Bacterial diversity of cosmopolitan Culex pipiens and invasive Aedes japonicus from Germany

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Abstract Symbiotic bacteria have gained significant attention in recent years. For example, microbiota of some mosquito species seems to influence the development and transmission of pathogens. Furthermore, several attempts using bacteria as a paratransgenetic tool have been made in order to assist the control of mosquito-borne diseases. In this study, we examined the bacterial diversity of wild-caught adult *Culex* (*Cx.*) *pipiens* and laboratory-reared adult *Aedes japonicus* (*Ae. japonicus*) in Germany using a culture-independent method. Genomic DNA was extracted from each specimen and submitted to PCR amplification of eubacterial 16S rDNA. After the cloning reaction, 28 bacterial transformants per sample containing the 16S rDNA inserts were selected per each sample for sequencing. The analysed specimens of *Cx. pipiens* as well as of *Ae. japonicus* showed a diverse bacterial community including some common bacterial genera. Blast analysis allowed to identify 21 bacterial genera belonging to 2 phyla among the 23 specimens of *Cx. pipiens*. The 14 analysed *Ae. japonicus* revealed 11 bacterial genera belonging to 3 phyla. In both mosquito species, identified isolates were mainly Proteobacteria. Only 4 of the bacterial genera were found in both mosquito species, with the most prevalent genera *Sphingomonas* and *Rahnella* in *Cx. pipiens* and in *Ae. japonicus* respectively. Most of the bacterial genera found in our study have been identified in other mosquito species before. Due to the currently scarce data situation, ongoing examinations on the very abundant bacterial genera or species are strongly required to determine their relevance for the biology and adaptiveness of mosquitoes including pathogen-host relationship.

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Introduction

Over the last few years, the interest in microorganisms associated with other organisms has increased rapidly, which can be partly explained by the recognition of their impact for vertebrates as well as invertebrates has been recognized. It has now been recognized that host organisms are not dissociable from their microbial partners; together they form the holobiont (Guerreo et al. 2013). For instance, the digestion of xylophagic termites mainly relies on intestinal microorganisms (Hongoh et al. 2005; Köhler et al. 2012). Another benefit for the host may be the provision of colonization resistance in insects, with indigenous microbiota inhibiting the settlement of pathogens in the gastrointestinal tract (Berg 1996). Different studies on *Drosophila*, locusts and also bumblebees found links between different bacterial endosymbionts and the protection of the host organism against viral infections (Dillon et al. 2005; Hedges et al. 2008; Koch and Schmid-Hempel 2011). Other studies showed that endosymbionts can lead to sexual aberrations of the host insect, such as feminization, masculinization or cytoplasmatic incompatibility (reviewed in Kageyama et al. 2012).

Mosquitoes (Culicidae) are one of the most important vectors for disease agents, the latter causing infectious diseases like malaria, dengue, West-Nile, Chikungunya or yellow fever as well as different forms of encephalitis (Becker et al. 2014; WHO 2016). They are also currently involved in the spreading of Zika virus in the Americas and the Pacific region. Every year, about 700 million people get infected with disease agents transmitted by mosquitoes and over 1 million of these cases are fatal (Caraballo and King 2014). Due to the spread of mosquitoes like *Ae. albopictus* into new geographical areas, some of the mosquito-borne diseases can now be found outside their endemic range (Bonilauri et al. 2008; La Ruche et al. 2010).

In the context of vector and pathogen control strategies, some new approaches using microbiota seem very promising. Several microorganisms show an influence on the development, the pathogen infection rates or the vector competence of mosquitoes (Werren 1997; Kambris et al. 2009; Minard et al. 2013). Natural bacterial endosymbionts of mosquitoes could also serve as a subject of paratransgenesis, i.e., the application of genetically modified microorganisms, which could be used to inhibit pathogens within the vector or pathogen transmission (Coutinho-Abreu et al. 2010).

Overall, studies on bacterial microbiota diversity in mosquitoes are mainly dealing with the medically most relevant vectors such as *Anopheles gambiae*, *Aedes aegypti* and *Aedes albopictus* (Wang et al. 2011; Zouache et al. 2012; Moro et al. 2013). Although a variety of established German mosquito species are also known as potential vectors for disease agents, there are currently no studies about the composition and structure of their associated bacterial communities. *Culex pipiens* (*Cx. pipiens*) is one of the most widespread mosquito species in Europe and North America and a potential vector for West-Nile virus (Fonseca et al. 2004; Diaz-Badillo et al. 2011; Hesson et al. 2014). In addition, pathogens like *Wuchereria bancrofti*, *Dirofilaria repens*, *Dirofilaria immitis* as well as Usutu and Sindbis virus have already been detected in this species (Ramzy et al. 1997; Cancrini et al. 2007; Jöst et al. 2010; Jöst et al. 2011; Yildrim et al. 2011). *Aedes japonicus* (*Ae. japonicus*, Asian bush mosquito) is an invasive mosquito species originally from Japan and Korea which can be found in Germany since 2008 (Tanaka et al. 1979; Schaffner et al. 2009) and is known as vector for West-Nile virus as well, but also for more tropical viruses such as Chikungunya or dengue virus (Sardelis and Turell 2001; Schaffner et al. 2011). *Aedes japonicus* is a mammalophilic mosquito, whereas *Cx. pipiens* is more known as an ornithophilic mosquito. As both species are often found in human settlements, they are of strong interest for human and veterinary medicine (Becker et al. 2011; Werblow et al. 2014; Melaun et al. 2015). This study is the first study to investigate the bacterial microbiota diversity of the common mosquito species *Cx. pipiens* and the non-indigenous species *Ae. japonicus* in Germany.

Methods

Sampling and morphological identification

The analysed specimens of *Ae. japonicus* were collected during a survey in October 2014 and April and May 2015 at two locations (Backnang and Remshalden-Geradstetten, both urban environments) in Baden-Wuerttemberg, Germany. The sampling location is situated in the south of Germany, approximately 130 km north of the Swiss border. Larvae were caught with small hand nets from buckets in a private garden (Remshalden-Geradstetten) and flower vases on a cemetery (Backnang) and raised in the laboratory. After hatching, adults ($n = 14$) had no access to nutrition sources like sugar or blood, and were killed by freezing them at −20 °C for at least 20 min. The adult *Culex* ($n = 23$) were sampled at the Federal Environmental Agency's premises in Berlin-Marienfelde (urban environment) between May and September 2014 with an EVS-Trap, and were stored at −20 °C until further analysis. The sampling site is situated in the east of Germany, about 175 km south of the Baltic Sea.

The morphological identification of *Culex* (to genus level) and *Ae. japonicus* (to species level) was carried out with a stereomicroscope using the identification keys of Gutsevich et al. (1974), Tanaka et al. (1979) and Becker et al. (2010).

DNA extraction

Before DNA extraction, all mosquitoes were individually surface disinfected following the protocol of Zouache et al. (2011). The samples were then homogenized in 150 µl of sterile 0.8% sodium chloride solution with a tissue mill (MM400, Retsch GmbH, Germany) and two of 3 mm stainless steel beads (VWR, Germany) for 2 min at 25 Hz. Out of the homogenate, 75 µl was taken for DNA extraction using the peqGold Microspin Tissue DNA Mini kit (Peqlab Biotechnology GmbH, Erlangen, Germany), with additional 50 µl of lysozyme. DNA was eluted with 60 µl elution buffer.

Amplification of cox1 genes

In order to verify the morphological species results, the cytochrome c oxidase subunit 1 (cox1) gene fragment was amplified for mosquito species identification. The reaction was performed with primers LCO 1490/HCO 2198 for all *Aedes* samples and 13 *Culex* individuals (Folmer et al. 1994). For the remaining *Culex* individuals (*Cx.* 6, *Cx.* 15 – *Cx.* 23, $n = 10$), a different set of primers BC-kumar forward/reverse (Kumar et al. 2007) was used as they did not show any positive results with primers LCO 1490/HCO 2198. All PCR reaction mixtures contained 12.5 µl HotStart Mix Y (Peqlab Biotechnology GmbH, Erlangen, Germany), 1 µl of each primer (10 pmol μl^{-1}), as well as varying amounts of template DNA and ddH_2O, in a total volume of 25 µl. The amplification was performed in a thermocycler (Eppendorf, Germany) with the following cycle parameters for LCO 1490/HCO 2198: 1 cycle of 94 °C, 3 min; 40 cycles of 94 °C, 45 s; 37 °C, 45 s and 72 °C, 60 s followed by terminal extension of 72 °C, 10 min and a final ramping to 8 °C. The cycling parameters for primers used by Kumar et al. (2007) were 1 cycle of 94 °C, 2 min; 40 cycles of 94 °C, 60 s; 59 °C, 60 s and 72 °C, 60 s followed by terminal extension of 72 °C, 5 min and also a final ramping to 8 °C. Quality and yield of DNA was checked by Midori Green (Nippon Genetic EUROPE GmbH) staining and agarose gel-electrophoresis. Positive samples were purified using the peqGOLD Cycle-Pure Kit (Peqlab Biotechnology GmbH, Erlangen, Germany).

Amplification of 16S rDNA genes

The amplification of the bacterial 16S rDNA genes was carried out with eubacterial-specific primers pA/pH (Edwards et al. 1989; Moro et al. 2013) and the same reaction mix as described for the cox1 amplification was used. PCR reactions were performed as described in Moro et al. (2013) with terminal extension of 72 °C, 12 min. The expected fragment length of about 1500 bp was again checked by Midori Green staining and agarose gel-electrophoresis. Positive samples were used for cloning reactions.

Cloning reaction and plasmid purification

The cloning reaction (one per mosquito) was carried out with the pGEM-T vector system (Promega Corporation, USA) using the standard reaction protocol for ligation and transformation as described in the manufacturers manual. In preparation for the cloning reaction, we produced chemically competent cells out of a stock of 50 µl One Shot® Top 10 chemically competent *E. coli* cells (Invitrogen Life Technologies, USA) following the protocol described in Mülhardt (2009). The cells were portioned into 200 µl aliquots, shock frosted with liquid nitrogen and stored at −80 °C until further use. Clones, which were positive by blue/white screening, were checked for the proper insert length by amplification of the manufacturer recommended primer pair M13 forward/reverse. We used not more than 28 positive colonies from each plate to check for insert length. Each colony was picked with a sterile toothpick, added to a master plate and 20 µl ddH_2O. The solution of bacterial material and water was used for a cell lysis which was performed at 95 °C for 2 min followed by 2 min incubation on ice (step performed twice) before centrifuged at 4000 rpm for 10 min. The supernatant was used as template DNA for the amplification with M13 forward/reverse in the same reaction mix as described for cox1 and 16S rDNA. The amplification steps were 1 cycle of 94 °C, 12 min; 35 cycles of 94 °C, 60 s; 50 °C, 60 s and 72 °C, 60 s followed by terminal extension of 72 °C, 10 min and a final ramping to 8 °C. The insert length was checked by Midori green staining and agarose gel-electrophoresis. Clones with inserts of the expected size (about 1500 bp) were purified with the peqGOLD Plasmid Miniprep Kit according to the manufactures protocol without the third and optional washing step.

Sequencing reaction

For subsequent Sanger-sequencing reactions with product specific forward primers, PCR products were purified using the peqGOLD Cycle-Pure Kit (Peqlab Biotechnology GmbH, Erlangen, Germany). Each obtained sequence was edited using BioEdit (Hall 1999) and compared with sequences deposited in GenBank using the BLAST algorithm (Altschul et al. 1997).

Results

Molecular identification of mosquito species

The identification of the female adult *Culex* mosquitoes to species level resulted in 23 members of the *Cx. pipiens* complex. As neither the morphological differentiation nor DNA barcoding is utterly reliable, no distinction was made between *Cx. pipiens* biotype *pipiens* and *Cx. pipiens* biotype *molestus*. All 14 laboratory-reared female individuals of *Ae. japonicus* were determined to species level. The sequences of the molecular species identification are given under accession numbers KX260917-KX260953 in the GenBank database. All of the 23 *Culex* and 14 *Ae. japonicus* mosquitoes were used for subsequent bacterial analyses.

Bacterial diversity in *Culex pipiens* and *Aedes japonicus*

After the cloning reactions not more than 28 colonies per mosquito specimen (positive by blue/white screening, *Cx. pipiens* = 550, *Ae. japonicus* = 384) were picked and checked for the correct insert. Among them, we only used sequences with more than 800 bp and a query cover and identity in GenBank of 98–100%, resulting in a total of 282 analysed clones. Sequence-based identification classified bacteria in 2 phyla, 4 classes, 14 families and 21 genera for *Cx. pipiens* (Table 1). Although fewer samples of *Ae. japonicus* were analysed, a higher rate of positive clones was identified including one additional phylum, the Actinobacteria. Overall,

Table 1 Taxonomic assignments of bacterial clones from *Culex pipiens.* In total, 123 clones were sequenced with eubacterial-specific primer pA for bacterial 16S *rrs* genes. Only results with a sequence length >800 bp and a query cover and identity in NCBI GenBank between 98 and 100% are shown

Phylm	Class	Family	Nearest genus acc. to BLASTn	Nearest species acc. to BLASTn	Ident [%]	Sequences
Firmicutes	Bacilli	Carnobacteriaceae	*Carnobacterium*	*Carnobacterium maltaromaticum*	99	1
		Enterococcaceae	*Enterococcus*	*Enterococcus* sp.	99	1
		Staphylococcaceae	*Staphylococcus*	*Staphylococcus warneri*	99	1
		Streptococcaceae	*Streptococcus*	*Streptococcus thermophilus*	99	5
Proteobacteria	α-Proteobacteria	Bradyrhizobiaceae	*Afipia*	*Afipia* sp.	100	1
			Bosea	*Bosea vestrisii*	99	1
		Methylobacteriaceae	*Methylobacterium*	*Methylobacterium* spp.	99	2
		Phyllobacteriaceae	*Mesorhizobium*	*Mesorhizobium* spp.	99	3
		Rhizobiaceae	*Agrobacterium*	*Agrobacterium tumefaciens*	100	1
		Sphingomonadaceae	*Sphingomonas*	*Sphingomonas echinoides*	99	39
				Sphingomoas leidyi	99	1
	β-Proteobacteria	Comamonadaceae	*Acidovorax*	*Acidovorax* spp.	99	8
			Curvibacter	*Curvibacter* spp.	99	12
		Ralstoniaceae	*Ralstonia*	*Ralstonia* spp.	99	7
				Ralstonia insidiosa	99	15
	γ-Proteobacteria	Enterobacteriaceae	*Escherichia*	*Escherichia coli*	99	1
			Hafnia	*Hafnia* paralvei	98	1
			Kluyvera	*Kluyvera* sp.	99	1
			Pantoea	*Pantoea coffeiphila*	98	6
			Rahnella	*Rahnella aquatilis*	99	4
			Tatumella	*Tatumella* spp.	99	3
				Tatumella ptyseos	99	1
		Moraxellaceae	*Acinetobacter*	*Acinetobacter* sp.	99	1
		Pseudomonaceae	*Pseudomonas*	*Pseudomonas* spp.	99	2
				Pseudomonas psychrotolerans	99	3
Unknown	Unknown	Unknown	Unknown	Uncultured bacterium clone	99	2
∑2	4	14	21	26		123

the 159 bacterial clones of *Ae. japonicus* could be subdivided into 3 phyla, 5 classes, 9 families and 11 genera (Table 2). The most abundant classes found in *Cx. pipiens* were the α-Proteobacteria (no. of sequences, $n = 48$) with the genus *Sphingomonas* ($n = 40$) and the species *Sphingomonas echinoides* ($n = 39$) as well as the β-Proteobacteria ($n = 42$) with the most abundant genera *Ralstonia* ($n = 22$) and *Curvibacter* ($n = 12$). Within the γ-Proteobacteria only 23 bacterial isolates were found, however, this class had the highest number of genera ($n = 8$). The lowest number of bacterial isolates ($n = 8$) was found within the class Bacilli (Table 1). Similar to *Cx. pipiens*, the phylum Proteobacteria was also the most abundant phylum ($n = 145$) in *Ae. japonicus* with the genus *Rahnella* ($n = 104$) representing the highest number of isolates (Table 2). The genera *Afipia*, *Mesorhizobium*, *Sphingomonas* as well as *Rahnella* were shared between *Cx. pipiens* and *Ae. japonicus*, whereas all other genera ($n = 28$) were present in only one of the mosquito species. The bacterial sequences obtained were deposited in the GenBank database under accession numbers KX260635-KX260916.

Discussion

In this study, the bacterial microbiota of the common house mosquito *Cx. pipiens* and the non-indigenous species *Ae. japonicus* in Germany were investigated using a culture-independent method.

Most of the sequences (91.9%) obtained in *Cx. pipiens* belonged to the gram-negative phylum Proteobacteria (class: α-, β- and γ-Proteobacteria). Most of the remaining sequences (6.5%) belonged to the gram-positive phylum Frimicutes (class: Bacilli). High numbers of sequences were affiliated with *Sphingomonas echinoides*, a bacterium which was first isolated from plants and exists in seawater as well

Table 2 Taxonomic assignments of bacterial clones from *Aedes japonicus.* In total, 159 clones were sequenced with eubacterial-specific primer pA for bacterial 16S *rrs* genes. Only results with a sequence length >800 bp and a query cover and identity in NCBI GenBank between 98 and 100% are shown

Phylum	Class	Family	Nearest genus acc. to BLASTn	Nearest species acc. to BLASTn	Ident [%]	Sequences
Actinobacteria	Actinobacteria	Microbacteriaceae	*Microbacterium*	*Microbacterium oxydans*	99	9
				Microbacterium maritypicum	99	3
		Propionibacteriaceae	*Propionibacterium*	*Propionibacterium acnes*	99	1
Firmicutes	Bacilli	Carnobacteriaceae	*Granulicatella*	*Granulicatella adiacens*	99	1
Proteobacteria	α-Proteobacteria	Bradyrhizobiaceae	*Afipia*	*Afipia* sp.	99	1
		Caulobacteraceae	*Caulobacter*	*Caulobacter* spp.	98	2
		Phyllobacteriaceae	*Mesorhizobium*	*Mesorhizobium* spp.	99	17
				Mesorhizobium plurifarium	99	1
		Sphingomonadaceae	*Sphingomonas*	*Sphingomonas echinoides*	99	2
				Sphingomonas melonis	99	1
	β-Proteobacteria		*Aquincola*	*Aquincola* sp.	98	1
	γ-Proteobacteria	Aeromonadaceae	*Aeromonas*	*Aeromonas salmonicida*	100	1
		Enterobacteriaceae	*Ewingella*	*Ewingella americana*	99	15
			Rahnella	*Rahnella* spp.	99	57
				Rahnella aquatilis	99	47
∑3	5	9	14	15		159

(Heumann 1960; De Vos et al. 1989; Kim et al. 2006). *Sphingomonas* spp. were already detected in other mosquito species (i.a. Moro et al. 2013; Dhayal et al. 2014; Ngo et al. 2015). Minard et al. (2015) found that the bacterial family Sphingomonadaceae is very prevalent in mosquitoes. These bacteria have the ability to produce hydrolases involved in the degradation of oligosaccharides, so that there might be a link between the bacteria and the provision of plant sugar to the mosquito host (Minard et al. 2015). Demaio et al. (1996) identified eight bacterial species in a culture-dependent study of microbiota of *Cx. pipiens* in the USA. Two of those were also found in our study belonging to the genera *Acinetobacter* and *Pseudomonas* (both γ-Proteobacteria). The differences regarding the total number of genera found in our study ($n = 21$, culture-independent) and the study by Demaio et al. (1996) ($n = 8$, culture-dependent) might be explained by the choice of method which influences the number of species detected, e.g., only a few bacteria grow on culture media (Hugenholtz et al. 1998; Charan et al. 2013).

The most abundant bacterial phylum in *Ae japonicus* was also the gram-negative Proteobacteria (class: α-, β- and γ-Proteobacteria) with 91.2% of sequences. The remaining sequences belonged to the gram-positive phyla Actinobacteria and Firmicutes (class: Actinobacteria and Bacilli). The most abundant bacterial sequences were affiliated with *Rahnella aquatilis*, a species that is known to occur in different environments, including fresh water and soil (Guo et al. 2012; Martinez et al. 2012) and has been found in mosquitoes, e.g., within larvae of *Anopheles stephensi* (Dinparast Djadid et al. 2011).

Kim et al. (2015) examined the microbiota of larval as well as adult *Ae. japonicus* from two different sampling sites in the USA. In their culture-independent approach, they found that the relative abundance of bacterial taxa varies between habitats as well as between mosquitoes' developmental stages. In their culture-dependent attempt, they detected 10 bacterial genera in larval *Ae. japonicus* and 2 genera in newly emerged adults. Two of the bacterial genera found by Kim et al. (2015) in the USA were identified in our study as well. Those were *Aeromonas* and *Microbacterium* which were found by Kim et al. (2015) in adults (*Aeromonas*) and larvae (*Microbacterium*). Interestingly, another invasive mosquito species (*Aedes albopictus*, Asian tiger mosquito) showed a reduced bacterial microbiota in recently established populations (France) compared to autochthonous populations (Vietnam), possibly due to an altered/reduced availability of plant nutrition sources within the new habitats (Minard et al. 2015). Such comparative analyses on the invasive rock pool mosquito *Ae. japonicus* are still lacking.

Great differences regarding the bacterial diversity between individuals from laboratory breeding and wild-caught specimens can occur as shown by Rani et al. (2009) for *Anopheles stephensi*. In their study, only 7 bacterial taxa were present in laboratory-reared female mosquitoes in contrast to 36 taxa in wild-caught specimens. This might explain differences in bacterial taxa between the examined mosquito species here (21 genera and 11 genera found in *Cx. pipiens* and *Ae. japonicus* respectively). Individuals of *Ae. japonicus* were laboratory-reared to adults from field-caught larvae and were examined shortly after hatching, whereas specimens of *Cx. pipiens* were wild-caught as adults. Furthermore, it has been shown that metamorphosis in mosquitoes leads to reduced bacterial content and diversity in newly emerged adults (Demaio et al. 1996; Moll et al. 2001; Wang et al. 2011).

The 7 bacteria genera *Afipia*, *Aquincola*, *Bosea*, *Caulobacter*, *Curvibacter*, *Granulicatella* and *Mesorhizobium* were recorded for the first time in mosquitoes, while the remaining 21 genera in our study have been found in other studies before, mainly in species that are medically highly relevant, like *Aedes aegypti, Ae. albopictus, Anopheles gambiae* and *An. stephensi* (Minard et al. 2013; Dhayal et al. 2014; Ngo et al. 2015). Overall, more than 90% of the analysed isolates were gram-negative bacteria of the phylum Proteobacteria in both mosquito species, *Cx. pipiens* and *Ae. japonicus*. The dominance of this phylum was observed in different mosquito species before (Pidiyar et al. 2004; Osei-Poku et al. 2012; Moro et al. 2013).

The detection of different bacteria species of the same genus could imply a high association of these bacteria genera with mosquitoes, possibly providing a better adaptability to the environment (Moro et al. 2013). However, the research of the natural microbiota of mosquitoes is only at an early stage. Further studies are necessary to better understand and differentiate the importance of different bacteria for mosquitoes, which could also foster further research of paratransgenetic approaches to assist the control of mosquito-borne diseases. There might be general interspecific variations in associated bacterial diversity of mosquito species based on different ecological and biological habits; however, those cannot be sufficiently evaluated due to the currently scarce data situation.

Conclusion

In this study, the microbiota of two mosquito species, *Cx. pipiens* and *Ae. japonicus*, in Germany were investigated. While 21 bacterial taxa have been also reported from other mosquitoes, 7 identified genera have not been recognized before and are therefore first time records. Further analyses are strongly needed to decipher the significance of associated symbiotic bacteria regarding mosquito biology, adaptiveness and the impact on vector-pathogen-interactions. This knowledge could provide new approaches for mosquito control and the decrease of mosquito-borne infectious diseases.

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