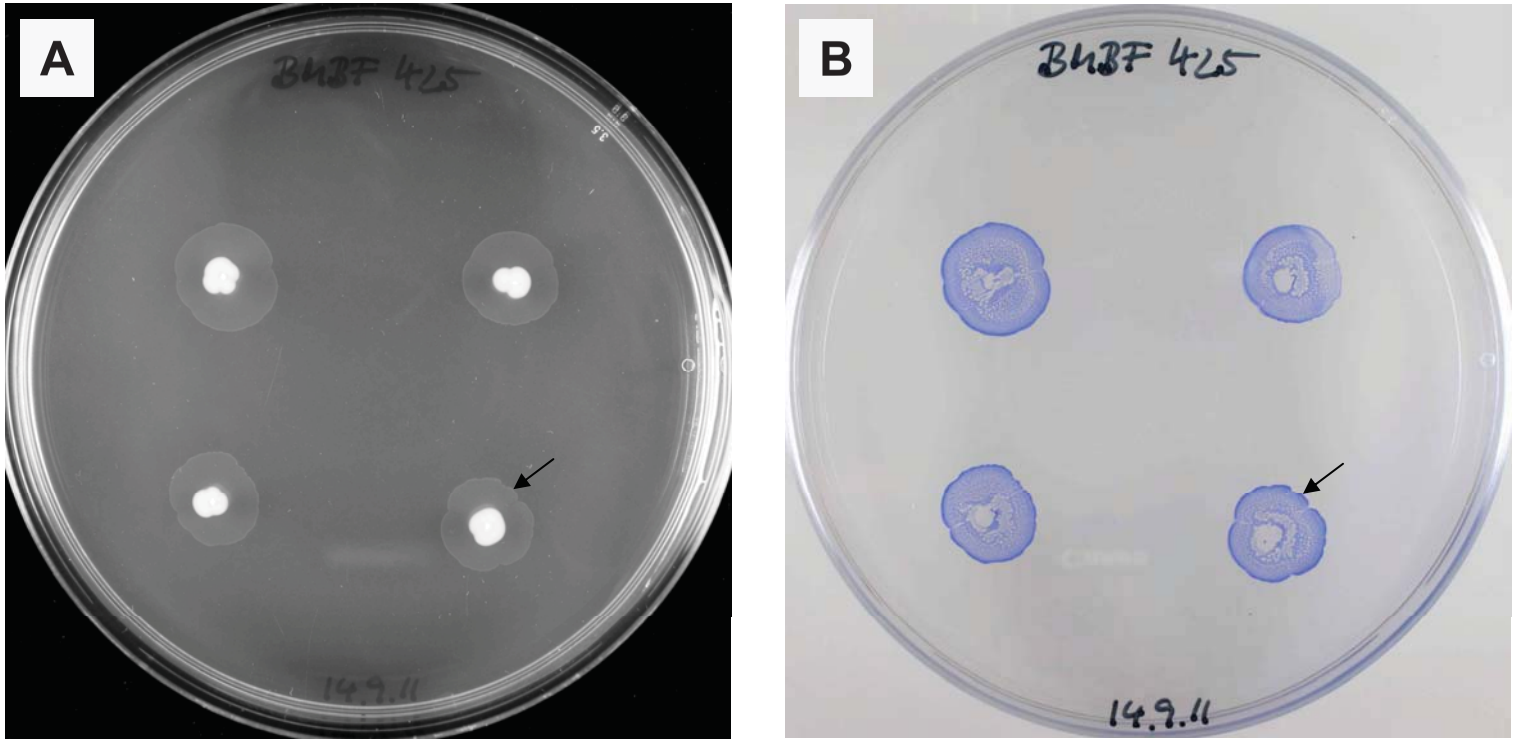
Supplementary Table 2

Skiebe et al.

		µmol/g dry mass				
No.	Strain	DAP	PUT	CAD	SPD	HSPD
1	ATCC 17978 *	73,56	0,14	0,09	0,93	0,02
2	ATCC 17978	81,32	0,08	-	1,68	-
3	Mut. 129/*ddc*	0,50	0,35	-	7,66	0,05
4	Mut. 129/*ddc* + p*dat-ddc*	79,82	0,13	-	2,50	-
5	Mut. 277/*dat*	1,84	0,21	-	7,67	0,05
6	Mut. 277/*dat* + p*dat-ddc*	81,10	0,19	-	2,75	0,01
7	07-095	71,51	0,11	-	5,34	0,03
8	07-095 *ddc*	0,59	0,13	-	2,30	0,01
9	07-095 *dat*	1,28	0.08	-	5,79	0,02
10	07-102	68,01	0,23	-	0,83	0.05
11	07-102 *ddc*	1,09	0,07	-	5,44	-
12	07-102 *dat*	5,17	0,12	0,03	8,23	0,06
13	*A. baylyi* ADP1	90,98	11,93	8,46	7,29	0,04
14	*A. baylyi* ADP1 ΔACIAD1210 (*dat*)	32,19	0,69	0,05	3,70	0,03
15	*A. baylyi* ADP1 ΔACIAD1211 (*ddc*)	135,89	0,87	0,02	3,76	0,02

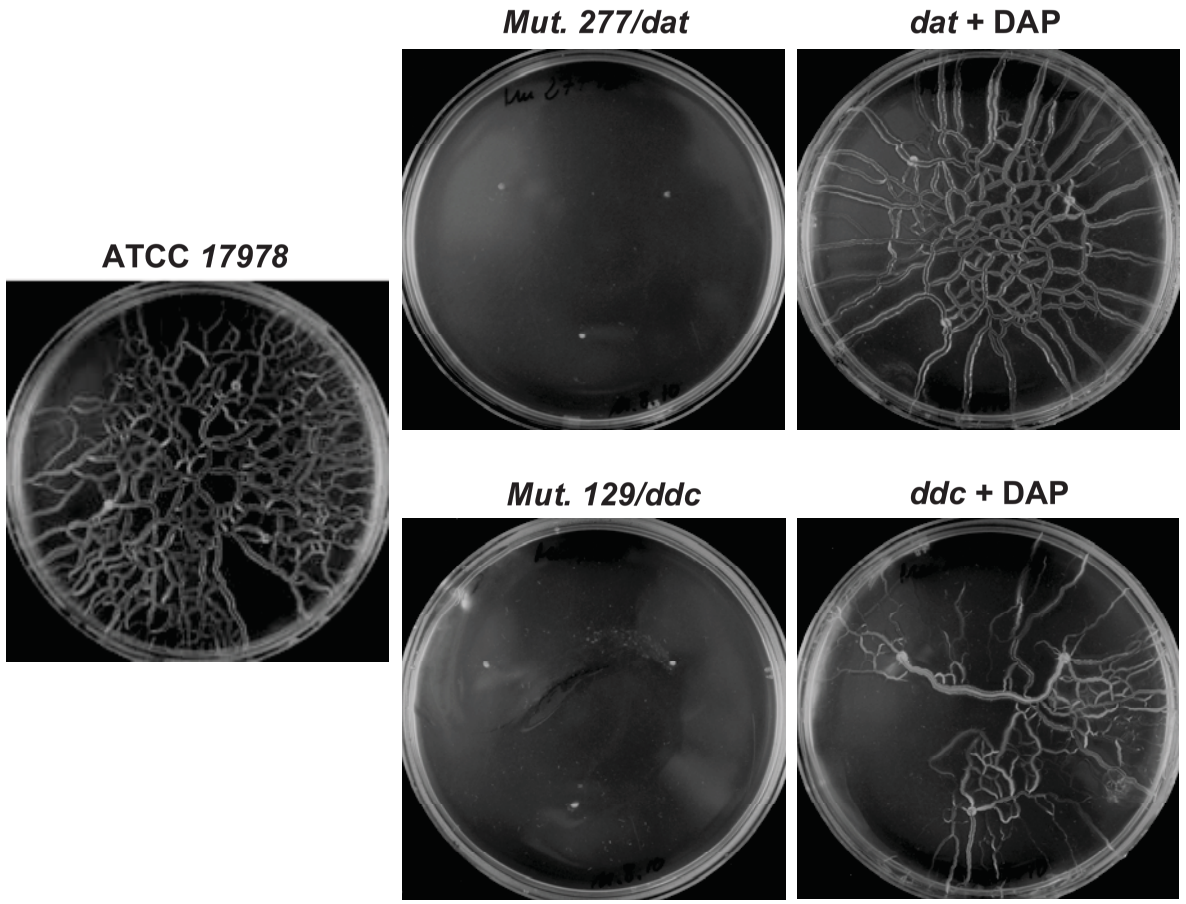
Bacteria were cultured in liquid broth (5 g/l tryptone, 2.5 g/l NaCl) except sample 1 (ATCC 17978 *) which was grown a motility plate overnight (0.5% agarose, 5 g/l tryptone, 2.5 g/l NaCl). Comparable results were obtained in an independent replicate experiment representing sample No. 2-6.

Supplementary Fig. 1 Skiebe et al.



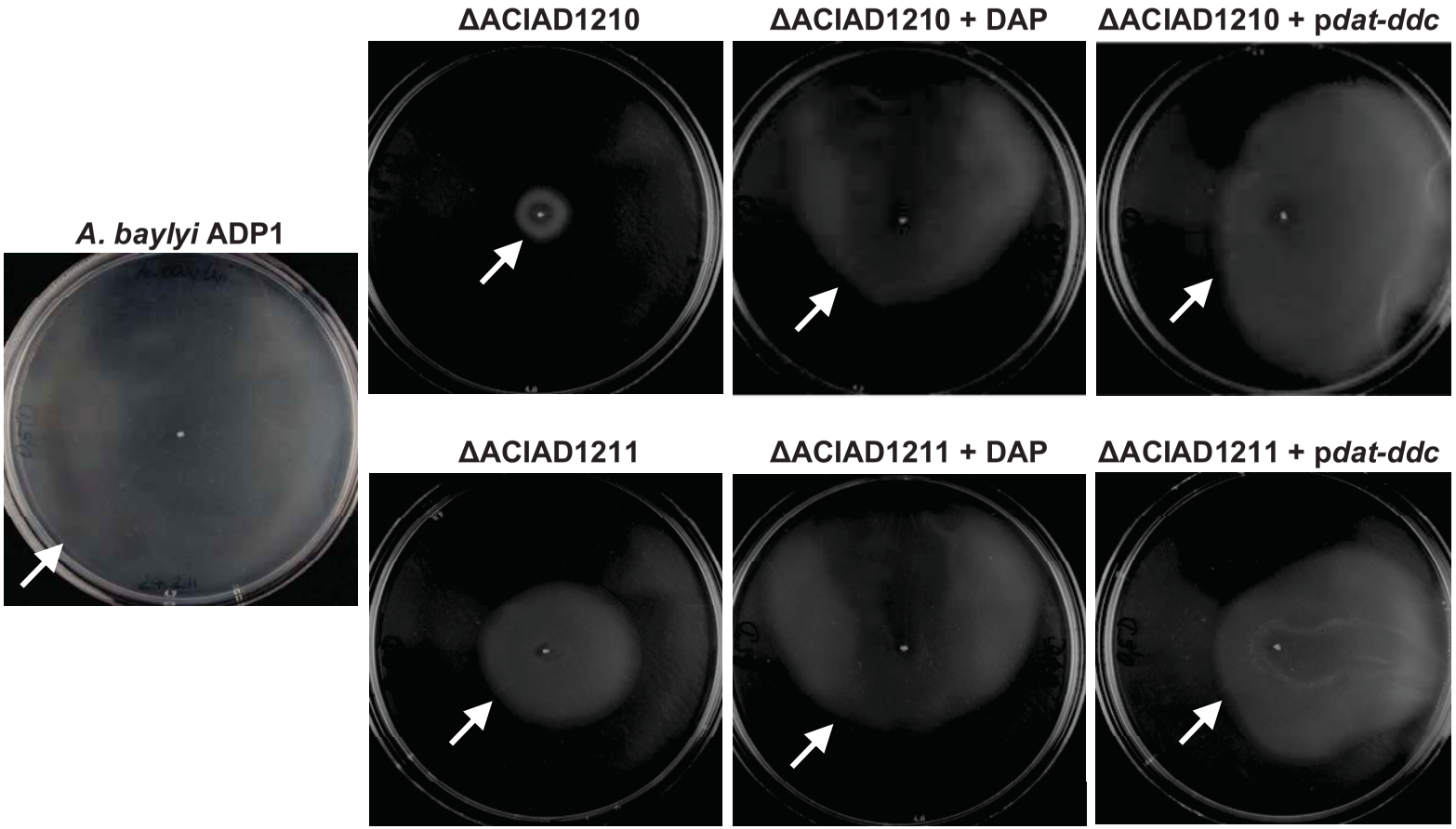
Motility at the interphase between petri dish and agarose. *A. baumannii* isolate BMBF 425 was stab-inoculated four times into 0.5% agarose supplemented with 5 g/l tryptone and 2.5 g/l NaCl and incubated for 16 h at 37°C. The colonies were photographed (A), subsequently the plate was opened under the biological safety-cabinet and dried for 10 minutes. Then, the agarose was removed from the petri dish and the bacterial biofilm sticking to the bottom was photographed after staining with Coomassie blue (B). The motility front-line of one of the colonies is indicated with an arrow.

Supplementary Fig. 2 Skiebe et al.



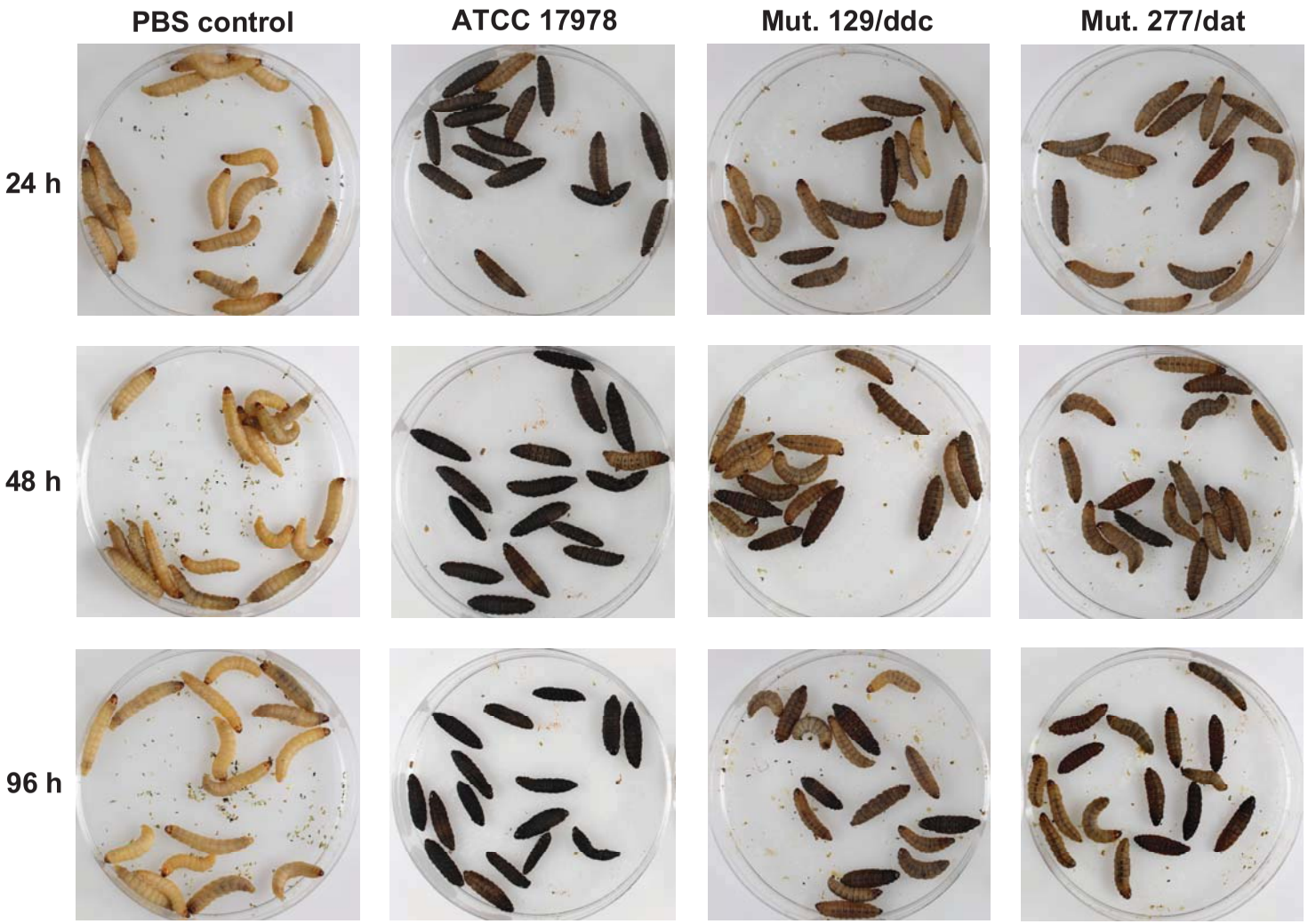
Formation of ditches on thread agar depends on 1,3-diaminopropane. Phenotype of parental strain *A. baumannii* ATCC 17978 and transposon insertion mutants 129/*ddc* and 277/*dat* on 0.8% thread agar incubated for 16 h at 37°C; DAP supplementation (0.001%) as indicated. Each strain was inoculated in triplicate and the whole experiment was repeated twice with comparable outcome.

Supplementary Fig. 3 Skiebe et al.



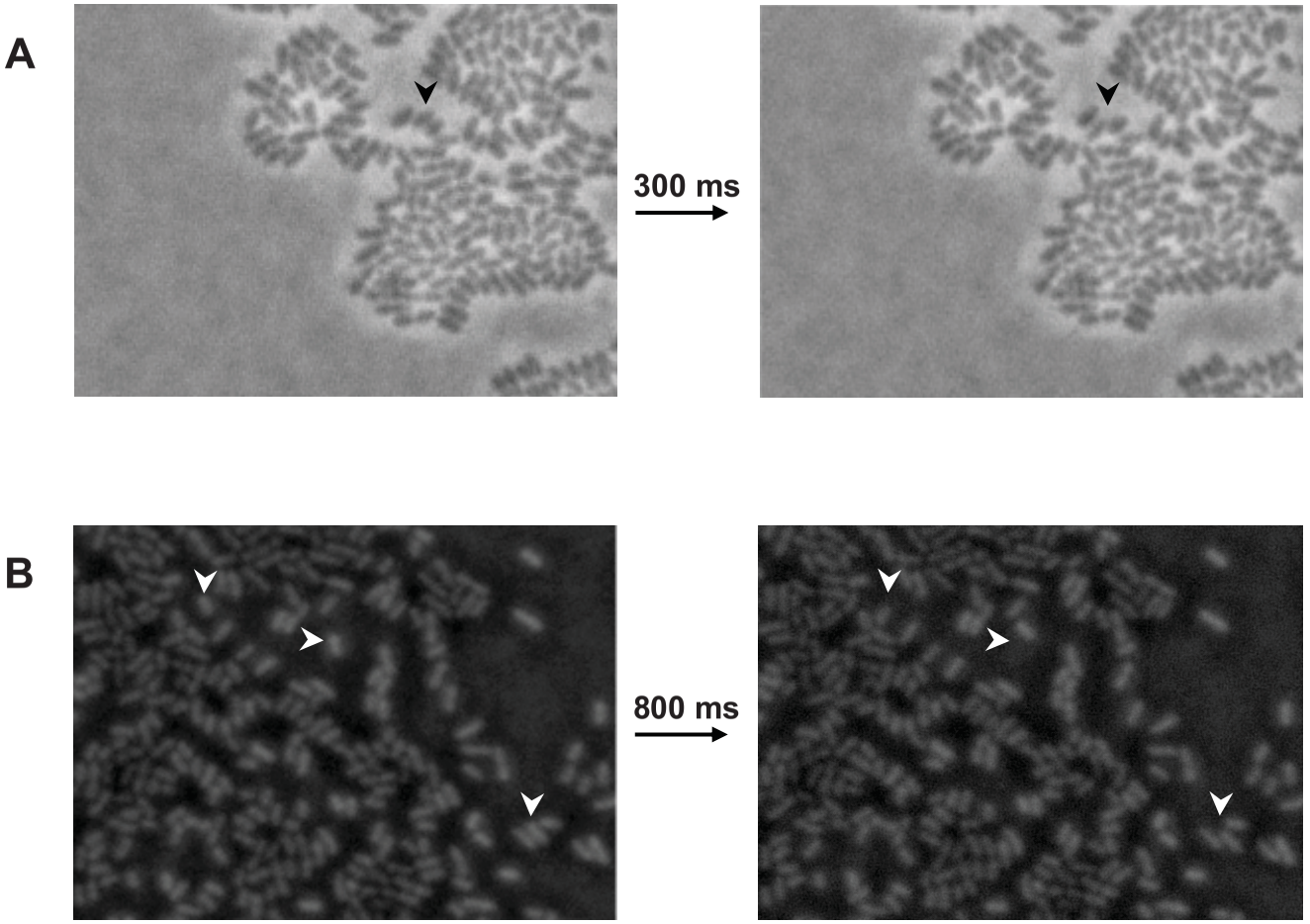
Motility of *A. baylyi* ADP1 is affected by deletion of the *dat* and *ddc* homologues, ACIAD1210 and ACIAD1211. Strains as indicated were inoculated at the centre of 0.5% agar motility plates and incubated for 16 h at 27°C. White arrows indicate the front lines of the moving colonies. Note that the parental strain *A. baylyi* ADP1 has covered the complete agar surface. Motility assays were performed three times yielding similar results.

Supplementary Fig. 4 Skiebe et al.



Mutants 129/ddc and 277/dat of ATCC17978 are attenuated in the *Galleria mellonella* caterpillar infection model. *G. mellonella* caterpillars purchased from Reptilienkosmos.de, Niederkrüchten, Germany, were infected with $3x10^5$ CFU. Bacteria were grown in LB broth overnight at 37°C, diluted 1:50 in LB broth and cultured for another 3 hours at 37°C. Bacteria were then washed and resuspended in sterile phosphate-buffered saline (PBS) and adjusted to an optical density (OD_{600nm}) of 0.2. 5 µl-aliquots were injected into the last left proleg of caterpillars. CFUs were determined by serial dilutions that were plated on Mueller-Hinton agar. Caterpillars were incubated at 37°C for 5 days in Petri dishes. Photographs were taken 24, 48 and 96 hours after infection/injection.

Supplementary Fig. 5 Skiebe et al.



Twitching-like movement of individual cells of *A. baumannii* at the interphase: Isolates 07-102 (A) and 07-095 (B) were inoculated into 0.5% agarose supplemented with 5 g/l tryptone and 2.5 g/l NaCl and incubated overnight at 37°C. The motility front at the interphase between agarose and bottom of the Petri dish was analysed by phase-contrast microscopy (400x magnification). Two photographs taken within 300 and 800 ms, respectively, illustrate the movement of individual cells designated with arrowheads.