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Parasite-specific immune response in adult *Drosophila melanogaster*: a genomic study

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Insects of the order Diptera are vectors for parasitic diseases such as malaria, sleeping sickness and leishmania. In the search for genes encoding proteins involved in the antiparasitic response, we have used the protozoan parasite *Octosporea muscaedomesticae* for oral infections of adult *Drosophila melanogaster*. To identify parasite-specific response molecules, other flies were exposed to virus, bacteria or fungi in parallel. Analysis of gene expression patterns after 24 h of microbial challenge, using Affymetrix oligonucleotide microarrays, revealed a high degree of microbe specificity. Many serine proteases, key intermediates in the induction of insect immune responses, were uniquely expressed following infection of the different organisms. Several lysozyme genes were induced in response to *Octosporea* infection, while in other treatments they were not induced or downregulated. This suggests that lysozymes are important in antiparasitic defence.

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INTRODUCTION

The majority of insect vectors for human parasites are found among dipterans. In an attempt to understand the immunological basis for *Anopheles* vector capacity, Schneider & Shahabuddin (2000) successfully used *Drosophila melanogaster* and the malaria parasite *Plasmodium gallinaceum* as a vector–parasite model system. Ookinetes injected into the fly haemocoel developed into sporozoites that were infective when injected into the chicken host. However, when feeding the flies with parasitized blood or ookinetes, parasite development was hampered, indicating that the important barrier for the parasite to develop resides in the gut of this insect. Either certain mosquito-specific invasion routes are not present in *Drosophila*, or the malaria parasites encountered immune response mechanisms, against which they have not evolved countermeasures as in *Aedes* or *Anopheles* hosts. None of the four immune genes tested were induced by the injected malaria parasites, and the question remains open as to whether antiparasitic response genes were induced.

In the search for genes encoding proteins involved in an antiparasitic response, we chose to infect *Drosophila* through a natural route with the protozoan parasite *Octosporea muscaedomesticae* and with fungi, bacteria and virus for comparison. To ensure the induction of genes that are needed to defeat the infection, our intention was to use previously known naturally infecting microorganisms that the fly would survive either completely or for a reasonably long time as compared to conditions in nature. In wildlife populations of *D. melanogaster*, the mean life expectancy for males is estimated as not more than 3 days (Rosewell & Shorrocks, 1987). Because the immune response in *Drosophila* has evolved under such premises, it seems logical to study the response during the first few days of its adult life. We assayed the responding genes at one time point that would cover the transcriptional activation of the most well-studied antimicrobial genes. Because in earlier studies, including oral feeding of *Drosophila* larvae with *Erwinia carotovora* (Basset *et al*, 2000), and oral feeding of adult *Drosophila* with *Crithidia* sp. (Boulanger *et al*, 2001) and *Serratia marcescens* (Kylsten *et al*, 1990), the assayed effector genes were expressed at 24 h, we chose to use this time point.

We selected a set of microorganisms that infect *Drosophila* in nature. The protozoan parasite *O. muscaedomesticae* (Microsporidia) was isolated from the proximal gut epithelium of infected houseflies, *Musca domestica*, and found to heavily infect, but not kill, *D. melanogaster* (Kramer, 1973). The fungus *Beauveria bassiana* infects by penetrating the exoskeleton and kills the host. Despite the fact that it kills within days, it was used as a standard in comparison with other genome-wide screens. The Gram-negative bacterium *S. marcescens* Db1140 is a derivative of the highly virulent strain Db11 isolated from *Drosophila* (Flyg & Xanthopoulos, 1983). The Db1140 strain was demonstrated by northern blots to induce expression of the *Cecropin A* gene in *Drosophila* when administered through the food (Kylsten *et al*, 1990). *Drosophila* C virus (DCV) is a picorna-like single-stranded RNA virus widely spread among *Drosophila.* When adult

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Drosophila were fed with DCV, 30–50% of the flies died within 6 days after infection (Gomariz-Zilber *et al*, 1995).

This is the first whole-genome study on antiparasitic response in *D. melanogaster*. We demonstrate that *Drosophila* responds by upregulating a new and specific set of genes on an oral infection with *Octosporea*. Many of the genes with unknown function have signal peptides and will be a subject for future analyses of antiparasitic activity.

RESULTS AND DISCUSSION

Genome data analysis

The *Drosophila* gene expression in response to different microbes was examined after 24 h of natural infection of adult males. The RNA was hybridized to Affymetrix *Drosophila* GeneChips, and Affymetrix MAS 5.0 software was used for the calculation of expression and statistical analyses of the chips (supplementary information table 1 online). Duplicates of each infection were compared to duplicates of normal flies in a 2×2 matrix (supplementary information text part A online). The genes that were significantly increased ($P < 0.0025$, Wilcoxon's signed ranks test) in all four comparisons were defined as induced genes. In total, 427 genes were induced and selected for further analysis (supplementary information table 2 online). The fungal infection generated the strongest response, with 298 genes induced, and the parasitic infection induced 127 genes. In the viral and bacterial infections, a low number of genes were significantly induced: 11 and 10, respectively. The significantly induced genes are found in many different functional classes (Fig 1). A common feature in the four infections was that many of the genes encode enzymes, in particular serine proteases: *Octosporea*, 35% enzymes (13% serine proteases); *Beauveria*, 24% (8%); *Serratia*, 60% (50%); and DCV, 36% (27%) (supplementary information table 4 online).

Unique or common induction of a gene was determined by comparing the expression of each induced gene selected in one treatment with its expression in other treatments (supplementary information text part A online).

The numbers of uniquely induced genes were 214, 59 and 2 in response to *Beauveria*, *Octosporea* and DCV, respectively; this constitutes 65% of the 427 induced genes and thereby demonstrates specificity in the immune response (Fig 2). Many genes were induced in several infections; 16 genes are designated as common in response to all four infections. The genes in common encode the antimicrobial proteins Attacin A, Cecropin A1, Cecropin A2, Drosomycin and Metchnikowin, as well as acetyl-CoA homeostasis (CG8628), one serine protease (CG6483) and nine genes with unknown functions (supplementary information table 3 online).

Confirmation of genes responding to *Beauveria* infection

The antifungal peptide genes *Drosomycin* and *Metchnikowin* (Ekengren & Hultmark, 2001, and references therein) were heavily induced by *Beauveria* in our study: 14.3- and 19.9-fold, respectively (Table 1). In a similar experiment, where the *D. melanogaster* strain Oregon^R was naturally infected with the same strain of *Beauveria*, the response at 24 h was lower compared to our results: *Drosomycin* 6.4-fold and *Metchnikowin* 4.4-fold (De Gregorio *et al*, 2001). The Canton S flies used in our study died within 5 days (Fig 3), whereas 90% of the Oregon^R flies used by De Gregorio *et al* (2002) were still alive at that time point. This may indicate that our flies were more heavily infected, or that there is a certain genetic difference between these two wild-type isolates of *D. melanogaster*.

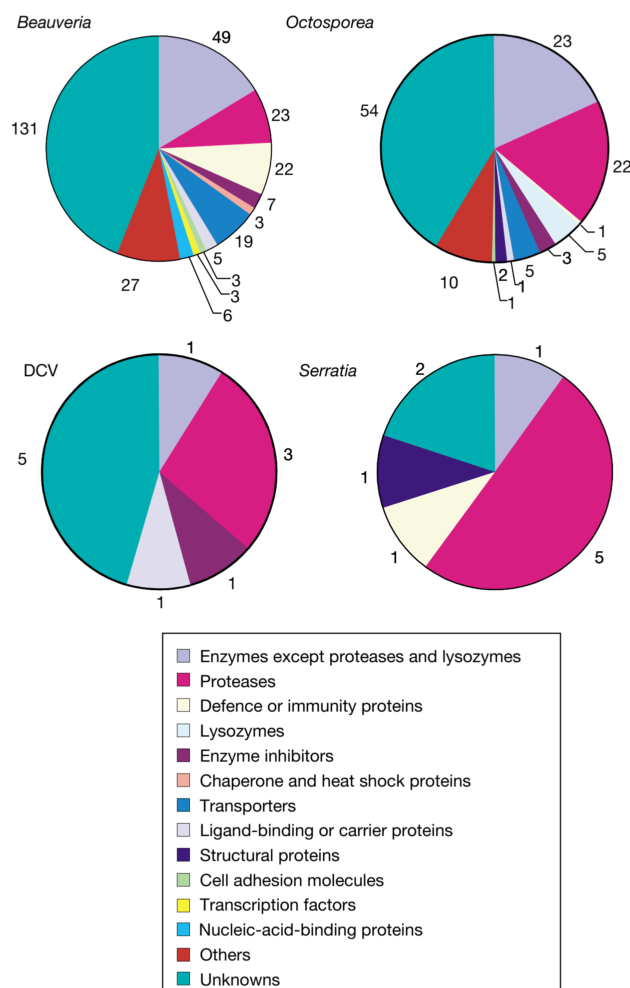

Fig 1 | Distribution of induced *Drosophila* genes according to their functional categories based on NetAffx^{TM} GO.

Turandot M (*TotM*) is a stress-induced humoral protein gene in *Drosophila,* earlier shown to be upregulated by the Gram-negative bacterium *Enterobacter cloacae* β12 when injected into adults (Ekengren & Hultmark, 2001). In our study, *TotM* is induced 13.7-fold by fungal infection (Table 1) and 2.4-fold by bacterial feeding. The strong fungal induction could reflect the stress response inferred by cuticular penetration. Notably, in De Gregorio's study TotM (CG14027) is, after 24 h, upregulated 3.6-fold by the fungal infection and 13.6-fold by septic injury. This is a recurring pattern of contrasting results on fungal versus bacterial infections, when we compare our results with theirs (see Speculation).

Serine proteases

Serine proteases and their inhibitors (serpins) are important early regulators in several invertebrate responses, including haemolymph coagulation, melanization and induction of antibacterial

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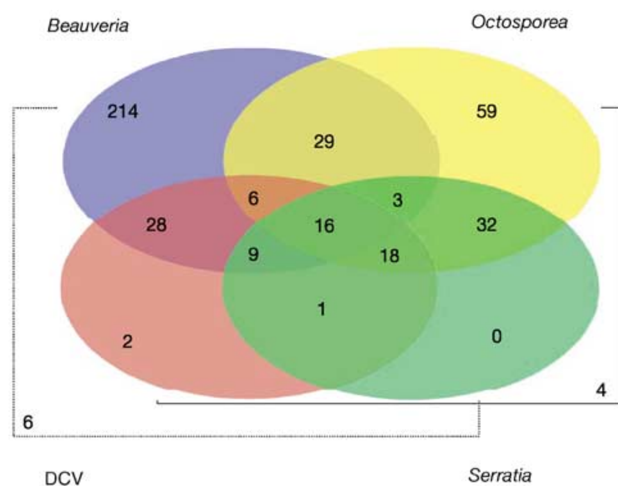

Fig 2 | Number of induced genes that are common or unique in the different infections. Significantly induced genes (cf. Fig 1) were compared with other infections for uniqueness.

peptide genes (Jiang & Kanost, 2000). In the present study, 37 of all 240 annotated serine protease genes (http://flybase.bio.indiana.edu) were induced. Interestingly, serine proteases induced by DCV and *Serratia* and some of those induced by *Octosporea* infections, Ser4, Ser99Da, Ser99Db, CG2229, CG8869, CG8871 and CG16749, were downregulated in the *Beauveria* infection (supplementary information table 4 online). It is possible that one set of serine proteases is induced to encounter infections through the gut and another set is induced on infection through the cuticle. Moreover, *Beauveria* and *Octosporea* also induced different sets of serpins (supplementary information table 5 online).

Among the serine protease genes that were significantly changed in response to the microorganisms used in our study (supplementary information table 4 online), ten were designated as *Drosophila* immune response genes also by De Gregorio *et al* (2001): Ser4, Ser99Dc, CG5909, CG6639, CG8215, CG8869, CG8871, CG11841, CG11842 and CG16749. The serine protease genes that are up- or downregulated after fungal infection in our study are changed in the same direction in their study, although at a lower level. On the contrary, some of the serine protease genes upregulated by bacteria in our study are found to be downregulated after septic injury in the study of De Gregorio *et al* (2001) (see Speculation).

Parasite response genes among immunity-related genes

After parasitic infection, 127 genes were recorded as significantly induced. Since the flies infected with the parasite survived, we can conclude that this insect can produce all the factors needed to mount the parasite invasion (Fig 3). The *Drosophila* flies infected with *Octosporea* gave a fundamentally different response by known immunity-related genes as compared to the other microorganisms used in this study. For instance, several lysozyme genes, *Lys B*, *C*, *D*, *E* and CG16756, were induced by *Octosporea*, which is contrary to the response of the fungal infection, in which lysozyme genes were downregulated (Table 1). Although lysozymes are induced in response to bacterial injection of Lepidoptera pupae (Sun *et al*, 1991), a similar induction in

Table 1 | Fold changes of selected immunity-related genes

Immunity-related genes	DCV (virus)	*Serratia* (G^- bacterium)	*Beauveria* (fungus)	*Octosporea* (protozoan)
Recognition proteins				
GNBP-like CG12780	—	—	—	2.8
GNBP-like CG13422	—	—	11.3	—
PGRP-SA	—	—	2.3	—
PGRP-SC2	—	1.5*	2.6	1.6*
PGRP-SD	—	—	17.8	—
Toll pathway				
Cactus	—	—	3.9	—
Necrotic	—	—	4.1	—
Pelle	—	—	1.7	—
Spätzle	—	—	3.0	—
Toll	—	—	2.7	—
Imd/NF-κB pathway				
Relish	—	—	2.2	—
Antimicrobial peptides/proteins				
Andropin	—	—	1.5	—
Attacin A	6.3*	—	5.2	—
Cecropin A1	2.6*	—	2.8	—
Cecropin A2	3.3*	—	3.5	—
Defensin	—	—	3.1	—
Drosomycin	3.0*	—	14.3	—
IM2 CG18106	—	—	4.2	**0.6***
IM2-like	—	1.5	3.2	**0.7**
Lysozyme B	—	—	**0.5**	1.8
Lysozyme C	—	—	**0.5**	2.2
Lysozyme D	—	—	**0.4**	2.0
Lysozyme E	—	—	**0.5**	2.1
Lysozyme CG16756	—	—	—	1.6
Metchnikowin	—	—	19.9	—
Other				
Lectin CG9978	—	—	—	4.0
Thor	—	**0.6***	1.8	**0.5**
TotM	—	2.4	14.6	—
Transferrin 1	1.8*	—	2.8	—
Transferrin 3	—	—	3.7	—
Scavenger receptor CG2736	—	—	—	1.8
CG11315	—	—	**0.5***	1.8

The fold change given is the mean of transformed $\log_2$ values of four significant comparisons, where all treatments are significantly different from the control. Downregulated genes are shown in bold. *Value based on four comparisons, where one treatment is not significantly different from the control. —, comparison where a maximum of two treatments are significantly different from the control. DCV, *Drosophila* C virus.

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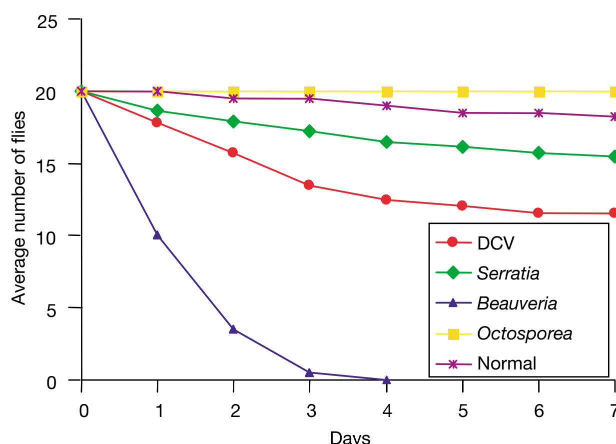

Fig 3 | Survival of 20 flies from each experiment after the 24-h infection period using different microbes in sugar solution. The flies were placed on normal fly food at day 0. Data represent the mean value of duplicates. The results correspond well to earlier studies (Flyg & Xanthopoulos, 1983; Gomariz-Zilber *et al*, 1995; Ekengren & Hultmark, 2001).

Drosophila has not been reported before. *Lys B*, *C*, *D* and *E*, which were upregulated by *Octosporea* in the present study, are closely related genes that are normally constitutively expressed in the midgut of *Drosophila* adults (Daffre *et al*, 1994). An important characteristic of many lysozymes is their chitinase activity. Recently, it was experimentally demonstrated that Lys D from *D. melanogaster* displays chitinase activity (Regel *et al*, 1998). Chitin is known as a major component of arthropod cuticle, fungal cell walls and the exoskeleton of some algae. It is also a component of *Octosporea* spores and, as such, a substrate and target for this activity. An interesting observation in this context is that transgenic expression of two *Plasmodium* sporozoite surface antigens in *Drosophila* strongly affects the lysozyme gene expression profile (M. Shahabuddin, personal communication).

We found that out of the 127 genes upregulated in response to *Octosporea*, 59 were uniquely expressed and, among these, 48 are newly retrieved immune responsive genes (Table 2), not upregulated in other extensive genome studies (supplementary information text part B online), of responses to bacteria and fungi in *Drosophila* (De Gregorio *et al*, 2001; Irving *et al*, 2001; Boutros *et al*, 2002). In the following, we highlight some of the genes in Table 2, which have been earlier known to possess domains with putative immune functions.

The parasite-induced gene CG2736 encodes a scavenger receptor with a CD36 family domain (http://flybase.bio.indiana.edu). A common feature of scavenger receptors is their ability to bind a broad range of ligands, including different microbes, indicating a potential role in innate host defence (Gough & Gordon, 2000). The scavenger receptor CD36 is involved in diverse cellular activities, like recognition and phagocytosis of apoptotic cells. Notably, CD36 is also a major receptor for the adherence of the *Plasmodium falciparum*-infected erythrocyte on endothelial cells (Tachado *et al*, 1997, and references therein). We speculate that the induction of the scavenger receptor gene could be due to the interaction between *Octosporea* and the endothelial cells.

The parasite-specific gene CG11315 is of particular interest, because it was earlier shown to contain two ML domains (MD-2-related lipid recognition), recognizing lipid moieties (Inohara & Nunez, 2002). In mammals, MD-2 serves as a cofactor for TLR4 (Toll-like receptor 4) and is essential for the response to lipopolysaccharides (LPS). Similar to bacterial LPS, several lipid-containing parasite antigens, like GPI links, are highly immunogenic in mammals (Tachado *et al*, 1997). Such molecules may be important targets for the recognition and signalling machinery also in insects. Apart from CG11315, seven other genes containing ML domains have been earlier identified in *Drosophila* (Inohara & Nunez, 2002).

Lectins are carbohydrate-binding proteins suggested to participate in agglutination of cells, glycoprotein clearance and innate immunity. One *Drosophila* gene (CG9978), which is parasite specific, contains a galactose-specific C-type lectin domain (CTLD) (Theopold *et al*, 1999). Similarly, a putative galactose-specific lectin was induced in the gut of *Anopheles gambiae*, on feeding with malaria-parasitized blood (Dimopoulos *et al*, 1997).

Most of the uniquely expressed genes encode gene products that lack known domains or other similarities to known proteins. To predict interesting candidates among these, to be tested for antiparasitic activity, we performed an *in silico* screen for the presence of signal peptide (http://www.cbs.dtu.dk/services/SignalP) and the absence of transmembrane domains (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html). From this screen, nine genes were found and, of these, eight have unknown functions and will be further characterized (Table 2).

The overall scope of this study was to identify parasite-specific response factors, and ultimately effector molecules that act on vector-borne protozoan parasites. Such molecules could serve as structural models for antiparasitic drug design. Another approach is the introduction of antiparasitic genes into bacterial symbionts residing in the gut of insect vectors. For this purpose, our search for strictly antiparasitic molecules is important.

SPECULATION

When we compared our results with the study of De Gregorio *et al* (2001), who also used Affymetrix *Drosophila* GeneChips, we observed striking differences. Out of 28 immune genes that are in common, 26 responded more strongly to bacteria than to fungi in their study, whereas our results were the opposite. Similarly, the three serine protease genes that were upregulated in our study after bacterial feeding were downregulated in their study. They challenged adult flies with nonpathogenic bacteria (*Micrococcus luteus* mixed with *Escherichia coli*) by septic injury or externally with pathogenic fungi, which means that both microorganisms entered through the cuticle. We used the same fungal infection, but oral feeding of bacteria (*S. marcescens*). The opposing results suggest that the method of infection is crucial for the specificity of the response. However, it is difficult at this point to draw firm conclusions, because a sterile injury control was not included in the study of De Gregorio *et al* (2001) and different bacterial species were used in the two studies, which could have given different responses.

METHODS

Microbes. *S. marcescens* Db1140 (obtained from A. Boman, Karolinska Institutet, Sweden) was grown to OD_{600} 0.5 in Luria–

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Table 2 | Parasite-specific genes

CG number	Molecular function	Amino acids	Fold change	CG number	Molecular function	Amino acids	Fold change
CG1742	Glutathione transferase	152	1.4	CG10849	Unknown	302	1.4
CG2505	Carboxylesterase	554	1.7	CG10943	Unknown	308	3.3
CG2736	Scavenger receptor	507	1.8	CG11151	Estradiol 17 β-dehydrogenase	115	1.5
CG3285	Unknown	466	1.5	CG11315	Unknown*	157	1.8
CG3724	Phosphogluconate dehydrogenase	481	1.6	CG11671	Unknown	140	1.9
CG3850	Unknown	384	5.3	CG12350	Trypsin§	272	1.2
CG3868	Unknown	308	1.9	CG12628	Glutathione transferase	152	1.6
CG3987	Unknown*	404	1.8	CG12990	Unknown	663	1.6
CG4986	Unknown*	103	1.5	CG13091	Unknown	523	1.6
CG5171	Trehalose phosphatase	273	1.6	CG13607	Unknown	546	1.9
CG5853	ATP-binding cassette (ABC) transporter	689	1.6	CG13639	Unknown*	69	1.5
CG5999	UDP-glucuronosyltransferase	512	5.7	CG14259	Unknown*	289	1.6
CG6084	Aldehyde reductase	316	2.0	CG14629	Unknown	319	1.7
CG6261	Unknown*	150	1.6	CG15155	Unknown	258	3.7
CG6726	NOT aminoacylase	401	1.9	CG15199	Unknown*	116	1.9
CG6733	NOT aminoacylase	401	1.9	CG15531	Stearoyl-CoA desaturase	334	1.6
CG7801	Glucose transporter§	857	1.7	CG16775	Unknown	208	3.2
CG8036	Transketolase	221	1.3	CG16834	Galactose-binding lectin†	118	2.3
CG8054	Unknown	507	1.6	CG16904	Unknown	262	1.4
CG8322	ATP-citrate (pro-S)-lyase	1,086	1.3	CG16926	Unknown	127	1.7
CG8630	Stearoyl-CoA desaturase	408	1.5	CG17101	Triacylglycerol lipase	1,073	1.3
CG9232	UTP-galactose 1-phosphate uridylyltransferase	350	1.3	CG17224	Uridine phosphorylase	300	2.4
CG9564	Trypsin	266	1.6	CG17734	Unknown	101	1.4
CG9978	Lectin*	295	4.0	CG18493	Unknown*	480	1.9

The selected genes are uniquely expressed after infection with *O. muscaedomesticae* as compared with other infections and with other studies (supplementary information text part B online). The fold change given is the mean of transformed $\log_2$ values of four significant comparisons, and the molecular function is based on NetAffx™ and Flybase (†) gene ontology (GO) in May 2003.
*Presence of signal peptide and absence of transmembrane domain.

Bertani broth (LB) supplemented with streptomycin at 30 μg ml^{-1} (Flyg & Xanthopoulos, 1983).

DCV EB strain was obtained from P.D. Christian (NIBSC, UK) (Johnson & Christian, 1998) and the infectivity was verified in *Drosophila* Schneider S2 cells.

O. muscaedomesticae (parasitic protozoan) isolated from *Sarcophaga bullata* was obtained from L. Solter (Illinois Natural History Survey, Illinois, USA). *B. bassiana* (fungus) was obtained from B. Lemaitre (Centre de Genetique Moleculaire, Gif-sur-Yvette, France) and was sporulated on malt-agar plates.

Natural infections. Flies of the Canton S strain of *D. melanogaster* were obtained from M. Rasmusson (Umeå University, Sweden) and were maintained on cornmeal and yeast food at 25 °C, at 70% humidity and a 12 h light/dark cycle. We collected 1–3-day-old male flies under ether anaesthesia and placed them in vials with a piece of wettex covering the bottom and soaked in 2 ml of 1% sugar solution or 1% sugar solution containing the microbe of interest. Flies were infected with *B. bassiana* by gently shaking them in spores according to Lemaitre *et al* (1997) and then transferring them to the 1% sugar solution diet. Control flies were fed on 1% sugar solution only. After 24 h, flies were ether anaesthetized and frozen in liquid N_2 or kept in vials containing normal food for survival experiments.

Feeding and starvation. To determine the efficiency of feeding, approximately 100 flies were fed with 0.05% bromophenol blue (Schmid, Köngen, Germany) in 1% sugar solution for 30 or 150 min. As judged by their bluish abdomens, 75% of the flies had ingested sugar solution after 30 min and 95% after 150 min.

The survival of flies feeding on 1% sugar was compared with survival on normal food. Six vials with 20 flies in each were used for each treatment. No difference in survival was detected in the first 3 days, indicating that the flies were not heavily starved.

Sample preparation and analysis. For each infection as well as for the control, two independent experiments were performed, each of them used for one genome array analysis. For each experiment, total RNA was isolated from 100 male flies and hybridized to the

Affymetrix *Drosophila* GeneChips in the Affymetrix core facility at NOVUM (Karolinska Institutet, Sweden), according to Affymetrix's protocols (supplementary information text part C online).

Supplementary information is available at *EMBO reports* online (http://www.emboreports.org).

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