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Relatedness of wildlife and livestock avian isolates of the nosocomial pathogen *Acinetobacter baumannii* to lineages spread in hospitals worldwide

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Summary

The natural habitats and potential reservoirs of the nosocomial pathogen *Acinetobacter baumannii* are poorly defined. Here, we put forth and tested the hypothesis of avian reservoirs of *A. baumannii*. We screened tracheal and rectal swab samples from livestock (chicken, geese) and wild birds (white stork nestlings) and isolated *A. baumannii* from 3% of sampled chicken ($n = 220$), 8% of geese ($n = 40$) and 25% of white stork nestlings ($n = 661$). Virulence of selected avian *A. baumannii* isolates was comparable to that of clinical isolates in the *Galleria mellonella* infection model. Whole genome sequencing revealed the close relationship of an antibiotic-susceptible chicken isolate from Germany with a multidrug-resistant human clinical isolate from China and additional linkages between livestock isolates and human clinical isolates related to international clonal lineages. Moreover, we identified stork isolates related to human clinical isolates from the United States. Multilocus sequence typing disclosed further kinship between avian and human isolates. Avian isolates do not form a distinct clade within the phylogeny of *A. baumannii*, instead they diverge into different lineages. Further, we provide evidence that *A. baumannii* is constantly present in the habitats occupied by storks. Collectively, our study suggests *A. baumannii* could be a zoonotic organism that may disseminate into livestock.

Introduction

Acinetobacter baumannii is a Gram-negative bacterium which causes nosocomial infections worldwide and has the propensity to rapidly develop antibiotic resistance (Karageorgopoulos and Falagas, 2008; Howard *et al.*, 2012). It is assigned to the so-called ESKAPE pathogens (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella*

pneumoniae, *Acinetobacter baumannii*, *Pseudomonas aeruginosa* and *Enterobacter* spp.) which share an outstanding potential to escape current antimicrobial therapy posing the threat of loss of any therapeutic options (Rice, 2008; Pendleton *et al*., 2013). For unknown reasons the prevalence of *A. baumannii* infections in intensive care units differs considerably from hospital to hospital and between geographical regions, ranging from 3.7% in North America to 19.2% in Asia (Vincent *et al*., 2009). In tropical climates, *A. baumannii* is also a cause of community-acquired infections (Dexter *et al*., 2015; Pailhories *et al*., 2015). The natural reservoirs of *A. baumannii* and of other clinically relevant *Acinetobacter* species such as *A. nosocomialis* are poorly defined (Peleg *et al*., 2008; Eveillard *et al*., 2013) impeding effective prevention of transmission. Healthy humans only rarely carry *A. baumannii* on their skin (Seifert *et al*., 1997). Likewise, environmental sampling only sporadically yielded *A. baumannii* (Choi *et al*., 2012; Eveillard *et al*., 2013). The bacteria have also been isolated from the exoskeleton of the moth fly *Clogmia albipunctata* in German hospitals suggesting a role of insects as potential vectors (Faulde and Spiesberger, 2013). Highest isolation rates so far have been identified in human head and body lice (La Scola and Raoult, 2004; Kempf *et al*., 2012a,b). However, *A. baumannii* strain SDF recovered from a human body louse represents an ecotype clearly distinct from clinical isolates (Vallenet *et al*., 2008; Peleg *et al*., 2009; Antunes *et al*., 2011). In recent studies on the environmental prevalence of *Acinetobacter* in Lebanon, *A. baumannii* and *A. pittii* were isolated from water, sewage and soil samples and also from various animals at a rate up to 8%, notably from cow feces (Rafei *et al*., 2015; Al Atrouni *et al*., 2016). Also recently, raw meat samples in Switzerland were found to be contaminated with *A. baumannii* at an overall rate of 25% (Lupo *et al*., 2014).

There is a worldwide spread of different clonal lineages of *A. baumannii* in hospitals (Dijkshoorn *et al*., 1996; Diancourt *et al*., 2010; Higgins *et al*., 2010). However, parts of the clinical isolates do not cluster with these worldwide clonal lineages or their distribution is geographically restricted (Karah *et al*., 2012; Schleicher *et al*., 2013; Bocanegra-Ibarias *et al*., 2015), suggesting a perpetual influx of novel strains from unknown reservoirs into the clinical setting. Recent whole-genome sequencing of almost 50 isolates from a single hospital in the United States provided evidence of a large reservoir of strains that interact with each other via horizontal gene transfer suggesting that isolates unrelated to described clonal lineages contribute significantly to evolution of hospital lineages (Wright *et al*., 2014).

A. baumannii and some related *Acinetobacter* species easily grow at 41–44°C (Nemec *et al*., 2011). This could reflect adaptation to hosts with core body temperatures above 37°C such as birds in particular (Line *et al*., 2010). *A. baumannii* has only sporadically been isolated from birds so far (Ahmed *et al*., 2007; Muller *et al*., 2010; Zordan *et al*., 2011; Rafei *et al*., 2015). However, workers in hatcheries show elevated immunoprecipitation reactions to *A. baumannii* (Skorska *et al*., 2007; Brauner *et al*., 2017). In line with this finding, *A. baumannii* could be cultivated from air samples collected in a duck hatchery (Martin and Jäckel, 2011). Based on these findings we have hypothesized that birds could be a reservoir of *A. baumannii*.

Results

Isolation of A. baumannii *from chickens*

To challenge our hypothesis of avian reservoirs of *A. baumannii* we initially collected samples from poultry livestock. We chose a culture-based approach using CHROMagar™ Acinetobacter to select *A. baumannii* from swab samples. In the year 2012, we took choana swabs from 220 chickens in Germany, including 21 at the fledgling stage, representing 37 flocks. We could isolate *A. baumannii* from six samples (2.7%). Speciation was based on partial 16S rRNA and *rpoB* gene sequencing as well as determination of the complete coding sequence of the $bla_{\text{OXA-51-like}}$ gene intrinsic to *A. baumannii* (Weisburg *et al*., 1991; Turton *et al*., 2006a; Nemec *et al*., 2009). The six *A. baumannii* isolates originated from three different farms. Three of the isolates (designated 65, 66 and 117) were from the same flock of chicks at the fledgling stage, two (designated 62 and 124) were from a flock of one day-old chicks of another farm, and one (designated 202) was from a hen of a third farm. ApaI macrorestriction analysis demonstrated that the three isolates (65, 66, 117) from the same flock were closely related (Fig. 1). The two isolates (62, 124) from the other farm were also closely related but distinct from isolates 65, 66 and 117. Strikingly, the single isolate 202 recovered from a hen of another farm was also closely related to the three isolates 65, 66 and 117 from the fledgling herd (Fig. 1). The close relationship of isolates 65, 66, 117 and 202 was further substantiated by DNA sequencing of the $bla_{\text{OXA-51-like}}$ gene which revealed the OXA-68 variant in all four cases. We found no relation between these samples other than sampling by the same person and originating from the same region in Germany. The two distinct isolates 62 and 124 recovered from another farm both harboured a novel $bla_{\text{OXA-51-like}}$ gene variant, OXA-385 (Table 1). The latter two isolates exhibited resistance to gentamicin and kanamycin which was not observed with the four isolates harbouring OXA-68. Sequence-based typing using multiplex PCR (Turton *et al*., 2007) and rep-PCR analyses (Zander *et al*., 2012) did not suggest a close relationship of the chicken isolates to any of the international clonal lineages (ICs) highly prevalent in hospitals worldwide (data not shown).

In addition, 22 pooled faecal samples representing 10 flocks of chicken were sampled in Germany. None of these faecal samples yielded *A. baumannii*.

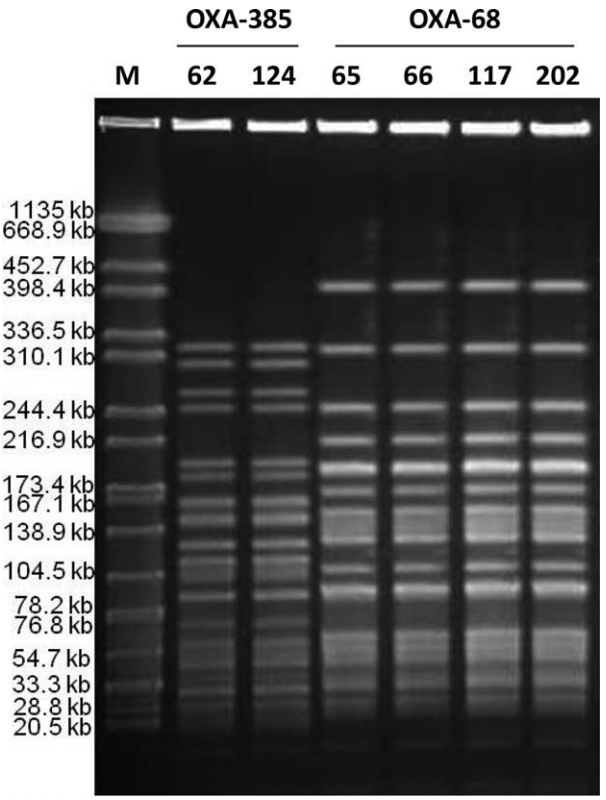

Fig. 1. ApaI macrorestriction analysis of six *A. baumannii* chicken isolates from Germany. DNA restriction pattern of six chicken isolates after ApaI digestion and subsequent pulsed-field gel electrophoresis. M, size standard *Salmonella* serotype Braenderup strain H9812, restricted with XbaI. OXA-variants are indicated above isolate numbers.

Isolation of A. baumannii *from geese*

We took choana swabs from 40 goslings from a single farm in Germany in the year 2013, and isolated *A. baumannii* from three of these chicks (7.5%). The three isolates (designated 3.3A, 3.5D and 4.1V) carried different variants of the $bla_{\text{OXA-51-like}}$ gene encoding OXA-314, OXA-71 and OXA-95, respectively (Table 1). ApaI macrorestriction analysis and sequence-based typing according to Turton *et al.* (2007) did not indicate a close relationship to international clones IC1–3 (data not shown). Antimicrobial resistance testing of one of the isolates (4.1V) showed intermediate susceptibility to kanamycin and resistance to sulfamerazine and sulfamerazine-trimethoprim suggesting a history of selection pressure of this strain in the context of livestock production.

Taken together these findings illustrate a first indication for dissemination of *A. baumannii* within poultry livestock. However, the relatively low incidence turned our attention towards wild birds especially since transmission from humans to livestock cannot be ruled out as an explanation for our findings in chicken and geese. Further, we speculated that migratory birds could contribute to the dispersion of *A. baumannii* from tropical high-incidence countries to low incidence regions with temperate climates (Vincent *et al.*, 2009).

Stork sampling in western Poland in the year 2013

In 2013, we sampled 72 white stork nestlings from different regions in western Poland (voivodships Lubuskie and Lower Silesia; see Supporting Information Fig. S1). Some storks were sampled up to four times (samplings on June 4th, 14th, 21st and July 1st) adding up to 136 choana swabs originating from 72 adolescent storks. Overall, 24 *A. baumannii* isolates were obtained from 72 individuals (33%) (Table 1, serial no. 18–41; Supporting Information Figs. S2–S4). Accordingly, the isolation rate in relation to the number of 136 samples was 18%. Not in a single case the same *A. baumannii* strain was isolated from a resampled individual. Also among chick siblings the diversity of isolates was striking. Partial *rpoB* and complete $bla_{\text{OXA-51-like}}$ gene sequencing as well as ApaI macrorestriction analysis revealed indistinguishable isolates among siblings in only two pairs of siblings; one pair from a nest in Kłopot (Lubuskie; isolates 151/1C and 152/1C in nest KLO_02/04; see Table 1 and Supporting Information Figs. S2 and S4) and another pair from a nest in Kamiona (Lower Silesia; isolates 284/2C and 285/2C; see Supporting Information Fig. S3 and Table 1). By contrast, in another nest in Kłopot (KLO_14/14) three out of five nestlings were found to be colonized at the same time, but each with a different strain (87D C, 88B C, 90/3C) (Table 1 and Supporting Information Fig. S4). Resampling of the two surviving chicks only a week later yielded one new strain (159/1C) in a previously colonized nestling but no recovery of any of the strains isolated before (Table 1 and Supporting Information Fig. S4). In addition to *A. baumannii*, we isolated *Acinetobacter variabilis* (Tjernberg and Ursing, 1989; Krizova *et al.*, 2015) from 21 out of 136 samples (15%). This speciation was based on *rpoB* partial gene sequence analysis which revealed a similarity to *A. variabilis* type strain NIPH 2171^{T} (EU477119) of $\geq$98% in all cases. As expected, recovery of the environmental species *Acinetobacter calcoaceticus* was high and found in 75 of 136 samples (55%). Other *Acinetobacter* species as classified by *rpoB* partial gene analysis (similarity $\geq$98% in comparison to the respective type strain) such as *A. schindleri*, *A. lwoffii*, *A. junii* and the recently described *A. gandensis* (Smet *et al.*, 2014) were only sporadically isolated. As the suitability of CHROMagar Acinetobacter for isolation of *Acinetobacter* species other than *A. baumannii* is unknown, their observed incidence is possibly an underestimate.

Stork sampling in northeastern Poland

In addition, in 2013 we sampled 50 white stork nestlings from the voivodship Warmińsko-Mazurskie in northeastern Poland and isolated *A. baumannii* from choana

Table 1. Characteristics of *A. baumannii* isolates of avian origin.

Serial no.	Strain no.	Year of isolation	Host	Sample type	Geographic region	Natural competence	Oxa-type	GenBank
1	62	2012	Chicken	Hen chick, 1 day; choana	Germany	KanR	385	
2	65	2012	Chicken	Hen chick; choana	Germany	yes	68	
3	66	2012	Chicken	Hen chick; choana	Germany	yes	68	
4	117	2012	Chicken	Hen chick; choana	Germany	yes	68	
5	124	2012	Chicken	Hen chick, 1 day; choana	Germany	KanR	385	KF986253
6	202	2012	chicken	Hen; choana	Germany	yes	68	
7	3.3A	2013	Goose	Choana	Germany	no	314	
8	3.5D	2013	Goose	Choana	Germany	no	71	
9	4.1V	2013	Goose	Choana	Germany	KanR	95	
10	I B1	2008	Duck*	Air sample; duck hatchery	Germany	no	120	
11	I B2	2008	Duck*	Air sample; duck hatchery	Germany	no	375	
12	I B3	2008	Duck*	Air sample; duck hatchery	Germany	no	386	KF986254
13	II B9	2008	Duck*	Air sample; duck hatchery	Germany	no	375	
14	II B12	2008	duck*	Air sample; duck hatchery	Germany	no	386	
15	I B25	2008	Duck*	Air sample; duck hatchery	Germany	no	375	
16	II B29	2008	Duck*	Air sample; duck hatchery	Germany	no	375	
17	III B30	2008	Duck*	Air sample; duck hatchery	Germany	no	386	
18	77II/1C	2013	White stork	Nestling; choana	Poland, Kłopot	no	93	
19	86II/2C	2013	White stork	Nestling; choana	Poland, Kłopot	yes	374	KF986255
20	87DC	2013	White stork	Nestling; choana	Poland, Kłopot	yes	314	
21	88BC	2013	White stork	Nestling; choana	Poland, Kłopot	no	67	
22	90/3C	2013	White stork	Nestling; choana	Poland, Kłopot	yes	375	KF986256
23	97/1C	2013	White stork	Nestling; choana	Poland, Kłopot	yes	376	KF986257
24	101II/1C	2013	White stork	Nestling; choana	Poland, Kłopot	no	377	KF986258
25	150/1C	2013	White stork	Nestling; choana	Poland, Kłopot	yes	388	KJ135343
26	151/1C	2013	White stork	Nestling; choana	Poland, Kłopot	no	378	KF986259
27	152/1C	2013	White stork	Nestling; choana	Poland, Kłopot	no	378	
28	156/2C	2013	White stork	Nestling; choana	Poland, Kłopot	yes	379	KF986260
29	159/1C	2013	White stork	Nestling; choana	Poland, Kłopot	no	65	
30	191/2C	2013	White stork	Nestling; choana	Poland, Rybaki	no	380	KF986261
31	192/2C	2013	White stork	Nestling; choana	Poland, Połęcko	yes	381	KJ135344
32	261/1C	2013	White stork	Nestling; choana	Poland, Kłopot	yes	non-functional	
33	268/2C	2013	White stork	Nestling; choana	Poland, Bytomiec	yes	382	KJ135345
34	276/2C	2013	White stork	Nestling; choana	Poland, Połęcko	no	383	KF986262
35	277/1C	2013	White stork	Nestling; choana	Poland, Połęcko	yes	389	KJ135346
36	278/3C	2013	White stork	Nestling; choana	Poland, Stary Raduszec	no	51	
37	279/3C	2013	White stork	Nestling; choana	Poland, Stary Raduszec	no	51	
38	280/1C	2013	White stork	Nestling; choana	Poland, Czarnowo	no	378	
39	284/2C	2013	White stork	Nestling; choana	Poland, Kamiona	no	384	KF986263
40	285/2C	2013	White stork	Nestling; choana	Poland, Kamiona	yes	384	
41	291/1C	2013	White stork	Nestling; choana	Poland, Moszowice	no	90	
42	638C	2013	White stork	Nestling; choana	Poland, Saduny	no	67	
43	658C	2013	White stork	Nestling; choana	Poland, Podlechy	no	390	KJ135342
44	661C	2013	White stork	Nestling; choana	Poland, Korsze	no	390	
45	8D1	2014	White stork	Nestling; choana	Poland, Kłopot	no	69	
46	9D1	2014	White stork	Nestling; choana	Poland, Kłopot	no	90	
47	15D1	2014	White stork	Nestling; choana	Poland, Kłopot	no	104	

Table 1. *cont.*

Serial no.	Strain no.	Year of isolation	Host	Sample type	Geographic region	Natural competence	Oxa-type	GenBank
48	29D2	2014	White stork	Nestling; choana	Poland, Bytomiec	yes	51	
49	31D1	2014	White stork	Nestling; choana	Poland, Bytomiec	yes	338	
50	32D1	2014	White stork	Nestling; choana	Poland, Rybaki	yes	429	KM979376
51	33D1	2014	White stork	Nestling; choana	Poland, Rybaki	yes	104	
52	41D1	2014	White stork	Nestling; choana	Poland, Wezyska	yes	413	
53	47D2	2014	White stork	Nestling; choana	Poland, Dabie	yes	413	
54	49D1	2014	White stork	Nestling; choana	Poland, Dabie	yes	430	KM979377
55	73D4	2014	White stork	Nestling; choana	Poland, Siedlisko	yes	402	
56	29R1	2014	White stork	Nestling; rectal	Poland, Bytomiec	no	51	
57	42R3	2014	White stork	Nestling; rectal	Poland, Wezyska	yes	378	
58	1P1	2014	White stork	Pellet from stork	Poland, Kłopot	yes	208	
59	1PW1	2014	White stork	Pellet from stork	Poland, Kłopot	yes	431	KM979378
60	2P1	2014	White stork	Pellet from stork	Poland, Kłopot	no	69	
61	2P2	2014	White stork	Pellet from stork	Poland, Kłopot	no	432	KM979379
62	2P3	2014	White stork	Pellet from stork	Poland, Kłopot	no	379	
63	2PW1	2014	White stork	Pellet from stork	Poland, Kłopot	no	433	KM979380
64	4P1	2014	White stork	Pellet from stork	Poland, Kłopot	yes	431	
65	GaenseEi-1	2016	Goose	Egg shells from hatchery	Germany	n.d.	378	
66	Gaensemehl-2	2016	Goose	Down and dust from hatchery	Germany	n.d.	64	
67	BAuABod-3	2015	Turkey	Down and dust from hatchery	Germany	n.d.	64	
68	GB1–2	2015	Chicken	Down and dust from hatchery	Germany	n.d.	68	
69	LoGeIst3-1	2015	White stork	Pellet from stork	Germany, Isterbies	n.d.	378	
70	O1D3-2	2016	White stork	Pellet from stork	Germany, Rosian	n.d.	374	
71	PLG9P835	2016	White stork	Nestling; choana	Poland, Steblów	n.d.	374	

Kan^R, minimal inhibitory concentration of kanamycin $\geq$32 μg/ml in a microbroth dilution assay; *Probably of duck origin since the isolates were recovered from air samples collected in a duck hatchery; n.d., not determined; prototypic new OXA-51 types indicated in light grey; whole genome sequenced isolates are indicated by dark grey background.

swabs from only three of these nestlings (6%; Table 1 and Supporting Information Fig S5). This comparably low prevalence can probably be attributed to an accidental delay of sample processing. After all, this finding demonstrates that the appearance of *A. baumannii* in white stork nestlings is not restricted to western Poland. The three isolates from Warmińsko-Mazurskie were obtained from three different nests in neighbouring villages. Two isolates from different nests (658C and 661C) were indistinguishable based on partial *rpoB* and full $bla_{OXA\text{-}51\text{-}like}$ gene (OXA-390) sequencing as well as ApaI macrorestriction analysis (Table 1 and Fig. 2). The distance between the two nests was approximately 2 kilometres. However, a close relationship of the latter two isolates to an isolate recovered in western Poland in 2014 (2P2) as suggested by identical *rpoB* partial sequences and similar $bla_{OXA\text{-}51\text{-}like}$ genes was not confirmed by ApaI macrorestriction analysis (Fig. 2).

Stork sampling in western Poland in the year 2014

To confirm our findings we repeated sampling of white stork nestlings in western Poland in the year 2014. We collected 87 choana swabs from 87 individuals and isolated *A. baumannii* from 11 nestlings (13%) (Table 1). *A. variabilis* was isolated from 24 individuals (30%). In addition, we identified four isolates of *Acinetobacter pittii*, another clinically relevant species which we had not identified in the previous year. Again, we observed a distinct diversity among the 11 isolates of *A. baumannii* (Table 1, no. 45–55) as indicated by the finding of nine different $bla_{OXA\text{-}51\text{-}like}$ variants at the translational level. All 11 isolates including six isolates recovered from three pairs of siblings were distinguishable on the basis of ApaI macrorestriction analysis (data not shown).

Rectal sampling in comparison to choana sampling

In 2014, we took rectal samples from 52 white stork nestlings in parallel to choana sampling. Only in two cases (4%) we isolated *A. baumannii* from rectal swabs (isolates 29R1 and 42R3, see Table 1), whereas parallel choana sampling of these 52 individuals revealed isolation of *A. baumannii* from ten nestlings (19%). Interestingly, in the case of rectal isolate 42R3 no isolate was recovered from the corresponding choana sample, and in the case of the

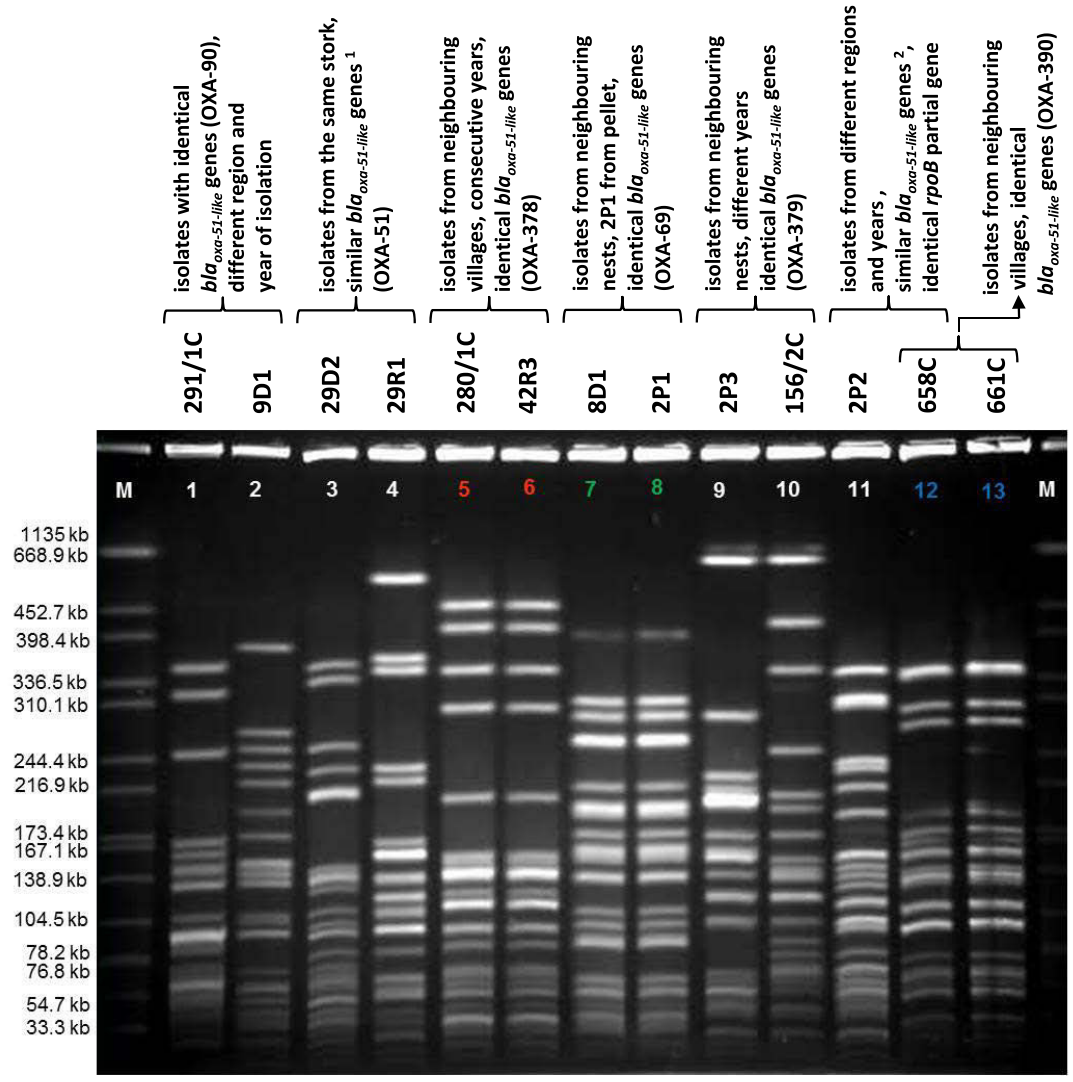

Fig. 2. ApaI macrorestriction analysis of selected *A. baumannii* isolated from white stork samples collected in Poland. DNA restriction pattern of 13 stork-associated isolates after ApaI digestion and subsequent pulsed-field gel electrophoresis. M, size standard *Salmonella* serotype Braenderup strain H9812, restricted with XbaI. Color code indicates pairs of isolates with closely related restriction pattern ($\leq$ 3 differences). [1]2 nucleotides (nts) different per 822 nts; [2]3 nts different per 822 nts.

second rectal isolate (29R1) the corresponding choana sampling revealed an isolate (29D2) clearly distinct from the rectal isolate (Fig. 2). In conclusion, while choana sampling yields higher isolation rates, rectal sampling can provide additional isolates independent of the yield of choana sampling.

Continuation of white stork sampling

Sampling of white stork nestlings in Poland was continued in 2015 and 2016 and expanded to additional regions located in the voivodships Opole and Greater Poland (Supporting Information Fig. S1). Table 2 summarizes our results of choana sampling of white stork nestlings in Poland from 2013 to 2016. The overall isolation rate based on the sampling of 661 individuals was 25%. Strikingly high isolation rates were found in the Opole region (48% and 47%) while variable rates were documented for the Greater Poland region (4% and 33%) indicating significant ecological differences.

Isolation of the same clone in consecutive years

When comparing isolates from 2013 to 2014 we realized that isolate 280/1C, recovered from the choana of a nestling in Czarnowo in the year 2013, was closely related to rectal isolate 42R3 recovered in the neighbouring village Wężyska in the year 2014 (Supporting Information Fig. S6). These isolates share the same ApaI macrorestriction

Table 2. Summary of choana sampling on white stork nestlings in Poland 2013–2016.

Region in Poland	Year	No. of storks sampled	No. of choana samples	No. of choana samples culture-positive for *A. baumannii*	No. of isolates[a]	Isolation rate %[b]
Lubuskie and Lower Silesia	2013	72	136[c]	24	24	18
	2014	87	87	11	11	13
	2015	69	89[d]	23	23	26
	2016	73	73	27	30	37
Warmińsko-Mazurskie[e]	2013	50	50	3	3	6
Opole	2015	96	96	46	58	48
	2016	53	53	25	31	47
Greater Poland	2015	85	85	3	3	4
	2016	76	76	25	29	33
Total of all regions	2013–2016	661	745	187	212	**25**

a. Multiple distinguishable *A. baumannii* isolates from some stork individuals.
b. No. of choana samples tested culture-positive for *A. baumannii* divided by no. of choana samples.
c. Multiple sampling of some nestlings within 4 weeks.
d. Twenty storks were sampled twice in the choana region.
e. Data possibly biased due to irregular sample transport (8 days without cooling).

pattern and the same $bla_{\text{OXA-51-like}}$ gene encoding OXA-378 (Fig. 2).

Stork pellets as a new sample source

Storks regurgitate undigested parts of their food in the form of pellets. In the year 2014, we collected four pellets from below a single nest in Kłopot village (Supporting Information Fig. S7). We isolated *A. baumannii* from three out of four of these pellets and were able to differentiate six strain types among the seven isolates based on $bla_{\text{OXA51-like}}$ gene sequences (Table 1, serial no. 58–64). On the contrary, we were unable to isolate any *A. baumannii* from choana and rectal swab samples drawn from the four chicks growing up in this specific nest. Of further interest, based on $bla_{\text{OXA-51-like}}$ gene sequences as well as ApaI macrorestriction analysis, one pellet isolate (2P1) was indistinguishable from the nestling isolate 8D1 which was recovered from a nest in close vicinity to the one from where we collected the pellets (Fig. 2 and Supporting Information Figs. S7 and S8). Taken together these findings suggest that stork pellets represent an easily accessible sample material for isolation of *A. baumannii*.

Diversity of the $\text{bla}_{\textit{OXA-51-like}}$ *gene*

Overall, the diversity of the $bla_{\text{OXA-51-like}}$ gene sequenced from stork isolates was high. Among the 47 isolates (including those obtained from pellets) isolated in 2013 and 2014 we identified a total of 30 variants of $bla_{\text{OXA-51-like}}$ on the translational level and 19 of these were novel in a sense that they were not represented by any database entry accessible via BLAST (see Table 1 for GenBank accession numbers). Only a single $bla_{\text{OXA-51-like}}$ gene was found to be non-functional due to introduction of an internal stop codon (Table 1, isolate 261/1C).

Avian isolates exhibit OXA variants known from international clonal lineages

It was described that typing of clinical isolates of *A. baumannii* belonging to international clones (IC) by rep-PCR and multilocus sequence typing (MLST) correlates well with specific variants of $bla_{\text{OXA-51-like}}$ (Zander *et al.*, 2012; Pournaras *et al.*, 2014). Here, we have identified a number of $bla_{\text{OXA-51-like}}$ genes in avian isolates encoding protein variants such as OXA-51, −65, −68, −69, −71 and −90 known to be associated with clinical *A. baumannii* lineages IC1, 3–6 and 8 (Zander *et al.*, 2012). However, on the nucleotide level none of the avian isolates carried a $bla_{\text{OXA-51-like}}$ gene identical to any database entry. Next, we have typed representatives of these OXA-variants by rep-PCR but found no significant similarity to any of the clinical *A. baumannii* IC lineages (Supporting Information Fig. S9). Taken together, identity of avian isolates' OXA proteins to that found in IC lineages does not necessarily indicate a close relationship to clinical strains.

Natural transformation competence is widespread among avian isolates

We recently demonstrated that natural transformation competence is widespread among clinical isolates of *A. baumannii* with about one third of the clinical isolates being competent under conditions that promote motility along wet surfaces (Wilharm *et al.*, 2013). Here, we have tested natural competence of all avian isolates that we had recovered between 2012 and 2014, with the exception of three isolates resistant to kanamycin, the antibiotic used for

selection of transformants. In addition, eight isolates recently recovered from air samples in a duck hatchery (Martin and Jäckel, 2011) were included (Table 1, serial no. 10–17). Overall, we found that 27 of 61 avian isolates (44%) were competent for transformation under the conditions tested (Table 1) which is even higher than the rate recently determined for kanamycin-sensitive clinical isolates (36%) (Wilharm *et al.*, 2013).

Antibiotic resistance

Apart from natural resistance to ampicillin and cephalosporins not a single *A. baumannii* stork isolate was resistant to kanamycin, gentamicin, amikacin, ciprofloxacin, sulfamerazine and meropenem, and no isolate was positive for an acquired carbapenemase gene indicating the absence of substantial anthropogenic selection pressure. Only some of the isolates from livestock showed signs of selection pressure such as chicken isolates 62 and 124, being resistant to kanamycin and gentamicin, and goose isolate 4.1V being intermediate to kanamycin and resistant to sulfamerazine. Similarly, two of the eight isolates previously obtained from air samples in a duck hatchery (Martin and Jäckel, 2011) exhibited resistance to sulfamerazine but were otherwise largely sensitive.

Virulence of avian isolates

Galleria mellonella larvae were used to compare the virulence of avian isolates to that from cases of human infection. All avian isolates tested ($n = 12$) exhibited virulence comparable to that of clinical isolates in the *Galleria* model. Supporting Information Fig S10 exemplifies the virulence of two chicken isolates and three stork isolates in comparison to widely used virulent strain ATCC 17978. The latter strain is known to be as virulent as epidemic clinical *A. baumannii* strains such as ACICU and AYE in the *Galleria* model (Antunes *et al.*, 2011). Lethal dose 50% (LD_{50}) values were determined after 24 h, revealing no significant difference between clinical isolates (ATCC 17978, ACICU and AYE) and twelve avian isolates ($7.4 \times 10^{-4} \pm 2.0 \times 10^{-4}$ cfu vs. $5.0 \times 10^{-4} \pm 4.3 \times 10^{-4}$ cfu).

Further, adhesion of selected avian isolates to A549 human lung epithelial cells was assessed in comparison to epidemic strains AYE and ACICU representing IC1 and IC2, respectively. All strains tested were able to adhere to host cells. The level of adhesion was different for some avian isolates, for example, goose isolate 3.5D exhibited significantly higher adhesion potential than strains AYE and ACICU, and adhesion of stork isolate 29D2 was significantly exceeding that of strain AYE (Fig. 3).

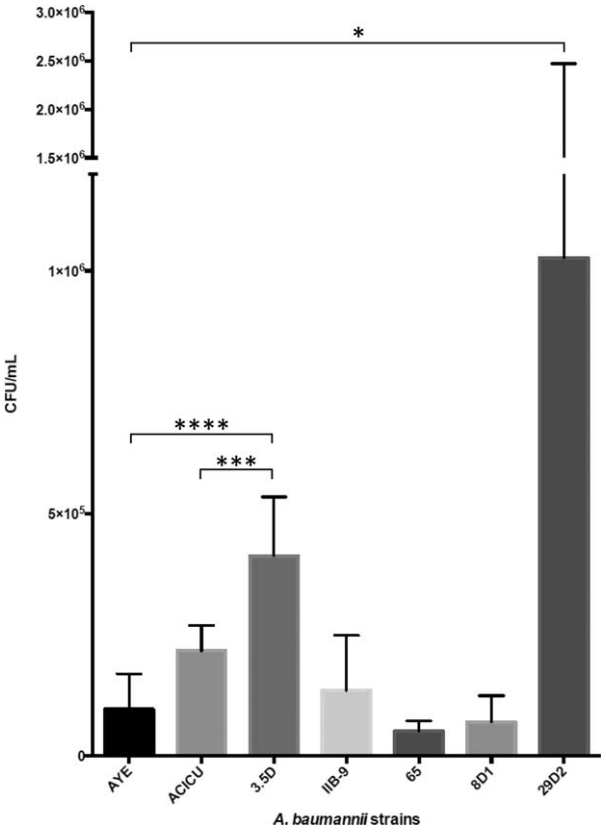

Fig. 3. Adhesion of avian and human clinical isolates of *A. baumannii* to A549 human epithelial lung cells. Significance based on unpaired student's *t* test: * 29D2 versus AYE significantly different with $P < 0.05$; *** 3.5D versus ACICU significantly different with $P < 0.0005$; **** 3.5D versus AYE significantly different with $P < 0.0001$.

Whole genome sequencing of selected avian isolates

Finally, to elucidate the standing of avian isolates within the species and in relation to hospital isolates, we determined the nearly complete genome sequence of 18 selected avian isolates, including one from a hen chick (65), one from a goose (3.5D) and ten from white stork nestlings (8D1, 29R1, 29D2, 31D1, 42R3, 192/2C, 280/1C, 86II/2C, 151/1C, PLG9P835; Table 1). Four sporadic isolates gained from poultry hatcheries in Germany and two sporadic isolates collected from white stork pellets in Germany were later included because the OXA-variants indicated a relationship to IC lineages or to strains previously isolated from stork samples (Table 1, serial no. 65–70). A phylogenetic tree based on 2182 genes with orthologues in all selected isolates is shown in Fig. 4. This analysis reveals that neither avian isolates in general nor stork isolates in particular form a distinct clade within the phylogeny of *A. baumannii*. Rather, they are highly diverse and show multiple early branching points with different clinical lineages. None of the avian isolates shows a close relationship to

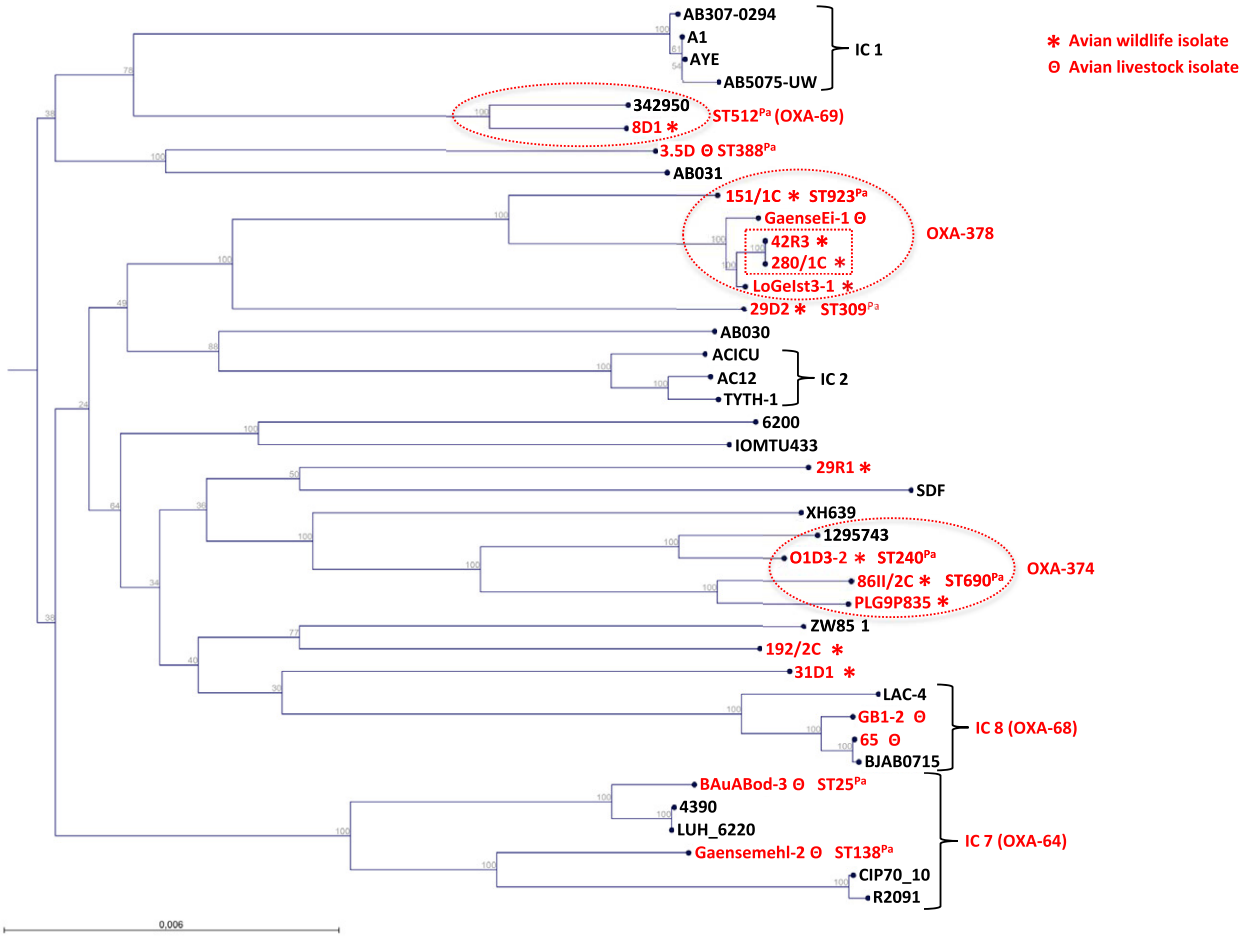

Fig. 4. Core genome-based phylogeny reveals the high diversity of avian isolates. Maximum likelihood tree based on 2181 orthologous genes present in all 40 *A. baumannii* strains included. Wildlife avian isolates are flagged with an asterisk (★), livestock-associated avian isolates are labelled with a theta (Θ); designation of all avian isolates in red. Strains representing international clones IC1, IC2, IC7 and IC8 are marked with brackets. The *scale* indicates substitutions per site. The red dashed ovals indicate clades outside of the IC nomenclature which include avian isolates. The red dashed box indicates two isolates from white stork nestlings recovered from neighbouring villages in consecutive years. Multilocus sequence types and OXA-types are indicated for isolates discussed in the main text.

international clones IC1 or IC2, however, stork isolate 8D1 shows early branching from the IC1 lineage and more recent branching from the human clinical isolate 342 950 collected in the United States. While we found more than 29 000 single nucleotide polymorphisms (SNPs) when comparing stork isolate 8D1 to different representatives of the IC1 lineage, we detected only 8575 SNPs in comparison to the clinical isolate 342 950 (see distance matrix in Supporting Information. Table S1). Of interest, isolate 65, collected from a hen chick in Germany in the year 2012, is closely related to a clinical isolate from China, BJAB0715, collected in 2007 (Zhu *et al.*, 2013). They show only 270 single nucleotide polymorphisms (SNPs). In relation to the overall alignment length of 1.857 megabases (Mb) this corresponds to only 145 SNPs/Mb. The most striking difference between isolates 65 and BJAB0715 is the acquisition of a resistance plasmid by the latter. In line with this, we found isolate 65 to be naturally competent (Table 1). Furthermore, isolate 65 and BJAB0715 share a common root with hypervirulent outbreak strain LAC-4, a representative of international clone 8 (IC8) (Ou *et al.*, 2015; Tomaschek *et al.*, 2016). This lineage also includes an additional isolate (GB1–2; see Fig. 4 and Table 1) that we recently isolated from a chicken hatchery in Germany.

Another clade, sharing the OXA-378 protein variant, includes four stork isolates from Poland and Germany as well as a goose isolate ('GaenseEi-1') from Germany, illustrating the relatedness of wildlife and livestock avian isolates (Fig. 4). In addition, a clade identified by the shared OXA-374 protein variant, comprises three stork isolates from Poland and Germany as well as the human clinical isolate 1 295 743 from the United States. Two livestock isolates from Germany which harbour the OXA-64 protein known from international clone IC7 actually cluster

with human clinical isolates within this deeply branching lineage (Fig. 4).

Moreover, our analysis confirms that isolates 42R3 and 280/1C, collected from white stork nestlings in consecutive years from neighbouring villages, are very closely related with a difference as low as 11 SNPs (Fig. 4 and Supporting Information Table S1).

Based on the genome data we were able to determine the sequence type (ST) of 17 out of 18 avian isolates according to the Pasteur MLST scheme (Diancourt *et al.*, 2010) and also according to the Oxford scheme (Bartual *et al.*, 2005) including 4 and 10 previously undescribed ST^{Pa} and ST^{Ox} sequence types, respectively (Supporting Information Tables S2 and S3). As expected, chicken isolate 65 belongs to the same sequence type as BJAB0715 from China in both systems ($ST23^{Pa}$ and $ST642^{Ox}$). Interestingly, $ST23^{Pa}$ is also shared by strain RUH 1316 isolated in 1964 from a mink in the Netherlands, by two strains isolated more recently in Sweden (one of which from a patient transferred from Serbia), by an additional strain from China, and by our isolate GB-1 suggesting they all belong to an international clone. In line with this, the $ST23^{Pa}$ is a single locus variant of $ST10^{Pa}$ which is associated with international clone 8 (IC8) (Ou *et al.*, 2015; Tomaschek *et al.*, 2016).

In addition, BAuABod-3 could be assigned to $ST25^{Pa}$ and $ST229^{Ox}$ which are associated with IC7 (Tomaschek *et al.*, 2016). In accordance with the above suggested relationship between stork isolate 8D1 and strain 342 950 isolated from a perirectal human sample in the United States, these two isolates share the same sequence type according to both MLST schemes ($ST512^{P}$ and $ST952^{Ox}$). Additional linkages were revealed between goose isolate 3.5D from Germany and two isolates from Taiwan ($ST388^{Pa}$), between stork isolate 29D2 from Poland and isolate LUH 15022 from Belgium ($ST309^{Pa}$), stork isolate 86II/2C from Poland and isolate 35–2002 from Spain ($ST690^{Pa}$) as well as stork isolate 151/1C from Poland and isolate A166B from Australia ($ST923^{Pa}$) (Fig. 4 and Supporting Information Table S3). Of specific interest, stork isolate O1D3-2 and goose-associated isolate Gaensemehl-2 share $ST240^{Pa}$ and $ST138^{Pa}$, respectively, with chicken meat isolates from Switzerland (Lupo *et al.*, 2014) and isolates from Japan and Taiwan, respectively (Fig. 4 and Supporting Information Table S3). We were unable to assign a ST to strain PLG9P835 because of a disrupted *gltA* allele. However, it shared 4 alleles with $ST1454^{Ox}$ from strain 86II/2C.

Discussion

A. baumannii*: where do they originally come from?*

Knowledge about the ecological context of pathogens is of utmost importance for elucidating their stratagem and for developing control measures. Unfortunately, we do not know much about the 'training grounds' of *A. baumannii* outside the hospital. Here, we studied birds as a potential reservoir of *A. baumannii* and found the bacteria in 25% of all choana samples taken from white stork nestlings in Poland. To the best of our knowledge, this is the highest rate documented for any endotherm to date. But is the stork really a reservoir?

Storks as samplers of A. baumannii *rather than colonized hosts?*

We demonstrated here that choana swabs from white stork nestlings are a reproducible source for *A. baumannii*. However, the nature of the interaction between *A. baumannii* and storks remains elusive. Our data suggest that colonization of the choana region is rather transient: (i) repeated sampling of nestlings did never recover the same strain, (ii) throughout the study only two pairs of siblings were found to carry the same strain, (iii) stork pellets collected below a single nest were highly contaminated with *A. baumannii* whereas sampling of the four nestlings within this specific nest at the same time yielded no *A. baumannii*. Taken together, we interpret these findings such that at least the choana region of young storks is possibly only transiently colonized with *A. baumannii* and that the feed of storks has to be considered as the true source of *A. baumannii* isolated from choanae and pellets. Studies are underway to monitor the recovery of *A. baumannii* from stork pellets during the breeding season to get a clearer picture on the colonization status of storks. Of course, we cannot rule out at present that body parts other than trachea and rectum are colonized more stably.

Evidence for residence of A. baumannii *in the Oder valley*

A close relationship between isolate 280/1C, collected from a choana swab in Czarnowo in 2013, and isolate 42R3, collected from a rectal swab in 2014 in the neighbouring village of Wężyska (approx. 3 km from Czarnowo; Supporting Information Fig. S6), was suspected due to identical $bla_{\text{OXA-51-like}}$ genes as well as indistinguishable ApaI macrorestriction patterns and confirmed by whole genome sequencing (Fig. 4). Given that adolescent storks typically stay in Africa for two years after their first migration, it is highly unlikely that the stork nestling sampled in 2013 came back in 2014 for breeding in the neighbouring village and transmitted a colonizing strain to its offspring. We cannot rule out that an adult stork breeding in Czarnowo in 2013 and in Wężyska in 2014 was the carrier and transmitter of both isolates. However, this scenario is unlikely in light of nest-site fidelity of white stork being close to 90% (Barbraud *et al.*, 1999) and considering the lack of evidence for transmission routes from stably

colonized parents to their nestlings (see above). Moreover and as discussed above there is evidence of storks' feed as the true source of *A. baumannii* in storks. Thus, the most likely explanation for the close relationship between isolates 280/1C and 42R3 is the stable circulation of this *A. baumannii* lineage in a habitat situated in the vicinity of these villages where storks are foraging. This scenario is also supported by additional findings from other sampling sites, for example, closely related isolates 658C and 661C recovered from nestlings that grew up within a distance of 2 kilometres. In conclusion, it is now possible to study *A. baumannii* in a native environmental context with a high probability to consecutively isolate specific clones. It should thus be possible to estimate evolutionary rates and timescales in a natural context and to compare to data obtained in a clinical context or related to animal production.

In the light of Poland giving home to the world's largest population of white storks it will be interesting to compare clinical isolates from Poland not belonging to the international clones and stork isolates to learn if transfer occasionally may occur.

Evidence for convergent evolution of the bla$_{OXA\text{-}51\text{-}like}$ *gene*

Another interesting aspect concerns the evolution of the intrinsic *bla*$_{OXA\text{-}51\text{-}like}$ gene in its natural context. Under anthropogenic selection pressure such as imposed by the use of antibiotics in the hospital setting, *bla*$_{OXA\text{-}51\text{-}like}$ can turn into a clinically relevant carbapenemase gene after insertion of IS*Aba1* into the promoter region (Turton *et al.*, 2006b; Zander *et al.*, 2013). However, we could not detect an IS*Aba1* insertion upstream of *bla*$_{OXA\text{-}51\text{-}like}$ in any of our avian isolates. Interestingly, among 47 different stork isolates obtained in 2013 and 2014 we found 30 different variants at the protein level. These protein variants included OXA-51, −65, −69, −71 and −90 commonly found in well-described clinical *A. baumannii* international clonal lineages (Zander *et al.*, 2012). However, at the nucleotide level the *bla*$_{OXA\text{-}51\text{-}like}$ genes of these avian isolates were never identical to that of clinical IC lineages and a close relationship of any isolate to an IC lineage could also not be demonstrated by rep-PCR suggesting convergent evolution of the *bla*$_{OXA\text{-}51\text{-}like}$ gene. We also found striking examples of apparently convergent evolution of the *bla*$_{OXA\text{-}51\text{-}like}$ gene among avian isolates. For instance, isolate 291/1C was collected in the year 2013 from a nestling near Glogów, and isolate 9D1 was collected in 2014 from a nestling in Kłopot, about 100 km away from Glogów. These isolates are clearly distinct as determined by ApaI macrorestriction analysis (Fig. 2) and partial sequences of *rpoB* and *gyrB* (data not shown) but share the same *bla*$_{OXA\text{-}51\text{-}like}$ gene (encoding OXA-90). As another example, isolates 41D1 and 47D1, collected from nestlings in neighbouring villages in the same year, are distinct by macrorestriction analysis and partial sequences of *rpoB* and *gyrB* (data not shown), nonetheless they are identical in the *bla*$_{OXA\text{-}51\text{-}like}$ gene (encoding OXA-413). Conversely, some new OXA-types such as OXA-378 and OXA-374 but also well-known types such as OXA-64 and OXA-68 may act as indicators for clonal affiliation (Fig. 4). Collectively, our data suggest that *bla*$_{OXA\text{-}51\text{-}like}$ is expressed and responsive to selection pressure in the environment.

Similar to what we found, Rafei *et al.* described 31 different OXA-51-like protein variants among 42 *A. baumannii* of non-human origin collected in Lebanon (Rafei *et al.*, 2015). By and large, they found a good correlation between MLST sequence types and *bla*$_{OXA\text{-}51\text{-}like}$ types but they also described some cases of distinct MLST types sharing the same OXA proteins in line with our observations.

The linkage between avian and human isolates

Whole genome-based phylogeny revealed a high diversity of avian isolates and the relatedness of several avian isolates both of wildlife and livestock origin to human clinical isolates including international clonal lineages IC7 and IC8 (Fig. 4). Especially intriguing, one clade characterized by carriage of OXA-374, comprises human clinical isolate 1 295 743 from the United States, stork isolates from Poland and Germany and exhibits relationship to clinical isolates from Spain and Japan as well as poultry meat isolates from Switzerland (Lupo *et al.*, 2014). In the light of an overall isolation rate of 25% in white stork nestlings, it is hard to conceptualize a scenario which links these findings but does not include an affinity of *A. baumannii* to avian host systems.

Our avian isolate closest to any human clinical isolate is chicken isolate 65 from Germany which is related to human clinical isolate BJAB0715 from China. Additional isolates related to isolate 65 were recovered in 2012 from two other chicks within the same flock and from a hen of another seemingly unrelated flock (Table 1 and Fig. 1) suggesting successful spread of a clone within poultry livestock. At present we can only speculate on the transmission path linking these chicken isolates from Germany to the human isolate from China. At least, a temporal estimate of their divergence is possible, based on the recent determination of a mutation rate of 5 SNPs per year per genome for the IC1 clade of *A. baumannii* (Holt *et al.*, 2016). Assuming comparable rates here, the most recent common ancestor of chicken isolate 65 and human isolate BJAB0715 existed around 100 years ago (270 SNPs per 1.857 Mbp corresponds to ~580 SNPs per genome which yields a calculated divergence 116 years ago). In conjunction with the fact that additional isolates, the oldest of which was recovered in 1964 from a mink in the

Netherlands, share the same MLST sequence type as 65 and BJAB0715 (Supporting Information Table S3), is much in support of their belonging to an international clone that was already spread worldwide before the use of antibiotics.

The recent description of a virulent strain isolated from diseased chicks in China not only demonstrates the presence of *A. baumannii* in poultry livestock worldwide but also suggests it to be a zoonotic pathogen (Liu *et al.*, 2016). The recent demonstration of *A. baumannii* in raw meat samples in Switzerland at an overall rate of 25% and in poultry meat in particular that was found contaminated at a rate of 48% (Lupo *et al.*, 2014) suggests a high abundancy in poultry livestock and a specific affinity for birds. The fact that our isolation rates from chicken (3%) and geese (8%) are lower than that reported for poultry meat may result from crosscontamination during meat processing but could be also due to differences in the isolation methods (CHROMagar Acinetobacter vs. Chrom ID ESBL agar; direct streaking of swabs vs. enrichment culture from meat homogenates). It may well be that more sophisticated methods would reveal in the future a greater avian impact on the epidemiology and spread of *A. baumannii*. We would like to emphasize again that our study links avian isolates ($ST138^{Pa}$ and $ST240^{Pa}$) to isolates of the same sequence types found in poultry meat by Lupo *et al.* (2014) and clinical isolates from Taiwan and Japan.

Recently, wild birds' feces was described as a source of *A. baumannii* in Nigeria with an average isolation rate of more than 30% (Dahiru and Enabulele, 2015). Unfortunately, the species identification was based on biochemical tests which are considered unreliable (Peleg *et al.*, 2008), leaving the question whether the isolates are true *A. baumannii*. By contrast, in a study on the cloacal and pharyngeal bacterial flora of free-living birds in Germany no *A. baumannii* was recovered (Stenkat *et al.*, 2014). The prevalence of *A. baumannii* in birds may thus largely depend on the bird species and the geographical region.

Concluding remarks

Collectively, our data in conjunction with previous work of others is much in support of the dissemination of *A. baumannii* in poultry livestock with possibly far-reaching consequences for public health. The diversity of avian isolates illustrated here by whole genome sequencing matches well with the diversity found among clinical isolates.

The prevalence of *A. baumannii* in poultry livestock and in the food production chain is underexplored and should be further investigated. Efforts should be made to screen avian populations worldwide including their environmental context.

Experimental procedures

Sample collection and processing

COPAN Amies agar gel medium transport swabs (COPAN 108C and 110C; HAIN Lifescience, Germany) were used for sampling. Swabs were immediately transferred to Amies transport medium and stored at 4°C until direct plating on CHROMagar Acinetobacter. CHROMagar™ Acinetobacter (CHROMagar, France) was prepared according to the manufacturer's description without addition of the CHROMagar MDR supplement CR102. In preliminary studies we had determined that all *A. baumannii* isolates of our collection were able to grow on CHROMagar Acinetobacter suggesting no major bias. Preliminary studies in our lab also revealed a higher specificity of CHROMagar Acinetobacter compared to Leeds Acinetobacter Medium and a higher sensitivity compared to Simmons Citrate Agar (data not shown).

Bacterial species identification

Speciation of isolates recovered from CHROMagar Acinetobacter was based on PCR detection of $bla_{OXA\text{-}51\text{-}like}$ (Turton *et al.*, 2006a), partial 16S rRNA gene sequencing (Weisburg *et al.*, 1991) and partial *rpoB* sequencing using primers Ac696F and Ac1598R as described (Nemec *et al.*, 2009). To determine $bla_{OXA\text{-}51\text{-}like}$ variation the coding region was fully sequenced as described (Zander *et al.*, 2012). New OXA-51 variants were deposited at GenBank (see Table 1) and registered at Lahey database now hosted at NCBI (https://www.ncbi.nlm.nih.gov/pathogens/beta-lactamase-data-resources/).

Infection in Galleria mellonella *caterpillars*

Larvae of *Galleria mellonella* were infected as described (Skiebe *et al.*, 2012). *G. mellonella* caterpillars were purchased from tz-terraristik.de, Germany. Bacterial strains were cultured in 5 ml LB media at 37°C. Overnight cultures were diluted 1:50 in LB and cultured for another 3 h at 37°C. Bacteria were then washed and resuspended in sterile phosphate-buffered saline (PBS). Optical density ($OD_{600\ nm}$) was adjusted to 0.2 and 5 μl of the bacterial suspension corresponding to 3×10^5 CFU was injected into each *G. mellonella* larva through the last left proleg. For each infection experiment, groups of 16 caterpillars were assigned to each of the mutant and parental strains and two control groups were used, one treated with PBS and one untreated. Then infected caterpillars were incubated at 37°C and vitality of the caterpillars was monitored every day for a period of 5 days by touching the larvae and survival was recorded. Caterpillars were considered dead when they showed no response to touching. Results were not considered valid when more than two dead caterpillars were found in control groups within five days. Three independent infection experiments were conducted. Lethal dose 50% (LD_{50}) values were determined after 24 h as described (Antunes *et al.*, 2011) inoculating and analysing triplicates of 12 larvae for each dilution (10^6, 5×10^5, 10^5, 5×10^4, 10^4 CFU in 5 μl injection volume).

Cell culture adherence

A549 cells were maintained in DMEM supplemented with 10% of fetal calf serum (FCS) at 37°C with 5% CO_2 atmosphere. The day before adhesion assay, cells were plated at 5×10^5 cells/ml. Cells were infected with different *A. baumannii* strains after overnight culture at a multiplicity of infection of 100, then the plate was centrifuged at 400 *g* for 5 min and incubated for 1 h at 37°C with 5% CO_2. Cells were washed 10 times with PBS to avoid unspecific adhesion to surface and lysed with sterile water. Lysed samples were diluted in 5x PBS so that serial dilutions could be performed in PBS before plating on LB agar to enumerate CFUs.

Natural transformation competence

The competence of avian isolates to take up naked DNA from the environment and to incorporate it into the genome by homologous recombination was analysed as described previously (Wilharm *et al*., 2013).

Macrorestriction analysis

Bacterial strain typing was performed by ApaI-macrorestriction and subsequent pulsed-field gel electrophoresis (PFGE), and results were interpreted according to the criteria suggested by Tenover *et al.* (1995).

Antimicrobial susceptibility testing

Susceptibility to different antibiotics (ampicillin, cefotaxime, ceftazidime, cefoxitin, meropenem, gentamicin, kanamycin, amikacin, sulfamerazine, sulfamerazine/trimethoprim and ciprofloxacin) was tested using the microbroth dilution method according to DIN58940 and EUCAST guidelines with result interpretation according to EUCAST v5.0. Additionally, a PCR screening for the presence of *Acinetobacter*-specific carbapenemase genes ($bla_{\text{OXA-23-like}}$, $bla_{\text{OXA-40-like}}$ and $bla_{\text{OXA-58-like}}$) was performed in all isolates of this study.[48]

Illumina sequencing

Libraries for Illumina short read sequencing were prepared from 1 ng of extracted DNA utilizing the Nextera XT DNA Library Prep Kit according to the manufacturer's recommendations (Illumina Inc., USA). Sequencing was carried out in paired-end (2×300 bp) on a MiSeq benchtop instrument. The whole genome shotgun project of 18 avian isolates has been deposited at DDBJ/ENA/GenBank under the accession of bioprojects PRJNA326058 and PRJNA390481.

WGS data processing

The determination of the maximum common genome (MCG) alignment was done comprising those genes present in all 40 genomes selected for the alignment (von Mentzer *et al*., 2014). To determine them we clustered the coding sequences based on the parameters sequence similarity (min. 70%) and coverage (min. 90%) and defined those genes that were present in each genome while fulfilling the threshold parameters as MCG containing 2182 orthologous genes for the 40 strains. Next, the allelic variants of these genes were extracted from all genomes by a blast based approach, aligned individually for each gene and then concatenated which resulted in an alignment of 1.857 Mbp for these 40 strains. This alignment was used to generate a phylogenetic tree with RAxML 8.1 (Stamatakis, 2014).

rep-PCR

Epidemiological typing of isolates was performed by rep-PCR (DiversiLab System; bioMérieux, Nürtingen, Germany) following the manufacturer's instructions. Results were analysed with the DiversiLab software using the Pearson correlation statistical method to determine distance matrices and the unweighted pair group method with arithmetic averages (UPGMA) to create dendrograms. Isolates that clustered below the threshold of 95% were considered unrelated (Higgins *et al*., 2012). Representative isolates belonging to *A. baumannii* international clones 1–8 were included as controls (Higgins *et al*., 2010).

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Supplementary Table S1: distance matrix indicating the number of single nucleotide polymorphisms (SNPs) within the set of 2182 genes
Supplementary Table S2: Wilharm *et al.*, MLST sequence types of *Acinetobacter baumannii* strains as indicated according to the Oxford and the Pasteur scheme (http://pubmlst.org/abaumannii/)
Supplementary Table S3: Additional information on MLST sequence types found in avian isolates
Supplementary Fig. S1: Overview of regions in Poland where white stork nestlings have been sampled
Supplementary Fig. S2: Sample sites along the Oder river (voivodship Lubuskie) where *A. baumannii* isolates have been obtained from white stork nestlings
Supplementary Fig. S3: Sample sites in the Glogów region (voivodship Lower Silesia) where *A. baumannii* isolates have been obtained from white stork nestlings
Supplementary Fig. S4: Sample sites in the village Kłopot (52° 7′ 51″ N, 14° 42′ 8″ O, voivodship Lubuskie, Poland) in the year 2013
Supplementary Fig. S5: Sample sites in the voivodship Warmińsko-Mazurskie where *A. baumannii* isolates have been recovered from white stork nestlings
Supplementary Fig. S6: Closely related isolates from consecutive years suggest that *A. baumannii* is resident in the Oder valley
Supplementary Fig. S7: Sampling of stork pellets and nestlings in the village Kłopot on June 30th, 2014: relatedness of pellet isolate 2P1 and nestling isolate 8D1
Supplementary Fig. S8: Sample sites in the village Kłopot (52° 7′ 51″ N, 14° 42′ 8″ O, voivodship Lubuskie, Poland) in the year 2014
Supplementary Fig. S9: DiversiLab rep-PCR analysis of selected avian isolates of *A. baumannii* and relationship to clonal lineages
Supplementary Fig. S10: The virulence of avian isolates of *A. baumannii* is comparable to that of clinical isolates in the *Galleria mellonella* infection model