Metagenomics, paratransgenesis and the *Anopheles* microbiome: a portrait of the geographical distribution of the anopheline microbiota based on a meta-analysis of reported taxa

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Anophelines harbour a diverse microbial consortium that may represent an extended gene pool for the host. The proposed effects of the insect microbiota span physiological, metabolic and immune processes. Here we synthesise how current metagenomic tools combined with classical culture-dependent techniques provide new insights in the elucidation of the role of the Anopheles-*associated microbiota. Many proposed malaria control strategies have been based upon the immunomodulating effects that the bacterial components of the microbiota appear to exert and their ability to express anti-*Plasmodium *peptides. The number of identified bacterial taxa has increased in the current "omics" era and the available data are mostly scattered or in "tables" that are difficult to exploit. Published microbiota reports for multiple anopheline species were compiled in an Excel® spreadsheet. We then filtered the microbiota data using a continent-oriented criterion and generated a visual correlation showing the exclusive and shared bacterial genera among four continents. The data suggested the existence of a core group of bacteria associated in a stable manner with their anopheline hosts. However, the lack of data from Neotropical vectors may reduce the possibility of defining the core microbiota and understanding the mosquito-bacteria interactive consortium.*

Key words: *Anopheles* - core microbiota - paratransgenesis - *Plasmodium* - metagenomics - databank

Insects are by far the most diverse and abundant clade of metazoans (Basset et al. 2012). Their success is partly attributable to the beneficial members of their associated microbiome. The term microbiota refers to the microbial communities that stably or transiently colonise the insect epithelium and their intracellular compartments and target organs. These communities vary from bacteria to viruses, yeasts and protists. The bacterial component of this ecosystem is the most studied and characterised (Ng et al. 2011b, Gendrin & Christophides 2013, Minard et al. 2013). These symbiotic microbiotas or consortia are beneficial to their insect hosts in many ways (Dillon & Dillon 2004, Azambuja et al. 2005, Engel & Moran 2013), including dietary supplementation, enhancement of digestive mechanisms, tolerance of environmental perturbations, protection from parasites (Degnan & Moran 2008, Weiss & Aksoy 2011) and pathogens (Nartey et al. 2013) and maintenance and/or enhancement of host immune system homeostasis. Furthermore, the

absence or elimination of the microbial fauna and even the modification of its composition can reduce the fitness of the host insect (Toft & Andersson 2010). This influence of the microbiota on its host has been referred as the "extended phenotype" (Koch & Schmid-Hempel 2011), which can range from mutualism to parasitism. Notably, bacterial communities appear to be associated with their invertebrate hosts in a highly specific manner that resembles the host phylogeny (Bauman 2005). How these associations are determined remains unclear, but species-specific antimicrobial peptides likely shape these co-evolving species-specific bacterial communities. The species that are vital to the host are stably associated with it even under different environmental conditions (Franzenburg et al. 2013).

Studies of microorganisms living in insect guts have recently increased considerably. In the last decade, multiple relevant studies have been published, ranging from diversity screening using metagenomic surveys (Baumann 2005, Lindh et al. 2005, Carpi et al. 2011, Djadid et al. 2011, Lindh & Lehane 2011, Ng et al. 2011a, b, Chavshin et al. 2012) to molecular studies of the interaction of gut bacteria with the host immune system and the response to infection (Azambuja et al. 2005, Chouaia et al. 2010, Cirimotich et al. 2011, Boissière et al. 2012). In addition to the native gut microbiota, symbiotic bacteria occupy other key organs, tissues or structures (e.g., salivary glands, ovaries, haemolymph). Primary endosymbionts, which are transmitted vertically from mother to offspring, are found in bacteriocytes, specialised cells that provide nutrients to bacteria (Wernegreen 2012). Secondary endosymbionts are transmitted horizontally, vertically or *via* the environment (Eleftherianos et al. 2013) and can be located in the haemocoel (Wernegreen

2012). Both surface-colonising consortia and intracellular symbiotic bacteria live under the pressure of an active immune system and therefore must devise strategies that enable them to withstand the adverse effects of the host immune response (Douglas 2011, Weiss & Aksoy 2011).

An exhaustive review of metagenomic tools or the architecture and dynamics of this microecosystem within *Culicidae* vectors is outside the scope of this analysis. Recent reviews have covered both of these topics substantially and creatively (Dillon & Dillon 2004, Abdul-Ghani et al. 2012, Suenaga 2012, Engel & Moran 2013, Gendrin & Christophides 2013, Minard et al. 2013, Segata et al. 2013). Rather, we summarise basic topics of this computational field and how its interaction with vector biology has expanded our knowledge of vector mosquitoes and their associated microbiota. From our perspective, computational sciences offer a means to coordinate, analyse and visualise the growing body of anopheline microbiota-related data in an organised manner that is readily available and understandable to the community. For instance, we propose to continue the effort of Minard et al. (2013), who generated a databank encompassing the reported microbiota data pertaining to several mosquito vectors. We could use this databank as a foundation for *Anopheles*-oriented expansion and, depending on the filtering criteria applied, the identification of underlying patterns.

First, we will briefly explore the concept of metagenomics and summarise recent advancements in malaria vector control based on the exploitation of the anopheline microbiota and its impact on vector competence traits. The use of metataxonomic computational tools has enabled the identification of insect-associated microbial species. Strategies have been developed based on these species to block malarial transmission *via* the expression of anti-*Plasmodium* peptides, immunomodulation or *via* induced dysbiosis (Coutinho-Abreu et al. 2010, Ricci et al. 2012, Wang & Jacobs-Lorena 2013).

Due to the vast amount of taxonomic profiling data provided by next-generation sequencing (NGS), the categorisation and filtering parameters selected for databank searching are critical for a better understanding of the mosquito as a holobiont (Brucker & Bordenstein 2013).

On metagenomics and insect vectors - Metagenomics emerged as a derivative of classic microbial genomics; the key difference between these disciplines is that metagenomics bypasses the requirement for obtaining pure cultures for sequencing (Olsen et al. 1986, Glass et al. 2010, Kim et al. 2013). Genomic information can be obtained directly from microbial communities in their natural habitats, enabling these communities to be studied in a concerted manner to describe their species composition and even predict their potential genomic functions and metabolic capabilities (Wooley et al. 2010, Huttenhower et al. 2012, Williamson & Yooseph 2012).

Metagenomics refers to the sequencing of whole-community DNA or a range of techniques; tools and approaches that attempt to identify microorganisms, genes and variants, metabolic pathways or functions within a community (Segata et al. 2013). The sequencing of whole-community DNA can be complemented by

metatranscriptomics (cDNA sequencing), metaproteomics and metabolomics (Wilmes & Bond 2006, Gilbert & Hughes 2011, McCarthy et al. 2011). Both metagenomics and metatranscriptomics aim to elucidate the genomic composition, diversity and potential within and across microenvironments by applying culture-independent techniques that include targeting rRNA gene sequences (16S for bacteria, 18S for eukaryotes and internal transcribed spacers for fungi) and whole-metagenomic shotgun sequencing (Huson et al. 2007, Segata et al. 2013).

Determining the identity and proportions of the members of a microbial community is the typical primary objective of amplicon sequencing investigations such as 16S rRNA gene surveys (Suenaga 2012). Such surveys are currently the predominant approach of insect vector metagenomics. According to Segata et al. (2013), when performing metagenomic profiling, shotgun-sequencing approaches can provide an information profile comparable to that obtained from 16S rRNA-based surveys, often with better resolution. Taxonomic profiling can be achieved by either the *de novo* binning of microbial sequences (based upon intrinsic sequence properties) or relying on information from sequenced microbial genome databases (extrinsic information) (Thomas et al. 2012). Taking into consideration sequencing coverage, read quality, read length and experimental design, a high-throughput shotgun-driven survey may enable the user to assess the diversity of genes and biochemical pathways that are represented in the consortium (Abubucker et al. 2012).

As NGS platforms develop, our potential to generate genomic data has increased exponentially (Ansorge 2009); benchmarking has thus gained relevance in providing guidance to experimental biologists confronted with a myriad of bioinformatic tools, which include GAAS (Angly et al. 2009), Glimmer (Delcher et al. 2007), CARMA3 (Gerlach & Stoye 2011), MG-RAST (Glass et al. 2010), MEGAN (Huson et al. 2007) and TAMER (Jiang et al. 2012). When designing experiments that encompass metagenomic data generation, it is imperative to consider factors such as sampling techniques, DNA/RNA extraction protocols, sequencing platforms, assembly, taxonomic binning and gene annotation tools, statistical analysis and data/meta-data sharing formats (Wommack et al. 2008, Tanenbaum et al. 2010, Wooley et al. 2010, Thomas et al. 2012). The availability of standardised procedures (Field et al. 2008, Tanenbaum et al. 2010) and platforms for data storage and sharing are increasingly important to ensure that the output of individual projects can be assessed, shared and compared (Thomas et al. 2012).

In this meta-omic era, NGS screening assays are used to determine the diversity of microorganisms and viruses residing in arthropod vectors of medical importance. This information enables human health agencies and research groups to monitor endemic infections, perform real-time surveillance of newly emerging zoonotic pathogens, identify etiological agents and determine how these agents associate with and within their hosts (Bishop-Lilly et al. 2010, Carpi et al. 2011, Ng et al. 2011a, b, Mokili et al. 2012, Hall-Mendelin et al. 2013).

Modulating effects of bacteria on the Plasmodium-anopheline interaction - Due to their importance as

malarial vectors, anopheline mosquitoes have been the targets of multiple microbiota profiling efforts (Gendrin & Christophides 2013). Underlying these efforts is the knowledge that bacteria living in the midgut can modulate the response of mosquitoes toward *Plasmodium* infection (Dong et al. 2009, Eappen et al. 2013). Thus, these bacteria have the potential to block infections and be used as genetic transformation vehicles (Pumpuni et al. 1996, Weiss & Aksoy 2011, Ricci et al. 2012).

Midgut-colonising microbial organisms have been identified in both laboratory-cultivated and field-collected *Anopheles* mosquitoes. For example, field populations of *Anopheles gambiae* and *Anopheles funestus* were found to contain 16 bacterial species spanning 14 genera (Lindh et al. 2005). Laboratory populations of *An. gambiae* and *Anopheles stephensi* have been found to harbour a wide variety of bacteria, particularly those belonging to the genera *Asaia*, *Enterobacter*, *Mycobacterium*, *Sphingomonas*, *Serratia* and *Chryseobacterium* (Favia et al. 2007, Dong et al. 2009, Rani et al. 2009).

Bacterial components of the anopheline midgut microenvironment modulate vectorial competence initially by inhibiting the sporogonic development of malaria parasites when infection is established (Pumpuni et al. 1993, 1996, Gonzalez-Ceron et al. 2003, Dong et al. 2009, Cirimotich et al. 2011). In addition, Gram-negative, but not Gram-positive bacteria inhibit oocyst formation in whole or in part (Pumpuni et al. 1993, 1996). Evidence regarding the influence of midgut microbiota in the life cycle of parasites has also been found in other insects such as sandflies and tsetse flies (Schlein et al. 1985, Welburn & Maldlin 1999).

Recent studies suggest that *Enterobacter* species isolated from *Anopheles arabiensis* from Zambia act directly on *Plasmodium falciparum*, hindering the development of the parasite in an *An. gambiae* model. This effect is due to the bacterial production of reactive oxygen species that interfere with the development of the parasite and cause parasite death before invasion of the intestinal epithelium (Cirimotich et al. 2011).

Bacteria colonising the midgut lumen modify the intestinal environment and inhibit the development of parasites by modulating the immune system and inducing the expression rate of immunity genes encoding antimicrobial peptides (Ratcliffe & Whitten 2004, Michel & Kafatos 2005, Meister et al. 2009). These peptides are likely to play key roles in suppressing pathogenic bacteria and modulating the abundance of symbiotic bacteria and may also play a role in the response towards parasite infections (Beard et al. 2001, Boulanger et al. 2004).

The inducible synthesis of nitric oxide metabolites is an important component of the immune response of anopheline mosquitoes to limit the development of *Plasmodium* parasites (Peterson et al. 2007). Using a comparative genomics approach, Wang et al. (2012) proposed that certain bacterial taxa may induce a reductive environment within the mosquito midgut, thus aiding in the detoxification of reactive oxygen and nitrogen species. This predicted bacterial redox capacity would enable a more aggressive mosquito immune response mediated by nitrogen oxides upon *Plasmodium* infection.

These observations have motivated interest in exploiting the genomic power of the bacterial passengers within the mosquito or modifying the inner environment of the mosquito midgut to develop novel transmission blocking tools. Briefly, we summarise the results of five examples of such control strategies.

Delivering anti-plasmodium effector molecules into the anopheline midgut - Paratransgenesis can be defined as the set of strategies for the use of engineered symbiotic bacteria from the mosquito midgut to deliver effector genes that encode molecules that inhibit parasite development (Durvasula et al. 1997). A recent literature review by Wang and Jacobs-Lorena (2013) (and references therein) lists known anti-*Plasmodium* molecules and classifies them as parasite killers, parasite-interacting, mosquito midgut or salivary gland epithelia-interacting and immunomodulators.

The potential success of such strategies relies on the fact that most of the mosquito microbiome resides in the same compartment in which the most vulnerable stages of malaria parasite development occur (Whitten et al. 2006); at that key intersection of space and time, bacterial loads increase due to blood meal ingestion. Thus, when expressed, the yield of anti-*Plasmodium* molecules should increase proportionally with this increase in bacterial load. Several research groups have explored paratransgenesis to interfere with the *Plasmodium* cycle within the anopheline host. The proposed paratransgenic vectors are discussed below.

Asaia - Bacteria from the genus *Asaia* have been evaluated as paratransgenic vectors to control malaria transmission (Favia et al. 2007, Damiani et al. 2010). Bacteria from this genus are considered viable candidates due to their stable association with anopheline mosquitoes throughout the body and different life stages; they are also easily cultivated and transformed. Engineered *Asaia* are capable of colonising laboratory-reared *An. stephensi* mosquitoes *via* vertical and horizontal transmission routes. Based on a series of experiments, Capone et al. (2013) reported three key conclusions. First, the physiological changes occurring in the mosquito midgut after an infected or uninfected blood meal had no negative effect and in fact appeared to benefit the *Asaia* population. Boissière et al. (2012) also observed a strong positive correlation between the presence of Enterobacteriaceae and an infected blood meal. Second, *Asaia* can act as an immunomodulator within the mosquito by inducing the production of antimicrobial peptides that may interfere with the course of infection, particularly during the invasion of epithelial tissues and translocation to the salivary glands. *Asaia* may also act as up-regulators of immunity genes. Third and more relevant to genetic manipulation strategies, Capone et al. (2013) demonstrated that modified strains of *Asaia* were widely spread in the midgut and salivary glands of infected mosquitoes and that they co-localised with *Plasmodium* parasites. Exogenous recombinant bacteria of this genus replicated and spread within the mosquito and colonised key organs in a sustained manner. When engineered to release anti-*Plasmodium* peptides, these

strains could interfere with the parasite's life cycle, thus reducing its vectorial competence.

Furthermore, the presence of this genus in laboratory-reared and field-collected *An. gambiae* from Burkina Faso (Damiani et al. 2010), *Anopheles maculipennis* from Italy (Favia et al. 2007) and in laboratory-reared *Aedes aegypti* (Crotti et al. 2009) suggests that *Asaia* cross-colonises species and genera. The bacterial association with widely distributed vectors could signify that, if used as a biological control strategy, *Asaia* would bypass genetic barriers among reproductively isolated mosquito populations (Damiani et al. 2010).

These are promising findings that support the efforts to identify a bacterial symbiont that negatively impacts vectorial competence without detrimental effects on the mosquito holobiont. However, Djadid et al. (2011) and Rani et al. (2009) did not identify bacteria from the genus *Asaia* in field-collected or laboratory-reared Asian malaria vectors (*An. stephensi* and *An. maculipennis* from Iran and India) using culture-dependent or culture-independent methods. Although it is possible that the predominant bacterial taxa in these anopheline species may have changed during the transition from their natural habitat to a laboratory colony (Gendrin & Christophides 2013), their apparent absence in field-collected Asian vectors could hinder the use of this model. The colony from which the main observations of these studies have been generated was established approximately two decades ago at the University of Camerino in Italy (1985-1988) (Favia et al. 2007, Damiani et al. 2008). What are the implications of a non-existent natural and steady relationship between this Acetobacteraceae and field-collected Asian anophelines? Both Djadid et al. (2011) and Rani et al. (2009) discuss the possibility of this absence being a "rounded zero" observation due to "low coverage" of the NGS data. Furthermore, the size of the continent and the ecological niches in which these two species are distributed across Asia and Eurasia in the case of the maculipennis subgroup (Di Luca et al. 2009, Sinka et al. 2010, 2012) could partly explain the absence of this genus in the sampled mosquitoes. It was not until 2013 that Manguin et al. reported *Asaia* in Southeast Asia anophelines (non-*An. stephensi* mosquitoes from Thailand and Vietnam). The intention of this narrative is to emphasise the need for a data repository to record such events and ease their interpretation upon publication as well as what we consider a fundamental step in vector metagenomics - the need for coordinated efforts to produce technically homogeneous and comparable data across continents, species or tested conditions regardless of the metataxonomic profiling technique. If *Asaia* is to become a successful component of the bacterial malaria control arsenal, further testing to determine if other relevant anophelines harbour it naturally (or in a laboratory model) would be beneficial. Neotropical anophelines are of particular interest and have been largely neglected when developing such strategies. There are no records of this genus in American anophelines.

Serratia - Strategies for controlling malaria through the genetic manipulation of midgut bacterial symbionts primarily involve mechanisms that act by killing *Plas-* *modium* ookinete/oocysts in the mosquito midgut, the "location" in which the cycle within the mosquito undergoes a severe bottleneck (Wang & Jacobs-Lorena 2013). However, resistance of the *Plasmodium* parasite to effector molecules may arise during paratransgenesis within the mosquito because the principal mode of action is to attack and kill the pathogen itself (Bando et al. 2013).

Serratia marcescens HB3, which was isolated from laboratory-reared *An. stephensi* mosquitoes, inhibits *Plasmodium* development within the mosquito midgut by interrupting ookinete invasion through the midgut epithelial cells. Phenotypic variation at the cellular and structural levels was observed and directly correlated with the ability to induce resistance against *Plasmodium* invasion (Bando et al. 2013).

To evaluate whether the intraspecific diversity of *S. marcescens* is associated with vectorial competency of human malaria vectors in endemic areas, Bando et al. (2013) profiled the microbiota in the midgut of wild *An. gambiae* mosquitoes collected in Burkina Faso. Six strains of *S. marcescens* were identified based on their 16S rRNA sequences. To determine if the isolated strains were able to influence *Plasmodium* development within the mosquito midgut, they were tested in the *An. stephensi-Plasmodium berghei* model. A significant inverse correlation between cell length and oocyst number was recorded for five different *Serratia* strains.

A blocking strategy denying parasite entry into the epithelial cells could be effective for *Plasmodium* development inhibition. This would broaden the arsenal of biological control strategies and enable these strategies to be used in synergy. Finally, the authors of this study noted that the *S. marcescens* HB3 strain must be continuously introduced to the vector to exert this action. This would indicate that there may be active stress factors, such as innate immunity, that modulate the dynamics of *Serratia* populations within the enclosed environment of the midgut.

Pantoea - The natural mosquito symbiont *Pantoea* can cross-colonise several mosquito species and is readily transformed and cultured; thus, *Pantoea* has been proposed for paratransgenic applications (Bisi & Lampe 2011, Djadid et al. 2011).

A new strategy to deliver anti-*Plasmodium* effector molecules into the mosquito midgut was recently described. Wang et al. (2012) engineered a *Pantoea agglomerans* strain secreting five different antimalaria proteins in the mosquito midgut. They observed suppression of the development of *P. falciparum* and *P. berghei* in their respective mosquito models (*An. gambiae* and *An. stephensi*) of up to 98% and 83%, respectively. The authors stressed that the data collected on prevalence are more important than the inhibition of oocyst formation. The authors observed a dramatic reduction of *P. falciparum*-infected mosquitoes when scorpine or (EPIP)4 were the expressed anti-*Plasmodium* effectors (from 90% in controls to 14% and 18%, respectively). This reduction represents an 80-84% transmission blocking potential. The authors aim to reduce the probability of resistant parasite selection by combining multiple effector proteins. In addition, the anti-*Plasmodium* ef-

fects of the tested molecules were equally effective when tested in *An. gambiae* (African vector) and *An. stephensi* (Asian vector). Thus, paratransgenesis is promising as a universal control strategy because the effectiveness of the molecules tested in this study was not influenced by the mosquito species. Introducing engineered *Plasmodium agglomerans* strains into multiple species in the wild and their establishment as dominant members of the midgut ecosystem is a future logistical challenge and an interesting field within micro-ecology studies.

The potential role of viruses - There is little information regarding the association of viruses with anophelines and the available information primarily pertains to the use of viruses as tools to control malaria. An early claim on the use of viruses to control *Plasmodium* infections was made by Bird et al. (1972), who reported the presence of cytoplasmic polyhedrosis virus in adult *An. stephensi* during the sporogonic phase of infection with *P. bergei yoeli*. They associated the presence of the virus with an observed reduction in the transmission of the parasite in this model and speculated on whether the virus had an impact on both the mosquito and developing parasite. According to their microscopic findings, the majority of the sporozoites exhibited deformed structure and/or vacuolation.

An entomovirus with high specificity towards anophelines would provide the research community with a new set of tools to develop and implement more integral vector eradication policies. Such viruses had not been reported abundantly and remained unexplored until 2008, when Ren et al. discovered and described the *An. gambiae* densonucleosis virus (AgDNV). This entomovirus has a narrow host range and is readily transmitted between generations in natural populations. AgDNV is transmitted vertically and horizontally from adult mosquitoes to larvae. AgDNV apparently exhibits replication tropism towards adult tissues over larvae and pupae tissue, making it a potential selective biopesticide of adult anophelines. In addition, an exogenous green fluorescent protein (EGFP) gene was expressed by infection of *An. gambiae* cells and adult mosquitoes with recombinant AgDNV (Ren & Rasgon 2010). The recombinant virions readily infected mosquitoes, expressed EGFP in key tissues involved in the infective cycle of *Plasmodium* parasites and were transmitted to offspring in a manner similar to that of the wild-type virus. Recombinant AgDNV could be used as a paratransgenic tool to express anti-*Plasmodium* peptides or insect-specific toxins (Ren & Rasgon 2010).

Wolbachia - *Wolbachia* is a genus of intracellular α-proteobacteria that has been estimated to infect 40-75% of all arthropod species and several nematodes (Hilgenboecker et al. 2008, Zug & Hammerstein 2012, Rainey et al. 2014). These bacteria propagate through insects partly through the modulation of host reproduction (Moreira et al. 2009). *Wolbachia* can inhibit the replication and spread of arbovirus in vector mosquitoes, particularly dengue, Chikungunya, West Nile and yellow fever viruses (Moreira et al. 2009, Bian et al. 2010, Walker et al. 2011, Lu et al. 2012, van den Hurk et al.

2012, Hussain et al. 2013). The mechanism underlying these antiviral effects is unknown (Rainey et al. 2014). Recently, Bourtzis et al. (2014) published a concise review of mosquito-*Wolbachia* symbiosis, with an emphasis on the *Aedes albopictus* model. The mechanism underlying *Wolbachia*-mediated pathogen interference is likely strain-specific and unique to each parasitic challenge. The authors suggest that the involved modes of action could be the priming of the mosquito innate immune system and competition for access to key metabolites (limiting the cellular resources that are required for pathogen and symbiont replication).

An inhibitory effect of *Wolbachia towards Plasmodium* infection has been reported in anopheline laboratory models. While exploring its use as a dengue virus-blocking agent, Moreira et al. (2009) tested the effect of this *Wolbachia* endosymbiont in an avian malaria transmission system. The *w*MelPop-CLA *Wolbachia* strain reduced *Plasmodium gallinaceum* infection *in Ae. aegypti*. Because *Anopheles* mosquitoes are not naturally infected with *Wolbachia*, transinfection and *ex vivo* models have been tested to determine if *Wolbachia* can establish an infection and replicate within human malaria vectors (Jin et al. 2009, Kambris et al. 2010, Hughes et al. 2011b, 2012). The virulent *Wolbachia* strain *w*MelPop can survive and replicate when injected into female *An. gambiae* adults to establish a somatic avirulent infection (Jin et al. 2009). When *Wolbachia* (*w*MelPop and *w*AlbB strains)-infected *An. gambiae* mosquitoes were challenged with *P. falciparum*, a significant reduction in *P. falciparum* oocyst levels was detected. This somatic bacterial infection disseminated widely throughout the mosquito, reaching the fat body, head, sensory organs and other tissues, with the notable exception of the gut and ovaries (Hughes et al. 2011b).

These results have increased interest in viable techniques for establishing stable transinfections in major human malaria vectors. Although not fully understood, this *Wolbachia*-infected *Anopheles* line possesses a reduced ability to maintain *Plasmodium* transmission due to multiple strain-dependent mechanisms (Hughes et al. 2011a). A *Wolbachia*-based approach would represent a new vector control option to target dominant malaria vectors, even though human malaria is transmitted globally by multiple, highly specialised and sometimes sympatric species.

Recently, Bian et al. (2013) described the stable establishment of *Wolbachia* infection in laboratory-reared *An. stephensi*. The *Wolbachia* *w*AlbB strain displayed maternal transmission and high levels of cytoplasmic incompatibility. Furthermore, the seeding of infected female *An. stephensi* into a population of uninfected mosquitoes resulted in *Wolbachia* invasion into the uninfected population. In agreement with previous observations of refractoriness towards malaria transmission, the stable infection of *w*Alb *Wolbachia* conferred *An. stephensi* resistance to *P. falciparum* infection. The immense potential of this development could aid in the gradual reduction of vectorial capacity amongst major anopheline vectors. Nevertheless, it remains unknown if *Plasmodium* parasites will develop resistance towards the inhibitory mechanisms triggered by the *Wolbachia*-mosquito association.

The findings of this study and the *Wolbachia*-mediated strategy were recently analysed by four experts in the field (Killeen et al. 2013), all of whom noted that the observed inhibitory effect is most likely too modest to block malaria transmission in the field. However, they consider this approach a promising biological weapon against malaria transmission if it can be established in other anophelines to induce similar infection outcomes.

Following this line of thought, all applicable possibilities should be explored and assessed. The capacity of *Wolbachia* endosymbionts to substantially affect *Plasmodium* infection was also reported by Zelé et al. (2012), who observed that the *Wolbachia pipientis* strains *w*Pip(SI) and *w*Pip(Mc) naturally found in *Culex pipiens* protected the mosquitoes from infection by *Plasmodium relictum* parasites. Interestingly, this research group later attempted to validate this observation in a natural infection model (Zelé et al. 2014). Their aim was to determine if natural *Wolbachia* infections have the same parasite-inhibiting properties observed in laboratory models. Natural partnerships between *Wolbachia* and mosquito species with a long evolutionary history are key to understanding the effects of several generations of co-evolution on *Wolbachia*-transfected mosquitoes. For this purpose, they used an entirely natural mosquito-*Wolbachia-Plasmodium* combination. In contrast to most previous studies centred on the quantification of the midgut stages of *Plasmodium*, they measured a parasitaemia value that related directly to transmission potential by following infections to the salivary gland stages. They found that the presence of *Wolbachia* (of any density inside the mosquito) increased the susceptibility of *Cx. pipiens* mosquitoes to *P. relictum*, significantly increasing the prevalence of salivary gland-stage infections. Strikingly, these results suggest that mosquitoes that have been naturally infected with *Wolbachia* may be better malaria vectors than *Wolbachia*-free mosquitoes.

The anopheline (dynamic) core microbiota - Our knowledge of the anopheline internal ecosystem indicates that there is no natural obligate endosymbiont in the *Anopheles* genus, in contrast to what has been observed in other haematophagous insects such as the tsetse fly (*Wigglesworthia* spp) (Aksoy 1995), head louse (*Riesia pediculicola*) (Kirkness et al. 2010), *Ae. albopictus-Wolbachia* (*w*albA and *w*AlbB) (Armbruster et al. 2003) and *Aedes polynesiensis-Wolbachia* (*w*PolA) (Andrews et al. 2012). Secondary symbionts may have more recently associated with their insect host and are thus not obligate, although they may fulfill a role in host biology (Minard et al. 2013).

Studies on the microcosmos of anophelines have generated information that can appear to be contradictory, such as the presence (Cirimotich et al. 2011) or absence (Boissière et al. 2012) of *Enterobacter* sp. in field-collected African anophelines or the unexpected absence of *Asaia* in Indo/middle eastern field-collected or laboratory-reared anophelines (Rani et al. 2009, Djadid et al. 2011, Chavshin et al. 2012). Notwithstanding, predominant taxa (at the family and sometimes genus level) have consistently been observed in both laboratory-reared and natural mosquito populations. These observations

suggest (Wang et al. 2012, Gendrin & Christophides 2013) that laboratory colonies may retain their association with the predominant bacterial taxons established prior to laboratory colonisation.

The proposed existence of a "core group" of anopheline gut-associated bacteria mirrors the concept of the bacterial human enterotype (Turnbaugh et al. 2007, Evans et al. 2013); this core group may be critical to the host mosquito both as a metabolic exchanger/enhancer (Russell et al. 2013) and as a key element of its immune response (Nyholm & Graf 2012). The identity of this core microbiota has been referred to in several recent publications (Boissière et al. 2012, Gendrin & Christophides 2013). Wang et al. (2012) determined that at a family taxonomic level, Enterobacteriaceae, Flavobacteriaceae and Pseudomonadaceae encompass 80.9% and 94.3% of the *An. gambiae* microbiota in laboratory-reared and field-collected Kenyan samples after a blood meal, respectively. However, many other bacterial genera or families may be involved; although multiple detection techniques have been utilised, they primarily focused on a single anopheline species and organ. Although *An. gambiae* may be the most important, efficient and anthropophilic malaria vector, it cannot be assumed that the true composition of this "core" consortium can be elucidated using a single species. Furthermore, the patterns of fluctuation in the bacterial genera abundance within a "core family", and among different anopheline species and life stages are unknown and should be explored in more depth to strengthen the hypothesis that the bacterial population is similar/stable among laboratory-reared and wild adult *Anopheles* mosquitoes.

It is enticing to suggest that anopheline mosquitoes harbour their microbiota in a selective manner. If microbiota equilibrium could be reached, bacteria from a single genus could be engineered to serve as a universal paratransgenic vehicle in multiple anopheline species. The results obtained and expertise involved in the Eliminate Dengue project [based upon the interaction of *Ae. aegypti* mosquitoes and a bacterial endosymbiont (*Wolbachia*)] suggests a feasible partnership. Nevertheless, recent observations oppose the existence of a constant inter-species bacterial consortium associated with the insect gut environment (Wong et al. 2013).

If the anopheline holobiont is to be understood and other potential dysbiosis techniques are to be developed in the arms race against the malaria parasite, multiple avenues of research may be required to address current questions. The data generated by Boissière et al. (2012) and Wang et al. (2012) demonstrate that this microbiome is dynamic and that some of its members correlate to particular characteristics of the *An. gambiae* model, such as laboratory rearing and the abundance of the Flavobacteriaceae family and the association of the genus *Enterobacter* with *Plasmodium* infection. Simple questions could be answered by compiling the data from the reported microbiome surveys and mining the relevant text. How many bacterial families or genera have been reported for anophelines to date? Is there an anopheline species with clear data enrichment? Can filtering and building groups reveal an underlying presence/absence pattern? Minard

et al. (2013) generated an index with microbiota data that encompassed multiple mosquito vectors. We undertook the challenge of reviewing this index and complementing it with additional recent and prior reports. The data collection is an ongoing process and may lack genera from other recently published sources (Supplementary data 1).

Filtering and visualising the anopheline microbiota data - The reports included in the databank were selected based on a simple criterion: that they accounted exclusively for bacterial genera in anopheline mosquitoes that were field collected or reared on the continent in which they act as a malaria vector. The listed taxa are accompanied by their respective citation. Key sources were employed as guides to track each of the included genera reports: Abdul-Ghani et al. (2012), Apte-Deshpande et al. (2012), Gendrin and Christophides (2013), Manguin et al. (2013) and Minard et al. (2013). For our purpose, the inclusion criterion excluded important observations of identified taxons in laboratory models such as those by Pumpuni et al. (1996).

By filtering the compiled data regarding bacterial genera in each anopheline species, we identified 109 unique genera among the 218 reports in the literature (Fig. 1).

The anopheline species with the most reported associated bacterial genera was *An. gambiae* (71 genera), followed by the Asian human malaria vector *An. stephensi* (46 genera). These findings are based on multiple detection techniques that range from plate culture to 16S rRNA surveys with multiple platforms. Although malaria transmission is a current burden for developing regions or countries in Latin America, there are only five reported bacterial genera representing two primary Neotropical vectors (*Anopheles darlingi* and *Anopheles albimanus*).

The recent publication of the *An. darlingi* genome (Marinotti et al. 2013) mentions the presence (as contamination or associated) of bacterial reads and genes. The assembly of the *Aeromonas hydrophila* genome amidst the anopheline NGS data should be explored further. Genome sequencing projects present opportunities for anopheline microbiome research that should be care-

fully exploited while fully considering the lack of information available for American malaria vectors.

The species-enriched composition of the compiled data motivated us to develop another strategy to "interrogate" it from a broader scope to identify patterns and a core bacterial assembly across the screened *Anopheles* species. A classification according to continent criterion was applied to the compiled data and the groups obtained represent the bacterial genera identified in the continents of Africa, Asia, Europe and America. The correlation between each continent and bacterial genera was plotted using the online tool Circos: mkweb.bcgsc.ca/tableviewer/visualise/ (Krzywinski et al. 2009). Due to constraints on the table size allowed by the tool, only a subset of the filtered data is shown in Fig. 2. The matrix built after filtering the data is shown in Supplementary data 2. This file is provided only to exemplify the format that Circos requires.

Tables and lists are often difficult to follow and interpret. Visual inspection of the available data permits the elucidation of relationships in a more efficient manner. The circular web plotted (Fig. 2) by "continent filtering" the compiled data not only synthesises the information, but also displays it in a useful biological and geographical sense. In examining circular web, we can ask: is there an interpretable presence/absence pattern? Could we use it to further deepen our knowledge of anopheline bionomics and identify other candidate bacterial genera for paratransgenesis?

As shown in Fig. 2, the paratransgenesis candidates *Serratia* and *Pantoea* are present in malaria-endemic countries in Africa, Asia and America. However, data regarding America remain limited due to the absence of the *Asaia* genus in American mosquito-related literature. Significant efforts and progress have been made in exploring the potential of this genus as a paratransgenesis vehicle and attention should now focus on identifying *Asaia* in Neotropical vectors.

By prospecting the metabolic potential within the genome of the "continent-specific" bacterial genera, we could explore if this potential is linked to specific functional relationships developed throughout mosquito-bacteria co-evolution in ecologically differentiated niches.

To further visualise and quantify the shared and exclusive genera relationships obtained with the "continent" filtering criteria (refer to Supplementary data 1, spreadsheet 2), a Venn diagram was generated (Fig. 3) using the online tool Venny: bioinfogp.cnb.csic.es/tools/venny/ (Oliveros 2007). The distribution of the 109 unique genera reported in the literature was as follows: Africa (82), Asia (58), America (5) and Europe (3) (46.78% exclusive to Africa, 0% exclusive to America, 24.77% exclusive to Asia, 22.01% shared by Africa and Asia, 3.66% shared by America, Africa and Asia, 1.83% shared by Africa, Asia and Europe and 0.91% shared by America, Africa, Asia and Europe).

Five genera are shared among Africa, Asia and America: *Enterobacter*, *Aeromonas*, *Pantoea, Pseudomonas* and *Serratia*. At the family taxonomic level, these core genera represent the Pseudomonadaceae, Aeromonadaceae and Enterobacteriaceae families. Wang et
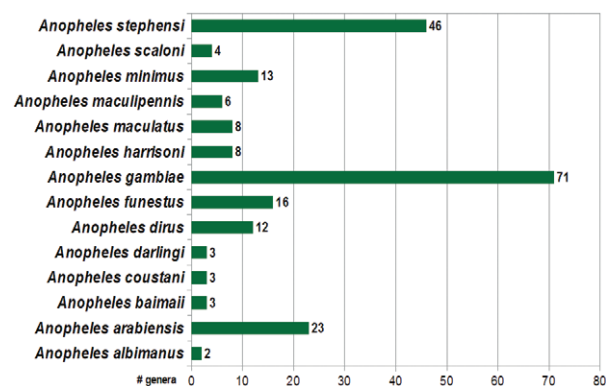

Fig. 1: number of bacterial genera reported per anopheline human malaria vectors. Current count on bacterial genera reported for multiple anopheline species according to our inclusion criterion. A total of 109 unique genera were identified amongst 218 published reports.
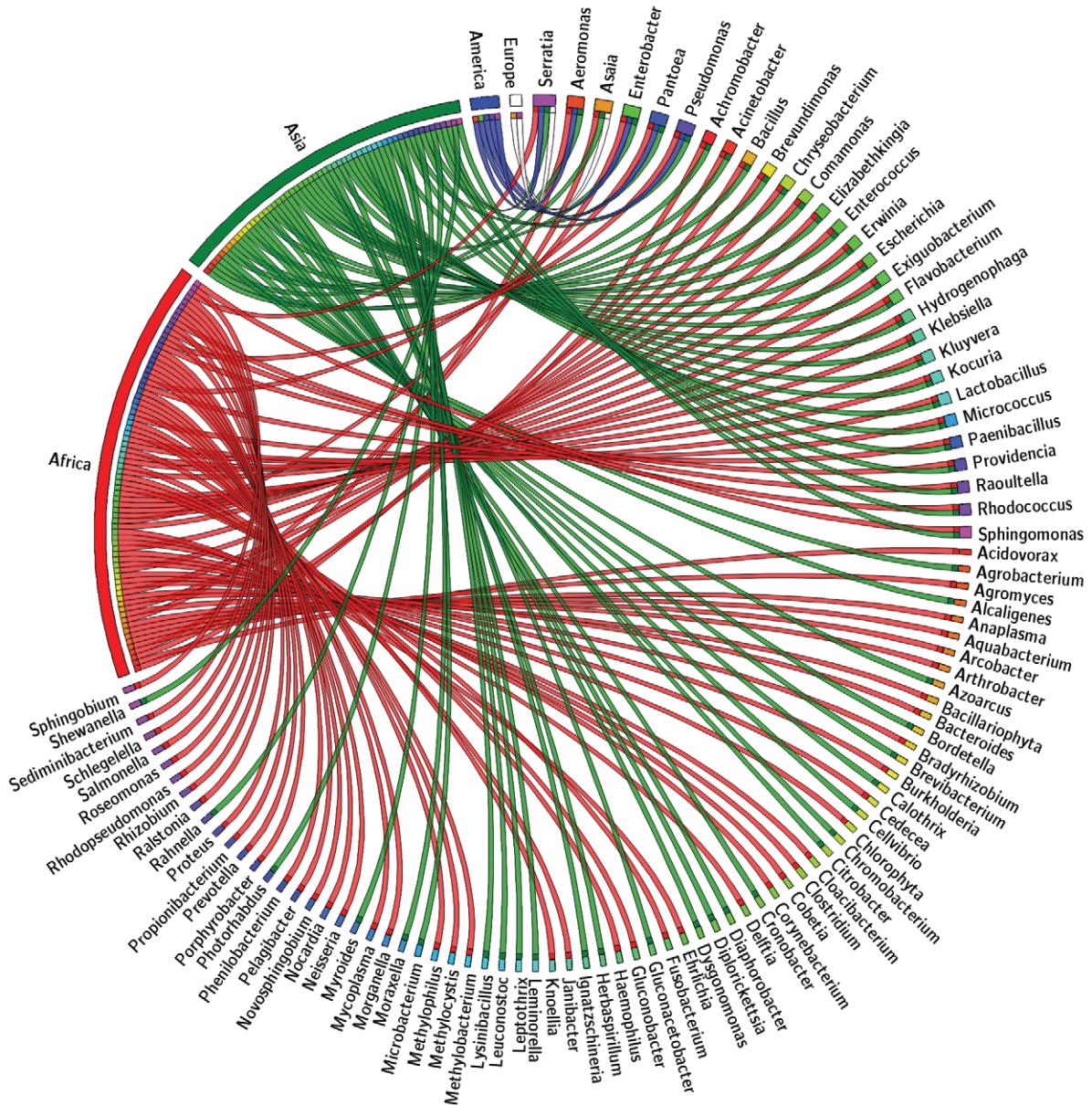
Fig. 2: *Anopheles*-associated microbiota distributed by continent. Circular web plotted from a subset of the anopheline microbiota databank filtered in order to show the correspondence between bacterial genus and its anopheline host according to the continent in which the mosquito exerts vectorial activity. The upper right quadrant contains the bacterial genera shared by at least two continents. The bacterial genera exclusive to each continent are not fully shown due to table size constrain on the online tool Circos (mkweb.bcgsc.ca/tableviewer/visualize/). By prospecting the metabolic potential within the genome of the "continent-specific" bacterial genera, we could explore if it is linked to specific functional relations built throughout mosquito-bacteria co-evolution in ecologically differentiated niches. To further depict and quantify the shared and exclusive genera relations obtained with the "continent" filtering criteria, a Venn diagram was generated (Fig. 3) with the online tool Venny: bioinfogp.cnb. csic.es/tools/venny/ (Oliveros 2007). The distribution of the 109 unique genera reported in the literature is the following: Africa (82), Asia (58), America (5), Europe (3), being 46.78% exclusive to Africa, 0% exclusive to America, 24.77% exclusive to Asia, 22.01% shared by Africa and Asia, 3.66% shared by America, Africa and Asia, 1.83% shared by Africa, Asia and Europe and 0.91% shared by America, Africa, Asia and Europe.

al. (2012) suggested a stable association between laboratory and field-collected *An. gambiae* and Enterobacteriaceae, Flavobacteriaceae and Pseudomonadaceae. Thus, these families may be present despite geographical barriers. However, the lack of data for Neotropical vectors could reduce this list of core bacterial families and, concomitantly, our understanding of this consortium and its impact on vector ecology.

Due to the skewed composition of the current data available regarding anopheline microbiota, we considered this broad filtering criterion to be suitable. Although it may deviate from the true nature of the anopheline-microbiome correlation, this exercise was primarily intended to draw the research community's attention to the construction of an online public repository of such information. This task is both necessary and attainable.
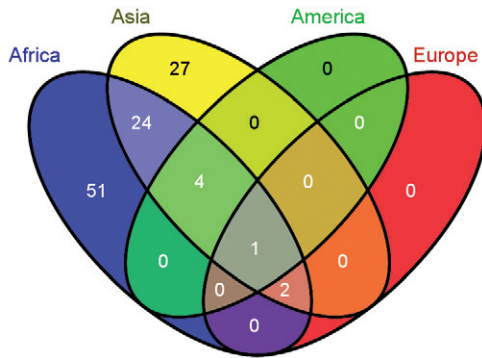
Fig. 3: shared and exclusive bacterial genera identified in anophelines by continent. Using a continent-oriented filtering criterion, the 109 unique bacterial genera reported to date in multiple anophelines distribute as follows: Africa (82), Asia (58), America (5), Europe (3), being 46.78% exclusive to Africa, 0% exclusive to America, 24.77% exclusive to Asia, 22.01% shared by Africa and Asia, 3.66% shared by America, Africa and Asia, 1.83% shared by Africa, Asia and Europe and 0.91% shared by America, Africa, Asia and Europe. Venn diagram generated with the online tool Venny (bioinfogp.cnb.csic.es/tools/venny/).

Understanding the microbial ecology within insect vectors is feasible as long as reliable and formatted data are made available to the research community.

As multiple anopheline metagenomic profiles become available, network association approaches to evaluate taxa co-occurrence and co-exclusion in microbial consortia across anopheline species, tissues or considering biogeographical parameters could be performed, as has been performed for the human microbiome (Faust et al. 2012, Friedman & Alm 2012).

Substantial advancements in vector (micro) biology have been made in recent years, many of which have been attained using "synergic" approaches in which computational science is a key element. The advent of high-throughput, meta-omic techniques ranging from metagenomics to metabolomics has exposed the large number of genes, transcripts, proteins and metabolites that compose the intricate web formed between the host and its inner ecosystem. Many interesting theories are now being discussed and explored regarding the hologenomic basis of speciation (Rosenberg & Zilber-Rosenberg 2011, Brucker & Bordenstein 2013) and how bacteria and viruses may have shaped and continue to impact the genome architecture and phenotypes of harbouring organisms (Gorski et al. 2003, Crochu et al. 2004, Nikoh et al. 2008, Klasson et al. 2009, Rosario & Breitbart 2011, Ni et al. 2012).

The Brazilian Malaria Network is currently focusing its efforts on establishing a viable malaria infection laboratory model using *Anopheles aquasalis* as the initial target species. Based on its bionomical characteristics and the fact that there are one million bacteria and 10 million viral particles per millilitre of surface seawater (Suttle 2005, Rosario & Breitbart 2011), maximising the NGS sequencing data generated in a genome or transcriptome project becomes an opportunity to explore many of these new avenues. The *An. aquasalis* genome project initiated a series of studies, one of which aims to elucidate and describe the composition of the potentially associated microcosmos. The diversity of this consortium and its impact on the genome during the co-evolution of the host and microbial members are unknown. In particular, we are interested in the extent to which larval development in brackish coastal waters may differentiate *An. aquasalis* from traditionally studied anopheline vectors.

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Combined databank generated by reviewing sources cited by Minard et al. (2013) and other authors cited within the DB as well

Africa (82)	Asia (58)	America (5)	Europa (3)
Achromobacter	*Achromobacter*	*Aeromonas*	*Asaia*
Acidovorax	*Acinetobacter*	*Enterobacter*	*Staphylococcus*
Acinetobacter	*Aeromonas*	*Pantoea*	*Serratia*
Aeromonas	*Agrobacterium*	*Pseudomonas*	
Agromyces	*Alcaligenes*	*Serratia*	
Anaplasma	*Asaia*		
Aquabacterium	*Azoarcus*		
Arcobacter	*Bacillus*		
Arthrobacter	*Bordetella*		
Asaia	*Brevibacterium*		
Bacillariophyta	*Brevundimonas*		
Bacillus	*Calothrix*		
Bacteroides	*Cellvibrio*		
Bradyrhizobium	*Chromobacterium*		
Brevundimonas	*Chryseobacterium*		
Burkholderia	*Citrobacter*		
Cedecea	*Comamonas*		
Chlorophyta	*Cronobacter*		
Chryseobacterium	*Diaphorobacter*		
Cloacibacterium	*Diplorickettsia*		
Clostridium	*Dysgonomonas*		
Cobetia	*Elizabethkingia*		
Comamonas	*Enterobacter*		
Corynebacterium	*Enterococcus*		
Delftia	*Erwinia*		
Ehrlichia	*Escherichia*		
Elizabethkingia	*Exiguobacterium*		
Enterobacter	*Flavobacterium*		
Enterococcus	*Gluconacetobacter*		
Erwinia	*Herbaspirillum*		
Escherichia	*Hydrogenophaga*		
Exiguobacterium	*Ignatzschineria*		
Flavobacterium	*Klebsiella*		
Fusobacterium	*Kluyvera*		
Gluconobacter	*Kocuria*		
Haemophilus	*Lactobacillus*		
Hydrogenophaga	*Leminorella*		
Janibacter	*Leptothrix*		
Klebsiella	*Leuconostoc*		
Kluyvera	*Lysinibacillus*		
Knoellia	*Microbacterium*		
Kocuria	*Micrococcus*		
Lactobacillus	*Moraxella*		
Methylobacterium	*Myroides*		
Methylocystis	*Paenibacillus*		
Methylophilus	*Pantoea*		
Micrococcus	*Photorhabdus*		
Morganella	*Providencia*		
Mycoplasma	*Pseudomonas*		
Neisseria	*Rahnella*		
Nocardia	*Raoultella*		
Novosphingobium	*Rhodococcus*		
Paenibacillus	*Serratia*		

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Africa (82)	Asia (58)	America (5)	Europa (3)
Pantoea	*Shewanella*		
Pelagibacter	*Sphingomonas*		
Phenilobacterium	*Staphylococcus*		
Porphyrobacter	*Stenotrophomonas*		
Prevotella	*Xenorhabdus*		
Propionibacterium			
Proteus			
Providencia			
Pseudomonas			
Ralstonia			
Raoultella			
Rhizobium			
Rhodococcus			
Rhodopseudomonas			
Roseomonas			
Salmonella			
Schlegelella			
Sediminibacterium			
Serratia			
Sphingobium			
Sphingomonas			
Spiroplasma			
Staphylococcus			
Stenotrophomonas			
Streptococcus			
Sulfurospirillum			
Thorsellia			
Vibrio			
Zymobacter			

Bacterial genera per continent				
Genera	255,0,0	0,128,0	0,0,255	255,250,250
Genera	Africa	Asia	America	Europe
Achromobacter	1	1	0	0
Acidovorax	1	0	0	0
Acinetobacter	1	1	0	0
Aeromonas	1	1	1	0
Agrobacterium	0	1	0	0
Agromyces	1	0	0	0
Alcaligenes	0	1	0	0
Anaplasma	1	0	0	0
Aquabacterium	1	0	0	0
Arcobacter	1	0	0	0
Arthrobacter	1	0	0	0
Asaia	1	1	0	1
Azoarcus	0	1	0	0
Bacillariophyta	1	0	0	0
Bacillus	1	1	0	0
Bacteroides	1	0	0	0
Bordetella	0	1	0	0
Bradyrhizobium	1	0	0	0
Brevibacterium	0	1	0	0
Brevundimonas	1	1	0	0
Burkholderia	1	0	0	0
Calothrix	0	1	0	0
Cedecea	1	0	0	0
Cellvibrio	0	1	0	0
Chlorophyta	1	0	0	0
Chromobacterium	0	1	0	0
Chryseobacterium	1	1	0	0
Citrobacter	0	1	0	0
Cloacibacterium	1	0	0	0
Clostridium	1	0	0	0
Cobetia	1	0	0	0
Comamonas	1	1	0	0
Corynebacterium	1	0	0	0
Cronobacter	0	1	0	0
Delftia	1	0	0	0
Diaphorobacter	0	1	0	0
Diplorickettsia	0	1	0	0
Dysgonomonas	0	1	0	0
Ehrlichia	1	0	0	0
Elizabethkingia	1	1	0	0
Enterobacter	1	1	1	0
Enterococcus	1	1	0	0
Erwinia	1	1	0	0
Escherichia	1	1	0	0
Exiguobacterium	1	1	0	0
Flavobacterium	1	1	0	0
Fusobacterium	1	0	0	0
Gluconacetobacter	0	1	0	0
Gluconobacter	1	0	0	0
Haemophilus	1	0	0	0
Herbaspirillum	0	1	0	0
Hydrogenophaga	1	1	0	0
Ignatzschineria	0	1	0	0

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Genera	255,0,0	0,128,0	0,0,255	255,250,250
Genera	Africa	Asia	America	Europe
Janibacter	1	0	0	0
Klebsiella	1	1	0	0
Kluyvera	1	1	0	0
Knoellia	1	0	0	0
Kocuria	1	1	0	0
Lactobacillus	1	1	0	0
Leminorella	0	1	0	0
Leptothrix	0	1	0	0
Leuconostoc	0	1	0	0
Lysinibacillus	0	1	0	0
Methylobacterium	1	0	0	0
Methylocystis	1	0	0	0
Methylophilus	1	0	0	0
Microbacterium	0	1	0	0
Micrococcus	1	1	0	0
Moraxella	0	1	0	0
Morganella	1	0	0	0
Mycoplasma	1	0	0	0
Myroides	0	1	0	0
Neisseria	1	0	0	0
Nocardia	1	0	0	0
Novosphingobium	1	0	0	0
Paenibacillus	1	1	0	0
Pantoea	1	1	1	0
Pelagibacter	1	0	0	0
Phenilobacterium	1	0	0	0
Photorhabdus	0	1	0	0
Porphyrobacter	1	0	0	0
Prevotella	1	0	0	0
Propionibacterium	1	0	0	0
Proteus	1	0	0	0
Providencia	1	1	0	0
Pseudomonas	1	1	1	0
Rahnella	0	1	0	0
Ralstonia	1	0	0	0
Raoultella	1	1	0	0
Rhizobium	1	0	0	0
Rhodococcus	1	1	0	0
Rhodopseudomonas	1	0	0	0
Roseomonas	1	0	0	0
Salmonella	1	0	0	0
Schlegelella	1	0	0	0
Sediminibacterium	1	0	0	0
Serratia	1	1	1	1
Shewanella	0	1	0	0
Sphingobium	1	0	0	0
Sphingomonas	1	1	0	0
Spiroplasma	1	0	0	0
Staphylococcus	1	1	0	1
Stenotrophomonas	1	1	0	0
Streptococcus	1	0	0	0
Sulfurospirillum	1	0	0	0
Thorsellia	1	0	0	0
Vibrio	1	0	0	0
Xenorhabdus	0	1	0	0
Zymobacter	1	0	0	0