ORIGINAL ARTICLE

The dual oxidase gene *BdDuox* regulates the intestinal bacterial community homeostasis of *Bactrocera dorsalis*

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The guts of metazoans are in permanent contact with the microbial realm that includes beneficial symbionts, nonsymbionts, food-borne microbes and life-threatening pathogens. However, little is known concerning how host immunity affects gut bacterial community. Here, we analyze the role of a dual oxidase gene (*BdDuox*) in regulating the intestinal bacterial community homeostasis of the oriental fruit fly *Bactrocera dorsalis*. The results showed that knockdown of *BdDuox* led to an increased bacterial load, and to a decrease in the relative abundance of Enterobacteriaceae and Leuconostocaceae bacterial symbionts in the gut. The resulting dysbiosis, in turn, stimulates an immune response by activating *BdDuox* and promoting reactive oxygen species (ROS) production that regulates the composition and structure of the gut bacterial community to normal status by repressing the overgrowth of minor pathobionts. Our results suggest that *BdDuox* plays a pivotal role in regulating the homeostasis of the gut bacterial community in *B. dorsalis*.

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Introduction

Insects have been shown to harbor an autochthonous bacterial community in the gut. These bacteria are adapted to the host gut environment, permanently colonizing the gut, and often establishing a symbiotic relationship that affects host development (Shin *et al.*, 2011; Storelli *et al.*, 2011), food digestion and energy extraction (Venema, 2010; Tremaroli and Bäckhed, 2012), defense of natural enemies (Teixeira *et al.*, 2008; Dong *et al.*, 2009) and maturation and development of the immune system (Weiss *et al.*, 2011; Broderick *et al.*, 2014). Therefore, gut epithelia must be able to tolerate a certain amount of proliferation of commensal microbes for beneficial gut–microbe interactions to occur while still competently eliminating detrimental microbes. This is not a simple task from an immunological point of view because commensal bacteria, like pathogenic bacteria, produce pathogen-associated molecular

patterns such as peptidoglycan that are reported to induce the gut immune response (Zaidman-Rémy *et al.*, 2006). The molecular mechanisms determining how the gut tolerates symbiotic bacteria without mounting a damaging immune response remain to be elucidated. To date, only few mechanisms have been identified that adjust the immune response in the gut.

The insect gut immune response relies primarily on two types of molecular effectors that act synergistically to restrict the growth and proliferation of invading microorganisms: the production of microbicidal reactive oxygen species (ROS) by dual oxidase (Duox) and production of antimicrobial peptides (AMPs) by the Imd pathway (Buchon *et al.*, 2013; Ruy *et al.*, 2006). The ROS is produced and regulated by Duoxes or nicotinamide adenine dinucleotide phosphate (NADPH) oxidases (Noxes) for various biological and pathological roles. Because of the highly reactive nature of ROS, they have microbicidal effects, and the microbicidal role of ROS in innate immunity has been best illustrated in the microbicidal effects of professional phagocytes (Rada and Leto, 2008). However, the overgeneration of ROS is cytotoxic, leading to the damage of important cellular building blocks, such as DNA, proteins and lipids (Bae *et al.*, 2010). In the human genome, there are five NOX enzymes (NOX1 to NOX5) and two DUOX enzymes (DUOX1 and

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DUOX2). Interestingly, the diverse NOX and DUOX family enzymes are not only expressed in phagocytic cells, but also in various nonphagocytic cells, including mucosal epithelial cells (Ha *et al.*, 2005; Allaoui *et al.*, 2009; Fischer, 2009), indicating their potential biological roles in these cells. In addition, several recent studies have shown that ROS are microbicidal effector molecules in the gut epithelia (El Hassani *et al.*, 2005; Ha *et al.*, 2005, 2009a). In *Drosophila*, there is only one *DuoX* gene and one *Nox* gene in the genome, and genetic analysis has shown that *DuoX*-knockout flies, but not *Nox*-knockout flies, cannot produce infection-induced ROS (Ha *et al.*, 2005). Structural analyses have demonstrated that DUOX consists of a gp91phox homology domain typical of all members of the NOX family responsible for H₂O₂ generation, as well as an EF-hand Ca²⁺ binding pocket and an extracellular peroxidase homology domain (PHD) that is not found in NOX (Bae *et al.*, 2010). The peroxidase homology domain has shown enzyme activity similar to that of myeloperoxidase, enabling the conversion of H₂O₂ to HOCl in the presence of chloride ion. DUOX lacking peroxidase homology domain cannot function normally, suggesting that peroxidase homology domain of DUOX is essential for the host defense system (Ha *et al.*, 2005). Beyond microbicidal effects, DuoX-dependent ROS are involved directly or indirectly in epithelial cell renewal through the activation of intestinal stem cells during gut infection (Buchon *et al.*, 2009). It is thought that stem cell proliferation is induced upon epithelia damage caused by ROS. In addition, DuoX plays important roles in the stabilization of the cuticle structure of *Drosophila* wings (Anh *et al.*, 2011), and DuoX together with peroxidase contributes to the peritrophic matrix of the *Anopheles gambiae* midgut, affecting gut permeability to immune elicitors (Kumar *et al.*, 2010).

The regulation of the intestinal Imd pathway has been extensively studied. With routine microbial burdens, such as those found in the absence of infection, the presence of a commensal-derived peptidoglycan constitutively activates the Imd pathway at a low level of activation. The basal activity of this pathway is maintained at a low level by various negative regulators, including peptidoglycan-recognition protein (PGRP)-LF (Maillet *et al.*, 2008), Pirk (Kleino *et al.*, 2008) and amidase PGRPs (notably PGRP-LB and PGRP-SC1a) (Bischoff *et al.*, 2006; Zaidman-Rémy *et al.*, 2006, 2011). However, acute infection with pathogenic bacteria results in the release of large quantities of peptidoglycan fragments during drastic bacterial division (Buchon *et al.*, 2013) that transiently increases the Imd pathway activity to trigger AMP production. In this model, the Imd pathway can maintain a low level of activation under basal conditions to enable the presence of symbiotic bacteria.

Fruit flies of the Tephritidae family, including the oriental fruit fly *Bactrocera dorsalis* (Diptera: Tephritidae), are important agricultural pests because of their proclivity to oviposit in fruit

(Li *et al.*, 2011). By combining microbial culture-dependent and -independent techniques, we found that adult flies harbored a stable bacterial community in which dominant members belong to Enterobacteriaceae with major species of *Klebsiella*, *Citrobacter*, *Enterobacter* and *Pectobacterium* (Wang *et al.*, 2011). These bacteria are also found in the gut of several other tephritids, such as *Ceratitis* (Behar *et al.*, 2005 and 2008), *Rhagoletis* (Howard *et al.*, 1985), *Dacus* (Drew and Lloyd, 1991), *Anastrepha* (Kuzina *et al.*, 2001), *Tephritis* and *Urophora* (Daser and Brandl, 1992). It has been hypothesized that these bacteria have an indirect contribution to host fitness by preventing the establishment or proliferation of pathogenic bacteria (Behar *et al.*, 2008).

In this study, we investigated the response of *BdDuoX* to opportunistic pathogens and autochthonal bacteria, and analyzed the effect of the *BdDuoX* gene knockdown on the gut bacterial community of *B. dorsalis* at 5, 15 and 20 days post RNA interference (RNAi) by MiSeq Illumina high-throughput sequencing (Shenzhen, China) to investigate the role of the *BdDuoX* gene on the regulation of bacterial community homeostasis of *B. dorsalis*.

Materials and methods

Rearing and dissection of Bactrocera dorsalis

B. dorsalis adults were raised in an insectary at the Institute of Urban and Horticultural Entomology, Huazhong Agricultural University (Wuhan, China) under a 14 h light/10 h dark cycle at 28 °C and 70–80% relative humidity. Adults were fed an artificial diet consisting of 2.5% yeast extract, 7.5% sugar, 2.5% honey, 0.4% agar and 87% H₂O, whereas larvae were raised in bananas (Li *et al.*, 2011). Before dissection, adult samples from different treatments were surface-sterilized with 70% ethanol for 3 min and rinsed three times in sterile distilled water. A number of adults were used for gut samples dissected with sterilized tweezers using a stereomicroscope. The dissected gut samples (from crop to hindgut without Malpighian tubes) were prepared for different experiments.

Total RNA isolation and cDNA synthesis

Total RNA was isolated from *B. dorsalis* at different developmental stages, including eggs, first-instar larvae, second-instar larvae, third-instar larvae, early pupae (48 h after pupation), old pupae (48 h before eclosion), adults before mating (3–4 days after eclosion) and adults after mating (13–15 days after eclosion) using RNAiso Plus reagent (TaKaRa, Otsu, Shiga, Japan). In addition, total RNA was isolated from different organs and tissues such as the head, hemocytes, crop, midgut, hindgut, Malpighian tubule, fat body, ovaries and testes. The experiments were performed in triplicate. The purity of the RNA was analyzed using a NanoDrop 2000

Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 260 nm. The first-strand complementary DNA (cDNA) of each pool was synthesized from 1 µg of total RNA using a two-step cDNA synthesis kit (Takara) with the gDNA eraser to remove residual DNA contamination.

Cloning and sequence analysis of full-length BdDuoX

Full-length *BdDuoX* was cloned using RACE (Rapid Amplification of cDNA Ends)-PCR with a SMARTer RACE cDNA Amplification Kit (Clontech, Mountain View, CA, USA) according to the manufacturer's instructions. Gene-specific primers, *BdDuoX* RACE-F and RACE-R, were designed on the basis of the fragment sequence from transcriptome of *B. dorsalis*. The first-strand cDNA template was synthesized from gut total RNA (1 µg) in the presence of the RACE primer in a standard reverse transcription reaction. Amplified products were inserted into the pMD-18 T vector for sequencing. The transmembrane domains in *BdDuoX* were identified using TMHMM online software (<http://www.cbs.dtu.dk/services/TMHMM-2.0/>), and the structural domains of *BdDuoX* were predicted using the simple modular architecture research tool (SMART; version 7.0) (<http://smart.embl-heidelberg.de/>).

Microbial oral infection

Adult flies (age: 3–4 days) were dehydrated for 10 h without food and then fed an artificial diet supplement with 5% sucrose solution containing concentrated microbe solution ($\sim 5 \times 10^8$ colony-forming units (CFUs) per ml). All of the bacteria used for oral infection were grown as a shaking culture in LB medium at 37 °C, 300 r.p.m. Exponential microbial culture (OD₆₀₀=1.0) was used for all of experiments as previously described (Ha *et al.*, 2005). The culture of 50–100 ml was pelleted by centrifugation (15 min at 3200 g) and adjusted to the concentration. The flies fed with a diet supplement with 5% sucrose only served as a control. For analyses of *BdDuoX* gene expression and ROS level changes after oral infection, the gut samples of different treatments were collected at different time points post oral infection (POI). The microorganisms used in this study were opportunistic pathogens *Escherichia coli* DH5α and *Staphylococcus aureus*, dominant members of gut bacteria *Citrobacter koseri*, *Enterobacter asburiae*, *Enterococcus faecalis*, *Enterobacter hormaechei*, *Klebsiella oxytoca* and minor gut bacteria *Bacillus cereus*. *S. aureus* was purchased from the American Type Culture Collection (ATCC25923, Rockville, MD, USA); *E. coli* was from the Institute of Urban and Horticultural Entomology; *B. cereus*, *C. koseri*, *E. asburiae*, *E. faecalis*, *E. hormaechei* and *K. oxytoca* were cultivable bacteria isolated from the gut of *B. dorsalis*.

Real-time quantitative PCR analysis

In all cases of gene expression analysis, three independent cohorts of ~14 flies each were collected for RNA extraction and cDNA synthesis. Real-time PCR was performed using a Bio-Rad IQ2 system (Bio-Rad, Hercules, CA, USA) and a 96-well plate. Each PCR mixture consisted of 10 µl of SYBR Green Mix (Bio-Rad), 100 nm of each primer and 2 µl of cDNA (diluted 1:10). The amplification program was preincubation at 95 °C for 30 s, followed by 45 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 30 s. Melting curve analysis was performed at the end of each amplification run to confirm the presence of a single peak. The thermocycler conditions for the melting curve analysis were 55 °C for 60 s, followed by 81 cycles starting at 55 °C for 10 s with a 0.5 °C increase each cycle. All of the samples were analyzed in triplicate, and the levels of the detected mRNA determined by cycling threshold analysis were normalized using *RpL32* as the control. The primer pairs used in quantitative PCR analysis are as follows: *BdDuoX* (forward (F) 5'-GACCACCACGTTTCTGGATG-3' and reverse (R) 5'-TAACATCGGAAGCAGCAGA-3'); a NADPH oxidase gene (*BdNox*) (GenBank accession number: XM_011203950) (F 5'-ACCTGTCCGCGTTGTCATTT-3' and R 5'-AATG;AGCGCTGATCACGGTT-3'); *RpL32* (F 5'-CCCGTCATATGCTGCCAACT-3' and R 5'-GC GCGCTCAACAATTTCTCT-3'). The relative gene expression data were analyzed using the $2^{-\Delta\Delta C_T}$ method as described before (Livak and Schmittgen, 2001). The target gene expression is presented as the relative expression levels after normalization.

The loads of total bacteria were quantified by real-time PCR using the 16S RNA gene-specific primers (Guo *et al.*, 2008) and normalized by real-time PCR data for the host β-actin gene according to the method as described previously (Petnicki-Ocwieja *et al.*, 2009). Primer pairs used in quantitative PCR analysis are as follows: 16 S rDNA gene primers (F 5'-ACTCCTACGGGAGGCAGCAG-3' and R 5'-AT TACCGCGGCTGCTGG-3') and β-actin (F 5'-TCGATC ATGAAGTGGGATGT-3' and R 5'-ATCAGCAATAC CGGGGTACA-3'). Real-time quantitative PCR was carried out in a volume of 20 µl. Each PCR mixture consisted of 10 µl of SYBR Green Mix (Bio-Rad), 100 nm of each primer and 5 ng of DNA. The amplification program consisted of (1) preincubation at 50 °C for 2 min and 95 °C for 10 min; (2) 45 cycles of denaturation at 95 °C for 15 s and annealing at 60 °C for 1 min; and (3) one cycle at 95 °C for 15 s, 53 °C for 15 s and 95 °C for 15 s.

Measurement of total in vivo ROS

The intestines of individual adult flies were rapidly hand dissected in phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄, 10 mM, at pH 7.4) containing aminotriazol (2 mg ml⁻¹, Sigma-Aldrich, St Louis, MO, USA). The dissected intestines were cut into small pieces

(~2 mm) and pooled in 50 μ l of phosphate-buffered saline containing aminotriazol (2 mg ml⁻¹). Three independent cohorts of six intestines were used for each measurement. The samples were centrifuged for 5 min at 3000 g, the resultant supernatant was further used for the colorimetric quantitative determination of diffused ROS. We used the common probe 2',7'-dichlorofluorescein-diacetate to detect ROS after a 30-min incubation of the diluted resultant supernatant (190 μ l) with the working solution (10 μ l) in the presence of 1 mM 2',7'-dichlorofluorescein-diacetate fluorescent dye (Sigma-Aldrich) in the dark at 37 °C, and measured with a Tecan Infinite M200 (Männedorf, Switzerland) using excitation at 485 nm and emission at 535 nm. The fluorescence intensity was corrected for the negative control without fluorescent dye.

dsRNA synthesis and delivery by injection

The target sequence fragments were amplified from the *BdDuoX* gene of *B. dorsalis* by nested PCR using template-specific primers conjugated with the T7 RNA polymerase promoter (F 5'-GGATCCTAATACGACTCACTATAGGATCGTCCGTCTACTCGTC-3' and R 5'-GGATCCTAATACGACTCACTATAGGCTGGGCTAAGCATCTTCATC-3'). PCR product at 1 μ g was used as the template for double-stranded RNA (dsRNA) synthesis using the T7 Ribomax Express RNAi System (Promega, Madison, WI, USA) according to the manufacturer's protocol. dsRNA was ethanol precipitated overnight, resuspended in RNase-free injection buffer (5 mM KCl, 0.1 mM sodium phosphate, pH 6.8) and quantitated at 260 nm using a NanoDrop 2000 Spectrophotometer (Thermo Fisher Scientific Inc.) before microinjection. The quality and integrity of dsRNA were determined by agarose gel electrophoresis. Needles were prepared with a puller at 60.8 °C (PC-10, Narishige, Tokyo, Japan). Microinjection was performed using an Eppendorf micromanipulation system (Microinjector for cell biology, FemtoJet 5247, Hamburg, Germany). The injection condition was set to a Pi of 300 hpa and a Ti of 0.3 s. Gene silencing experiments were performed injecting 1 μ l of a 2 μ g μ l⁻¹ solution of dsRNA into the ventral abdomen of each fly (3–4 days old).

Isolation of bacterial DNA from the gut and high-throughput sequencing

Total bacterial DNA from the intestines (dissected under sterile conditions) of 15 individuals per treatment was extracted using an E.Z.N.A. Soil DNA kit (Omega, Norcross, GA, USA) according to the manufacturer's instructions. DNA from the gut was isolated by treating the homogenate gut with 1 ml of buffer SLX Mlus and 100 μ l of buffer DS at 70 °C for 10 min. DNA was precipitated using a high-salt/ethanol precipitation method and was washed extensively with 70% ethanol. We amplified the V4

region (252 bp) of the bacterial 16S rDNA gene to assess the microbial diversity of the flies. The following primers were used for the PCR reaction: 515 F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806 R (5'-GGACTACHVGGGTWTCTAAT-3') (Caporaso *et al.*, 2012). PCR was performed in triplicate for each sample in a total reaction volume of 20 μ l. The PCR products were checked using 2% agarose gel electrophoresis, purified using the Axy-Prep DNA gel extraction kit and quantified using the fluorescence quantitation (Qubit 2.0 Fluorometer) and Qubit dsDNA HS assay kit (Invitrogen, Carlsbad, CA, USA). A total of 21 gut samples were subjected to high-throughput sequencing, including 3 gut samples of 3–4-day-old flies with no treatment before RNAi, and 18 gut samples of the *ds-egfp*-treated and *ds-duox*-treated flies from 5, 15 and 20 days post RNAi (DPR). The purified amplicons were sequenced on an Illumina MiSeq PE250 sequencer at the BGI (Beijing Genomics Institute, Shenzhen, China). The sequences have been deposited in the National Center for Biotechnology Information (NCBI) database with accession numbers from SAMN03443343 to SAMN03443363.

Data analysis was performed with Quantitative Insights Into Microbial Ecology (QIIME) (Caporaso *et al.*, 2010). We obtained a clean data of sequence reads by pre-processing the removal of the primer sequence; truncation of sequence reads not having an average quality of 20 over a 30-bp sliding window based on the phred algorithm; and removal of trimmed reads having <75% of their original length, as well as its paired read (Fadrosh *et al.*, 2014). These stringent criteria resulted in nearly 94% of the reads being retained. The FLASH, a fast computational tool to extend the length of short reads by overlapping paired-end reads for genome assemblies, was used (Magoč and Salzberg, 2011). For quality control purposes, no mismatches were allowed in the primer or barcode regions. Furthermore, tags with ambiguous bases (N) and screened potential chimeras were removed (Edgar *et al.*, 2011). To focus the analysis on bacterial taxa, nonchimeric sequences were mapped into operational taxonomic units by USEARCH (Edgar *et al.*, 2011), and 97% of operational taxonomic units were picked using a closed-reference operational taxonomic unit picking protocol against Ribosomal Data Project II database (Maidak *et al.*, 2001). Reads that did not match a reference sequence at >97% identity were discarded. Richness and diversity indices (Observed species, Chao, abundance-based coverage estimator (ACE), Shannon) and dissimilarity matrices (Bray–Curtis and weighted UniFrac) were estimated using the software package called mothur (Schloss *et al.*, 2009).

Vitamin C and paraquat feeding

B. dorsalis adult flies (age: 3–4 days) were fed with antioxidant, vitamin C (Sigma-Aldrich), that could

reduce ROS in the intestine (Molina-Cruz *et al.*, 2008) or with the ROS-producing compound paraquat (Sigma-Aldrich) (Buchon *et al.*, 2009) at various concentrations. The gut bacteria population and level of ROS were investigated at 24 h after the flies ingested vitamin C and paraquat. The gut bacterial population was investigated using both CFU counting (cultivable bacteria) and a culture-independent method (16S rRNA quantification by real-time PCR). Approximately 10 adults were used for guts dissected under sterile conditions, and the dissected gut samples (from crop to hindgut without Malpighian tubes) were transferred to a tube containing 1 ml of sterile phosphate-buffered saline and were then homogenized. A 100 μ l aliquot from these samples was further serially diluted $10\times$ to 10^{-9} and plated onto LB agar. Samples were incubated at 30 °C for 24–48 h, and the cultivable bacterial load was quantified by measuring CFUs in the dissected guts with different treatments. The bacteria 16S rRNA quantification by real-time PCR and ROS level detection were performed according to the method described above.

Statistical analysis

Results are shown as the average $\pm$ s.e.m. of three independent biological samples. Comparisons between the means of two independent groups were performed with Student's *t*-test, and multiple comparisons were carried out with one-way analysis of variance and Duncan's test using SPSS 20 (IBM Corporation, Armonk, NY, USA). Significance was considered when $P < 0.05$. The graphs were made using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA) or Microsoft Excel (Microsoft, Redmond, WA, USA).

Results

Sequence analysis and expression profiles of *BdDuox*

We cloned the *BdDuox* cDNA and found it to be 5.9 kb long and encoding for a 1528 amino acid length protein (Supplementary Figure S1). The cDNA sequence was deposited with GenBank (accession number KP824741). The protein contained seven transmembrane regions (Supplementary Figure S2) and binding domains for flavin adenine dinucleotide and NAD that are known to be functionally important in various electron transport systems (Bae *et al.*, 2010). These domains were found to be common in both Duox and Nox proteins. In addition to these domains, *BdDuox* harbors a peroxidase domain that is distinct from the Nox family (Brown and Griendling, 2009), a ferric-reductase domain and an N-terminal calcium binding domain containing three EF-hand motifs (Supplementary Figure S2), also found in Duox/Nox of many species. The structure of *BdDuox* is similar

to the previously characterized Duox of *Drosophila*, suggesting that it may have the same function.

The *BdDuox* gene was highly expressed in the third-instar larvae and adults and was weakly expressed in eggs, first- and second-instar larvae and pupae (Supplementary Figure S3a). The *BdDuox* gene was highly expressed in the head, hemocytes and ovaries, and low levels of expression were detected in the midgut, hindgut, Malpighian tubule, testis and fat bodies (Supplementary Figure S3b). Based on these results we suggest that *BdDuox* has an important function in the development of *B. dorsalis*.

The *BdDuox* gene is induced upon infection

To investigate the role of *BdDuox* in the mucosal response, we monitored *BdDuox* gene expression in a gut upon oral infection with the Gram-negative bacteria, *E. coli* DH5 α , and Gram-positive bacteria, *S. aureus*. The results showed that *E. coli* induced a 3.51-fold increase in *BdDuox* gene expression, with a peak at 6 h POI (Figure 1a). Consistent with this observation, an increased level of ROS was observed at 6 and 12 h POI (Figure 1b). Similar results were obtained when *S. aureus* was used to infect *B. dorsalis* (Figure 1). However, there was no significant change in expression of *BdNox*, another gene of NADPH oxidase family, upon oral infection with *E. coli* and *S. aureus* at 6 h POI (Supplementary Figure S4).

BdDuox is not induced upon infection with dominant members of the bacterial community

We next examined the profile of *BdDuox* expression and ROS production in the gut upon oral exposure to bacteria that are known to reside in the gut of *B. dorsalis*. Flies were fed a diet supplemented with either one of the five dominant bacteria (*C. koseri*, *E. asburiae*, *E. faecalis*, *E. hormaechei* and *K. oxytoca*) or with a minor bacteria (*B. cereus*) intestinal bacteria species. Strikingly, only *B. cereus* could significantly induce the *BdDuox* gene expression and increase ROS levels at 6 h POI (Figure 2). In contrast, there was no increase in *BdDuox* gene expression and the ROS production level when flies were fed with one of the five major symbiotic gut bacteria at the tested concentrations (Figure 2). Similar results were obtained in the experiment that fed intestinal autochthonous bacteria in flies after antibiotics treatment that only *B. cereus* could significantly induce the *BdDuox* gene expression and increase ROS levels (Supplementary Figure S5).

BdDuox regulates the gut bacterial density

Based on the above results of Figures 1 and 2, we conclude that *BdDuox* gene is activated differentially with opportunistic pathogens and autochthonal bacteria. Next, we tested the change in composition

Figure 1 The response of the *BdDuoX* gene in the gut during oral infection. (a) Expression levels of *BdDuoX* at different time points in whole guts (without Malpighian tubules) after oral infection with *E. coli* and *S. aureus*. (b) The total intestinal ROS levels were quantified with flies at different time points after oral infection. Data are representative of three independent experiments (mean±s.e.m.). Statistical comparison was based on Student's *t*-test (* $P < 0.05$, ** $P < 0.01$). Different letters indicate a significant difference in *BdDuoX* expression and ROS levels among the oral infection with *E. coli* or *S. aureus* ($P < 0.05$).

of different autochthonal bacteria to the silencing of the *BdDuoX* gene by injecting flies with *ds-duox* and monitoring the changes in the gut bacterial community. Before testing, we confirmed that the *ds-duox* treatment did not have off-target effects to *BdNox* gene (Supplementary Figure S6). We found that the level of *BdDuoX* gene transcript was inhibited during 1–7 DPR treatment, and the reduction of *BdDuoX* gene expression varied from 26% to 54% as compared with the *ds-egfp* control (Figure 3a). Surprisingly, the *BdDuoX* expression level was higher in *ds-duox*-treated flies from 9 to 17 DPR and then returned to the basal expression level at 20 DPR (Figure 3a). Consistent with *BdDuoX* gene expression changed, the level of ROS followed the same pattern with a decrease of 31–44% compared with *ds-egfp* controls at 1–7 DPR, an increase of 45–73% during 13–17 DPR and a return to the normal level after 20 DPR (Figure 3b). Interestingly, analysis of the overall bacteria densities by real-time quantitative PCR showed that *BdDuoX*-RNAi flies had more bacteria at 5 and 15 DPR compared with controls. Then, the bacteria load returned to the wild-type level at 20 DPR (Figure 3c). We also found that the silencing of *BdDuoX* gene blocked *BdDuoX* and ROS induction upon oral

infection with *E. coli*, suggesting that oral inoculation of pathobionts following RNAi could not quench the effects of *BdDuoX* knockdown (Supplementary Figure S7).

We conclude that the knockdown of the *BdDuoX* gene by RNAi reduced the ROS levels, resulting in an increase in the number of intestinal bacteria. We attribute the higher level of *BdDuoX* gene expression and ROS levels observed in the second phase to an increase in *BdDuoX* gene expression in response to an increase in bacteria at a time when RNAi was no longer effective. The observation that *BdDuoX* activity is regulated by bacterial load is suggestive of a key role of *BdDuoX* in the controls of load of bacterial community.

BdDuoX regulates the composition and structure of the gut microbial community

The experiment above indicates that the bacterial community density is regulated by *BdDuoX*, but it remains to be determined whether *BdDuoX* affects the gut bacterial community composition. We next investigated the bacterial composition in *ds-egfp*-treated and *ds-duox*-treated flies by MiSeq Illumina High-throughput sequencing. The results showed

Figure 2 Response of the *BdDuoX* gene to intestinal autochthonous bacteria. (a) Expression levels of the *BdDuoX* gene in flies 6 h after feeding a diet supplemented with autochthonous bacteria. (b) The total intestinal ROS levels in flies 6 h after feeding a diet supplemented with autochthonous bacteria. Values in (a) and (b) are the mean $\pm$ s.e.m. of three independent experiments. Statistical comparison was based on Student's *t*-test (* P < 0.05, ** P < 0.01). Different letters indicate a significant difference in *BdDuoX* expression and ROS levels among the oral infection with autochthonous bacteria (P < 0.05).

that the rarefaction curves tended toward saturation (Supplementary Figure S8), suggesting that all bacterial libraries from our samples represented the bacterial communities well. Analysis of the control *egfp-RNAi* samples confirmed previous observations that the indigenous bacterial community composition is of medium complexity and that the major members of the gut community of *B. dorsalis* were Enterobacteriaceae and Enterococcaceae, whereas the minor member was Bacillaceae.

Differences between the bacterial community of control and *BdDuoX-RNAi* samples were calculated using the UniFrac metrics that measures phylogenetic dissimilarities between microbial communities (Lozupone and Knight, 2005). Principal coordinate analysis based on UniFrac metrics showed a separation of *ds-duox*- and *ds-egfp*-treated samples along the first two axes that explained 68.38% and 12.71% of the data variation, respectively (Supplementary Figure S9). Interestingly, there was a significant variation in the composition of the gut microbial community upon *ds-duox* treatment (Figure 4a). At 5

Figure 3 Interference effects of RNAi of the *BdDuoX* gene. (a) The expression level of the *BdDuoX* gene at different time points after injecting *ds-duox* and *ds-egfp*. (b) The total intestinal ROS levels at different time points. (c) The total gut bacterial load at different time points. Data are representative of three independent experiments (mean $\pm$ s.e.m.). Statistical comparison was based on Student's *t*-test (* P < 0.05, ** P < 0.01).

DPR, *BdDuoX-RNAi* flies had a significantly lower relative abundance of Enterobacteriaceae and Leuconostocaceae than controls, with a decrease of 10.29% and 83.40%, respectively (Figure 4b). However, the relative abundance of others increased 2.79 times in the *BdDuoX-RNAi* flies than in the control flies. Among them, the relative abundance of Bacteroidaceae and Verrucomicrobiaceae that was too low to be detected in the control flies increased to detectable levels in the *BdDuoX-RNAi* flies. Furthermore, the relative abundance of Bacillaceae also increased in the *BdDuoX-RNAi* flies (Figure 4b). This indicates that flies with reduced *BdDuoX* gene expression display a profound difference in bacterial community composition, perhaps because of differences in the resistance of bacteria to ROS killing activity. At 15 DPR, we also noticed that the abundance of Bacillaceae, a minor bacteria, in

Figure 4 *BdDuoX* gene regulates the composition and structure of gut bacterial community. (a) Taxonomic breakdown at the family level grouped by *ds-egfp* and *ds-duox* treatments. (b–d) Relative abundance of different bacterial taxa after injecting *ds-duox* and *ds-egfp* at 5, 15 and 20 DPR. Data are representative of three independent experiments (mean+s.e.m.). Statistical comparison was based on Student's *t*-test (**P*<0.05). ND, not detected.

BdDuoX-RNAi flies increased significantly by two-fold compared with the controls. The relative abundance of Bacteroidaceae and Verrucomicrobiaceae was similar at 15 DPR to that observed at 5 DPR (Figure 4c). Finally, we observed a return to wild-type bacteria taxa composition in *ds-duox*-treated flies at 20 DPR, with the exception of Bacteroidaceae that remained high in *ds-duox*-treated flies (Figure 4d). Taken together, these results indicate that the *BdDuoX* gene has a pivotal role in regulating the structure of bacterial community in *B. dorsalis*. It also shows a high level of resilience, as the bacterial community composition returned to normal at 20 DPR.

Microbial community richness was altered by *BdDuoX* RNAi

We next investigated the impact of *BdDuoX* gene silencing on microbial diversity using a standardized approaches that assess community richness. At 5 DPR, the Observed species (Sobs) (56.33 ± 0.88), Chao index (73.38 ± 1.05), ACE index (78.70 ± 5.72) and Shannon index (1.64 ± 0.06) of the gut microbes in *BdDuoX-RNAi* flies were not different from the Sobs (58.67 ± 3.71), Chao index (66.53 ± 3.56), ACE index (73.66 ± 6.84) and Shannon index (1.67 ± 0.13) in the control flies (Figure 5a), whereas all of the richness metrics of *BdDuoX-RNAi* flies significantly increased at 15 DPR. In control flies, 46 observed species were identified, and this number increased (23.91%) to 57 in *BdDuoX-RNAi* flies (Figure 5b). The Chao, ACE and Shannon indices were 37.93%, 46.95% and 9.44% higher, respectively, in the *BdDuoX-RNAi* flies than in the control flies at 15 DPR (Figure 5b). Finally, there was no significant difference in the Sobs (59.33 ± 1.76 for *BdDuoX-RNAi* vs 57.67 ± 0.88 for controls), Chao index (70.03 ± 2.06 vs 69.74 ± 6.19), ACE index (77.76 ± 1.34 vs 70.27 ± 2.45) and Shannon index (1.77 ± 0.07 vs 1.69 ± 0.02) between *BdDuoX-RNAi* flies and controls at 20 DPR (Figure 5c). These results suggest that silencing of *BdDuoX* gene by RNAi has an increased bacterial community diversity compared with the controls, possibly because of the reduced ROS production.

The level of ROS regulates the density of gut bacterial community

The findings above point to a role of ROS in controlling the density and composition of gut bacterial community of *B. dorsalis*. To further confirm that *BdDuoX*-dependent ROS generation regulates the bacterial community in *B. dorsalis*, we fed flies an antioxidant vitamin C or ROS-producing compound paraquat and then monitored the bacterial load of the whole gut. The gut bacterial population was investigated using both CFU counting (cultivable bacteria) and a culture-independent method (16S rRNA quantification by real-time PCR). At 24 h after flies ingested vitamin C and paraquat

Figure 5 Effects of RNAi of the *BdDuoX* gene on diversity metrics. Richness measured as observed operational taxonomic units (OTUs; Sobs), Chao, ACE and Shannon indices of gut bacterial communities from different treatment at three time points. (a) 5 DPR. (b) 15 DPR. (c) 20 DPR. Data are representative of three independent experiments (mean+s.e.m.). Statistical comparison was based on Student's *t*-test (**P* < 0.05, ***P* < 0.01).

with the concentration of 100 mg ml^{-1} and 10 mM , respectively, the level of ROS in the intestine decreased by ~50% and increased twofold of the control, respectively (Figure 6a). Interestingly, the vitamin C treatment induced a fivefold increase in the load of cultivable bacteria, whereas the paraquat treatment induced a 70% decrease of the controls (Figures 6a and b). We next repeated this experiment using various concentrations of vitamin C. Figures 6c and d show that ingestion of a higher dose of vitamin C decreased ROS levels in a dose-dependent manner and increased the bacteria load, reaching threefold at the maximum concentration of 200 mg ml^{-1} . We performed the reverse experiment by monitoring

Figure 6 The level of ROS regulates the density of gut bacterial community. (a, b) The cultivable bacteria loads (CK, control; PQ, paraquat; VC, vitamin C) and ROS levels were detected in the guts of flies fed with antioxidant vitamin C and ROS generator paraquat after 24 h. (c–f) ROS levels and the total bacterial load in the guts of flies fed with different concentrations of vitamin C (c, d) and paraquat (e, f). Data are representative of three independent experiments (mean±s.e.m.). Statistical comparison was based on Student's *t*-test (* P <0.05, ** P <0.01, *** P <0.001). Different letters indicate a significant difference in ROS levels or CFU numbers among the vitamin C or paraquat treatments (P <0.05).

the bacterial loads in flies fed with various quantity of ROS generator, paraquat. Figure 6e confirmed that the level of ROS increased markedly in a dose-dependent manner after flies ingested paraquat. Interestingly, we observed a decrease in the bacterial loads at high doses of paraquat. The bacteria load in paraquat-treated flies was reduced to ~10% of the control levels at the maximum concentration of 40 mM (Figure 6f). These results show that the level of ROS regulates the density of gut bacteria loads in a dose-dependent manner.

Discussion

It is a delicate task for the host to mount potent gut immune responses to combat infectious microbes while maintaining the proper number of symbiotic microorganisms needed for healthy gut–microbe interactions (Ha *et al.*, 2009b; Bae *et al.*, 2010). In contrast to the systemic response including melanization, coagulation, phagocytosis and AMP production that ensures the sterility of the body cavity and hemolymph (Buchon *et al.*, 2013), the intestinal immune responses must tolerate the presence of the gut microbiota and dietary microorganisms while responding to and eliminating potential pathogens. Previous studies done in *Drosophila* have suggested that the fly's gut immune response relies mainly on two types of molecular effectors that act synergistically to restrict the growth and proliferation of invading microorganisms: the AMPs and ROS (Ryu *et al.*, 2006; Buchon *et al.*, 2013). Previous research has found that Imd pathway-mutant flies lacking AMP expression are usually resistant to gut infection except ROS-resistant bacteria (Ha *et al.*,

2005; Ryu *et al.*, 2006), and could still control the dietary yeast, *S. cerevisiae*. In contrast, the DUOX inactivation led to uncontrolled propagation of *S. cerevisiae* in the gut of *Drosophila* (Ha *et al.*, 2009a), underlying that ROS generation system has the primordial role in the gut antimicrobial response (Ha *et al.*, 2005, 2009a).

In this study, we found that *B. dorsalis* also used the *BdDuoX*-dependent immune response as a defense in the gut as previously characterized in *Drosophila* (Ha *et al.*, 2005; Bae *et al.*, 2010). Beyond the antimicrobial function of the *BdDuoX* gene, it may have other functions in the development of *B. dorsalis*, because of its expression levels at different development stages and tissues of adult. In this line, dDuoX has been described for playing important roles in the stabilization of the cuticle structure of the wings in *Drosophila* (Anh *et al.*, 2011), and mutation in *DuoX* is lethal (B Lemaître, personal communication). DuoX has been extensively studied for its role in the antibacterial defense against infectious bacteria; our study highlights the importance of *BdDuoX* in the control of intestinal bacterial community homeostasis in the intestine. The silencing effect of target genes by RNAi techniques lasts for only 4–6 days in *B. dorsalis*, as previously reported (Chen *et al.*, 2008; Li *et al.*, 2015), and the expression level of the *BdDuoX* gene was downregulated with the 1–7 DPR treatment. The silencing of the *BdDuoX* gene led to an increase in intestinal bacterial density and altered both the structure and richness of the intestinal indigenous bacterial community. This is consistent with a previous observation that DUOX inactivation led to uncontrolled propagation of the dietary yeast in the gut of *Drosophila* (Ha *et al.*, 2009a). The resulting

dysbiosis, in turn, stimulated an immune response by activating the *BdDuox* gene. This explains the finding that the *BdDuox* gene was strongly induced in *BdDuox*-RNAi flies from 9 to 17 DPR to repress the amount of noncommensal bacteria by increasing the ROS level. Finally, the composition and structure of the gut bacterial community were recovered to a normal status, and the *BdDuox* gene returned to the basal expression level at 20 DPR. The antagonist interaction between ROS produced by *BdDuox* and the bacterial load could explain the oscillation in the bacterial community with a slow return to basal levels. In addition, our experimental results using paraquat or vitamin C to change the amount of ROS in the gut also confirmed that the ROS level strongly affected the gut bacterial community. As *duox* is the main ROS-producing factor in the gut of *Drosophila*, our data support a model in which *BdDuox* plays a critical role in regulating the homeostasis of the gut microbial community.

The induction of *BdDuox* by bacteria provides a negative feedback mechanism to adjust the density of the indigenous bacterial community. This feedback loop is similar to that observed in the regulation of the antibacterial response by the Imd pathway that is tightly regulated by a negative regulator of the Imd pathway (Zaidman-Rémy et al., 2006; Paredes et al., 2011). The microbicidal role of *Duox* has been studied in *Drosophila*; to date, little is known about whether *Duox* regulates intestinal bacterial community homeostasis. In this study, we found that *Duox* expression was only weakly induced by most of the major components of the indigenous bacterial community, whereas it responded strongly to infectious bacteria and a minor intestinal component such as *B. cereus* to maintain the homeostasis of the intestinal bacterial community. The p38 mitogen-activated protein (MAP) kinase is a signaling pathway that is involved in both stress and immunity in various species from yeast to humans, and each MAP kinase pathway contains a three-tiered kinase cascade consisting of a MAP kinase kinase kinase (MEKK), a MAP kinase kinase (MKK) and MAP kinase (Qi and Elion, 2005). The pathway that regulates *Duox* gene expression has been characterized as requiring Mekk1 and p38c (Ha et al., 2009b; Chakrabarti et al., 2014). Whereas it was initially thought that *Duox* gene expression was regulated by peptidoglycan via the pattern recognition receptor PGRP-LC (Ha et al., 2009b), *Duox* expression remained similar to wild-type levels in PGRP-LC mutants (Chakrabarti et al., 2014). These results suggest that *Duox* expression is activated by another mechanism. To date, uracil is the only microbe-derived factor that modulates *Duox* activity in *Drosophila* (Lee et al., 2013). It is possible that *BdDuox* gene expression is also regulated by uracil in *B. dorsalis*. We hypothesize that the difference in the release of uracil between symbionts and opportunistic pathobionts could explain the difference in *BdDuox* gene expression described in Figure 2.

This is consistent with the observation of Lee et al. (2013) that uracil is released primordially by infectious bacteria but not by symbiotic bacteria. It was proposed that free uracil may originate from the breakdown of stable RNA, such as ribosomal RNA, and that uracil release could be considered to an indicator of a specific metabolic and physiological state of the bacterial community (Rinas et al., 1995).

An asset of *B. dorsalis* is the existence of stable bacterial community of medium complexity. This contrasts with *Drosophila*, in which the bacterial community, at least under laboratory conditions, is rather unstable and composed of few members (Wong et al., 2011; Broderick et al., 2014). In this study, we found that the composition and richness of the gut microbial community changed markedly in *BdDuox*-RNAi flies. In ds-*duox*-treated flies, the load of dominant members of the community, such as Enterobacteriaceae and Leuconostocaceae, decreased, whereas minor members of the community (Verrucomicrobiaceae, Bacillaceae, Bacteroidaceae and others) expanded when the *BdDuox* gene was knocked down by RNAi. A previous study has shown that Enterobacteriaceae were dominant intestinal commensal bacteria in the gut of *B. dorsalis* (Wang et al., 2011), as well as in several other tephritids, such as *Dacus* (Drew and Lloyd, 1991), *Anastrepha* (Kuzina et al., 2001) and *Ceratitidis* (Behar et al., 2008). These bacteria may contribute to nitrogen fixation (Behar et al., 2005), pectinolysis (Behar et al., 2008), male copulatory success (Ben-Yosef et al., 2008) and may also have an indirect contribution to host fitness by preventing the establishment or proliferation of pathogenic bacteria (Behar et al., 2008). Members of the *Leuconostoc* genus ferment fructose (Ljungdahl, 1962), suggesting that these microbes may play a symbiotic role in promoting insect digestion of fruits or other plant materials (Corby-Harris et al., 2007). Taken together, the decrease in Enterobacteriaceae and Leuconostocaceae in *BdDuox*-RNAi flies may have a damaging effect on the host. The concomitant increase in Bacillaceae, Bacteroidaceae and Verrucomicrobiaceae (*A. muciniphila*) in ds-*duox*-treated flies from 5 to 15 DPR suggests that silencing the *BdDuox* gene in the intestines allows the overgrowth of these bacteria. The bacterial composition of the ds-*egfp*-injected flies was also drastically changed from immature (5 DPR), middle age (15 DPR) and fully mature (20 DPR) *B. dorsalis*; the reasons might be because of the age change in the fly. In fact, the fluctuation of bacterial composition throughout adult life has been previously reported in *A. gambiae* (Wang et al., 2011) and *Drosophila melanogaster* (Wong et al., 2011).

Bacillaceae is a minor component in the gut of *B. dorsalis*, and feeding high doses of *B. cereus* reduced the fly's longevity (unpublished). As *B. cereus* was a potent inducer of *BdDuox*, the deleterious effect of *BdDuox* could be mediated by an increased ROS level in the gut. *B. cereus* is also a

common bacterial pathogen and many *B. cereus* strains have been isolated from insects (Lipa and Wiland, 1971; Broderick *et al.*, 2000). Collectively, our results suggest that a major role of *BdDuoX* is to regulate intestinal bacterial community homeostasis by promoting the growth of a few dominant symbiotic species while preventing the emergence and overgrowth of minor pathobionts. In this line, conditionally pathogenic autochthonous bacteria (that is, pathobionts such as *Gluconobacter morbifer*) of *Drosophila* are normally quiescent but are able to provoke chronic inflammation under dysbiosis conditions, as observed in *Caudal*-deficient *Drosophila* (Ryu *et al.*, 2008). The intestinal dysbiosis caused by the disruption of intestinal bacterial community are reminiscent of inflammatory bowel diseases, wherein some previously commensal resident bacteria cause gut pathology under certain condition (Garrett *et al.*, 2010; Sokol and Seksik, 2010).

Given that many mucosal inflammatory diseases in humans arise from abnormal mucosa–microbe interactions (Artis, 2008) and that ROS dysregulation plays a critical role in the pathogenesis of these diseases (Grisham, 1994; Rokutan *et al.*, 2008), the discovery that *BdDuoX* has an important role in maintaining the homeostasis of gut provides a new insight into the mechanism allowing the development of a healthy bacterial community.

Conflict of Interest

The authors declare no conflict of interest.

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