

Original article

Identification and characterization of novel natural pathogen of *Drosophila melanogaster* isolated from wild captured *Drosophila* spp.

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Received 16 February 2016; accepted 26 July 2016
Available online 1 August 2016

Abstract

Drosophila melanogaster is an emerging model system for the study of evolutionary ecology of immunity. However, a large number of studies have used non natural pathogens as very few natural pathogens have been isolated and identified. Our aim was to isolate and characterize natural pathogen/s of *D. melanogaster*. A bacterial pathogen was isolated from wild caught *Drosophila* spp., identified as a new strain of *Staphylococcus succinus* subsp. *succinus* and named PK-1. This strain induced substantial mortality (36–62%) in adults of several laboratory populations of *D. melanogaster*. PK-1 grew rapidly within the body of the flies post infection and both males and females had roughly same number of colony forming units. Mortality was affected by mode of infection and dosage of the pathogen. However mating status of the host had no effect on mortality post infection. Given that there are very few known natural bacterial pathogens of *D. melanogaster* and that PK-1 can establish a sustained infection across various outbred and inbred populations of *D. melanogaster* this new isolate is a potential resource for future studies on immunity.

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Keywords: DNA–DNA relatedness; Sequence similarity; Immunity; Survivorship; Pathogenicity

1. Introduction

Innate immunity is an important component of the anti-microbial defense mechanism of an organism and is evolutionarily conserved across taxa to a large extent (reviewed by [1]). *Drosophila* has emerged as a good model to study innate immunity because, apart from other practical advantages, signalling pathways and other cellular and humoral components of the innate immune system are shared with humans (for reviews, see [2,3]). In any study of the evolutionary ecology of immunity, it is desirable to use natural pathogens of the host. A number of bacteria associated with *Drosophila*

have been reported. Of these, some are gut symbionts (see Table 1 in Broderick and Lemaitre [4]) mostly belonging to the genera *Acetobacter* and *Lactobacillus* while very few are known to be pathogenic (*Erwinia cartovora*, *Erwinia Chrysanthemi*, *Enterococcus faecalis*, *Providencia rettgeri*, *Providencia alcalifaciens*, *Providencia burhodogranaria*, *Providencia sneebia* *Lactococcus lactis* *Pseudomonas entomophila* and *Pseudomonas aeruginosa*) [4–10]. A major limitation of using *Drosophila* for studies of innate immunity is the paucity of bacterial pathogens. Hence a large number of studies use non-pathogenic bacteria like *Escherichia coli* for the study of innate immunity. Additionally most of the bacteria that are known to cause mortality in adult *Drosophila* (and are hence used in studies) are not natural pathogens.

Our aim in this study was to isolate a natural pathogen of adult *Drosophila melanogaster*. Once we had isolated a pathogen, we investigated whether the:

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Table 1

Biochemical characteristics of PK-1 and other *Staphylococcus* species. Summary of biochemical results of PK-1 strain was compared with previously known results from other studies [19,22]. +, indicates more than 90% of the strain is positive; – denotes more than 90% of the strain is negative; ± showing between 25 and 75% strain are positive according to [22]; (+) weak positive, ND not determined, W suggests weak and not easily reproducible results (Place et al. [19]). Data from^a [19],^b [22]. AMG-D1 is a strain of *S. succinus* subsp. *succinus*, SB 72 is a strain *S. succinus* subsp. *casei*.

Biochemical assay	PK-1	AMG-D1 ^a	SB72 ^a	<i>S. xylosus</i> ^a	<i>S. saprophyticus</i> ^b
Nitrate reduction	(+)	–	+	+	–
Voges-Proskauer test	–	–	ND	±	+
Arginindihydrolase	+	–	–	–	–
Urease	+	+	+	+	+
Catalase	+	+	ND	+	+
Oxidase	–	–	–	–	–
Fermentation of:					
Raffinose	–	–	–	–	–
Lactose	–	+	+	±	±
Trehalose	+	+	+	+	–
Galactose	–	W	+	W	ND
Sucrose	+	+	+	+	+
Fructose	+	+	+	+	ND
Maltose	(+)	+	+	+	ND
Tween 40	–	W	+	W	ND
Tween 80	–	W	+	W	ND
Esculin hydrolysis	(+)	+	+	–	ND
Antibiotic sensitivity:					
Novobiocin resistance	+	+	+	+	+
Bacitracin resistance	+	+	ND	+	+

- initial dosage of the pathogen affects post infection survival of the host?
- mating status of the host affects its ability to survive post infection?
- mode of infection affects the survival of the host?
- pathogen grows within flies to establish a sustained infection?

We then also compared the pathogenicity of the new isolate with that of a known pathogen (see [SupInfo. S2](#)).

We cultured microbes from wild caught flies and screened them for pathogenicity. Out of the ~200 isolates screened, we were successful in isolating a new strain (*Staphylococcus succinus* subsp. *succinus*) of bacteria that was pathogenic to adult *Drosophila*. The new strain was named as PK-1 and deposited in culture collections (see below).

2. Materials and methods

2.1. Isolation of natural pathogen of adult *D. melanogaster* from wild caught *Drosophila* spp.

2.1.1. Fly collection

Flies were collected during (January–March, 2011) from a fruit market in Chandigarh, Punjab, India (Longitude 76° 47' 14E and Latitude 30° 44' 14N) using fly traps baited with

banana-yeast paste. Traps were harvested daily and the flies were processed immediately.

2.1.2. Isolation of bacteria from flies

Individual flies were rinsed in 70% ethanol for surface sterilization [11], transferred to micro centrifuge tubes and homogenized in 200 µL of 10 mM MgSO₄. Homogenates were serially diluted up to 10^{–5}, then 100 µL of each dilution was spread on Luria Bertani (LB) agar plate of pH 7 and 10, and incubated at 25 °C or 37 °C for 48 h. Each colony was preserved in 20% glycerol in –80 °C and notionally identified based on the colonial and morphological characteristics.

2.1.3. Generation of experimental flies to screen whether isolates are pathogenic or not

We used three outbred (PJB, BRB and LH) and two inbred (Canton-S and W1118) populations to screen pathogenicity. The outbred populations were unrelated to each other. Maintenance and origin of PJB, BRB, LH, Canton-S and W1118 populations used for this study has been described in Supplementary information (see [SupInfo. S1](#)).

All flies used for assay were reared at a density of 50 eggs per vial containing ~6 mL food of banana-yeast-jaggery hereafter referred to as the 'food' (for two outbred populations- PJB and BRB, and two inbred lines- Canton-S and W1118). For the LH population, the rearing density was 150 eggs/vial containing ~8 mL of cornmeal food. Twenty vials were set up for each population and reared at 25 °C and 50–60% Relative Humidity (RH). On 9–10th day post egg collection, virgin males or females were collected using light CO₂ anaesthesia at very young stage (approximately 4 h post eclosion). Five flies were housed in each vial (single sex groups) provisioned with 2 mL food. Every alternate day, flies were transferred into fresh food vials.

2.1.4. Screening of isolates and infection protocol

A single colony of each isolate was inoculated in LB broth, incubated at 37 °C and shaking at 170 rpm for overnight growth. The following day a sub-culture was started by inoculating 100 µL of overnight culture in 10 mL of LB broth and incubated (at 37 °C and at 170 rpm) until optical density (OD_{600nm}) reached 1 ± 0.050. Then 1 mL of culture was centrifuged (10,000 rpm for 4 min) and the pellet was resuspended in 1 mL of 10 mM MgSO₄ to yield bacterial suspension [12]. For screening if isolates are pathogenic or not, flies of known age (4–5 days old as adults), type and sex (see below for details) were mildly anaesthetized using CO₂. Flies were infected by poking the lateral thorax with a minuten pin (0.1 mm, fine Science Tools, Foster City, CA, USA) dipped in the bacterial suspension [13]. For sham infection, pin was dipped in sterile 10 mM MgSO₄ prior to poking the lateral thorax of the flies. All infections were done at the mid-point of the light phase of the 12:12 LD cycle of the flies.

2.1.5. Survivorship assay post infection

Forty to fifty male and female flies were infected separately for each population to screen whether the isolates were

pathogenic or not. Same numbers of flies were used for sham infection. Post infection, vials containing flies were monitored for the number of dead flies at every 3 h intervals until the first death in vial, after that at every one hour till 70 h post infection followed by 3 h intervals till 96 h post infection. All populations were infected between 2–4 PM (Indian standard time). These experiments were repeated twice (Data not shown).

2.2. Identification of the new isolate

After screening more than 200 isolates, we found one isolate (PK-1) that was pathogenic to the populations of *D. melanogaster*. Hence, we wanted to identify and further characterize PK-1. Initially PK-1 was identified on the basis of morphological and biochemical characters, fatty acid methyl esters (FAMES) and 16S-rDNA gene sequencing which were carried out by Microbial Type Culture Collection and Gene Bank at Institute of Microbial Technology (Chandigarh, India). The isolate was further characterized as described below.

2.2.1. DNA–DNA hybridizations

Genomic DNA of the pathogenic bacteria was isolated accordingly to modified procedure as described earlier [14]. DNA–DNA hybridizations were performed among strain PK-1 and *S. succinus* subsp. *succinus* (LMG 22185), *S. succinus* subsp. *casei* (LMG 22186), *Staphylococcus xylosus* (LMG 20217) and *Staphylococcus saprophyticus* subsp. *saprophyticus* (LMG 13350). DNA–DNA hybridizations were performed by BCCM/LMG Bacteria Collection at University of Gent, Belgium. Hybridizations were carried out in the presence of 50% formamide at 34 °C, a modification [15,16] of the method as described previously [17].

2.2.2. G + C content

G + C content was determined by BCCM/LMG Bacteria Collection at University of Gent, Belgium using HPLC technique [18]. The value that is reported is the mean of three independent analyses of the same DNA sample.

2.2.3. Isolation of genomic DNA for the *hsp60* gene sequencing

This was done to determine the identity of the pathogenic bacteria (Strain PK-1) at the subspecies level. Genomic DNA of the pathogenic bacteria was isolated using the protocol as described earlier [19] with minor modifications. Bacterial strain was cultured in 10 mL LB broth until $OD_{600nm} = 3 \pm 0.1$. Culture was harvested, centrifuged (6000 rpm/5 min/4 °C), pellet was washed and re-suspended in 250 μ L water. 125 μ L of 10% sodium dodecyl sulphate was added and mixed well. Then the tubes were incubated at 45 °C for 30 min in water-bath. After that 200 μ L glass beads were added to the tubes, vortexed 5 times for 30s each using bead beater and centrifuged at 13000 rpm. Supernatant was transferred into a fresh tube having 500 μ L PCI (Phenol: Chloroform: Isoamyl alcohol 25:24:1 saturated with 10 mM Tris, pH 8.0, 1 mM

EDTA) solution was added and inverted 5 times and centrifuged at 13,000 rpm for 10 min. Upper layer was collected in a fresh tube, isopropanol (twice the volume of the upper layer) was added to it and the tube was incubated at -20 °C for 5 min. After that it was centrifuged at 13000 rpm for 10 min. Pellet was washed with 500 μ L of 70% ethanol and dried at 37 °C. Finally it was dissolved in 30 μ L TERNase.

2.2.4. PCR amplification

The *hsp60* gene was amplified by PCR (Bio-Rad Thermal cycler) using Vent DNA polymerase (New England Biolab); 50 μ L of reaction mixture was prepared containing 100 ng of genomic DNA. Thermal cycler protocol for the amplification of *hsp60* gene was: 95 °C for 5 min for one cycle, after that 30 cycles at 95 °C for 45 s, 55 °C for 45 s, 72 °C for 1 min and finally at 72 °C for 10 min, 4 °C (infinite hold). Amplified DNA fragment was separated on 1% agarose gel and eluted from gel using a gel extraction kit (Thermo scientific). The *hsp60* gene sequencing was performed by Eurofins Genomic (Bangalore, India) using the dideoxy chain-termination method with the Big-Dye terminator kit using an ABI 310 Genetic Analyzer (Applied Biosystems). The *hsp60* gene sequence of strain (PK-1) generated in this work (900 bases) was aligned against *Staphylococcus* species. A sequence similarity search was performed with GenBank BLASTN using default parameters [20].

2.3. Characterization of the new isolate (PK-1) as a new model pathogen

2.3.1. Fly populations used for the study

We used PJB, BRB, LH, Canton S and W1118 flies to study PK-1 as a model pathogen. We specifically asked the following questions.

2.3.2. Does initial dosage of the pathogen PK-1 affect post infection survival of the host?

Single colony of PK-1 was inoculated into 10 mL LB broth and incubated at 37 °C for overnight growth. The next day it was sub-cultured in multiple batches of 10 mL LB broth until the culture attained $OD_{600nm} = 1 \pm 0.05$. To obtain cultures with $OD_{600nm} = 2$ and 3, multiple cultures with $OD_{600nm} = 1$ were combined and spun down till the desired OD_{600} was obtained. Flies were then infected (or sham infected) as described before. We used virgin males and females from various host populations: PJB, LH, BRB, Canton-S and W1118 for this assay ($N = 45$ – 50 for each sex by treatment by population combination). Flies were 4–5 days old at the time of infection.

2.3.3. Does mating status of the host affect its ability to survive post infection?

Flies from PJB, LH and BRB populations were isolated as virgins and randomly divided into two groups: virgin and mated. Males and females from the mated group were combined on the 12th day post egg collection and allowed to interact for two days prior to infection. Flies in the virgin

treatment were held as single sex groups till infection. Infections were carried out as described before on the 14th day post egg collection.

2.3.4. Can flies be infected with PK-1 through oral route?

Single colony of PK-1 was inoculated into LB broth, Incubated (at 37 °C and 170 rpm) until OD_{600nm} = 1 ± 0.05. Subsequently, 1.6 mL of the resultant culture was centrifuged and the pellet of bacteria was resuspended in 170 µL of 5% sterile sucrose solution. This slurry of bacteria was added on 2.3 cm Whatman filter paper disc and it was placed in a vial containing 5 mL of 1.2% agar. For controls only 170 µL of 5% sterile sucrose solution was transferred onto the Whatman filter paper discs. Virgin males and females from the W1118 population were transferred to the vials containing bacterial slurry (or only sucrose solution) at a density of 5 flies per vial. Ten such vials were set up per treatment by sex combination. The flies were allowed to feed for 5 days. Mortality was recorded at 6 h interval.

2.3.5. Does PK-1 grow within the flies?

We managed to isolate a pathogenic strain of bacteria which was named as PK-1 (see below for details). We carried out an assay to understand whether this pathogen (PK-1) can grow within the body of host and establish a sustained infection. Eighty male and female flies (4–5 days old adults) were infected with PK-1 (OD_{600nm} = 2.5 ± 0.1). Immediately after infection, flies were randomly divided into two groups (40 males and females in each group). Flies from the first group were frozen soon after infection. Flies from the second group were maintained under standard laboratory conditions for 24 h post infection after which they were frozen. These two sets of flies were used to estimate the number of CFUs at 0 h and 24 h post infection. Flies were surface sterilized with 70% ethanol using protocol as described previously [11] with minor modifications. Following this, set of 3 flies were homogenized in 100 µL of 10 mM MgSO₄. There were 10 sets per group for each sex. For 0 h post infection group, homogenate was diluted 10 times in 10 mM MgSO₄. In the 24 h post infection group, homogenate was diluted 1000 times in 10 mM MgSO₄. After that, 100 µL of the homogenate was plated on Petri plate of LB agar medium and incubated at 37 °C for 24 h. Following this, the number of colonies in each plate was counted. These counts were log₁₀ transformed and used as units of analysis.

3. Results

3.1. Isolation of bacteria

We collected wild flies during January–March, 2011 from a fruit market in Chandigarh, Punjab, India (Longitude 76° 47' 14E and Latitude 30° 44' 14N) using fly traps baited with yeast paste. To isolate bacteria, individual flies were surface sterilized, crushed and the homogenate was plated on LB media at different pH and incubated at different temperatures. We obtained 200 different isolates of bacteria (based on colonial and morphological characteristics) from wild *Drosophila* spp. To

test if a given bacterial strain is pathogenic, we infected 45–50 flies (roughly 4–5 days old as adults) from multiple fly populations. This was done with all 200 bacterial isolates. We found only one strain of bacteria that was pathogenic to multiple populations of *D. melanogaster*. We called this strain as PK-1.

3.2. Identification of strain PK-1

We found that PK-1 strain is a gram positive, coccus, non-motile bacteria with average cell size of 5 µm. Colonies start appearing after 18 h of streaking on LB agar plates and incubating at 37 °C. Colonies of this strain are raised, opaque, smooth, circular, have an off white pigment and aerobic in nature. Growth occurs at range of 4–42 °C temperature on LB medium. Optimal growth is obtained at 37 °C. While PK-1 shared several similarities with *Staphylococcus succinus* strains, PK-1 was different from both *S. succinus* subsp. *succinus* (AMG-D1) and with *S. succinus* subsp. *casei* (SB72) on the basis of the following tests—hydrolysis of arginine, lactose and galactose fermentation, tween 40 and tween 80 hydrolysis (Table 1). We further identified this strain on the basis of 16S rDNA gene sequence similarity. 16S rDNA gene sequence (accession number JQ988944) similarity result shows that PK-1 strain belongs to genus *Staphylococcus*. It shares 99.70% sequence similarity with *S. succinus* subsp. *succinus*, 99.57% sequence similarity with *S. succinus* subsp. *casei*, 99.20% sequence similarity with *S. xylosus*, 99.01% with *S. saprophyticus* subsp. *saprophyticus* and less than 99% with other species of *Staphylococcus*. To further confirm whether the PK-1 belongs to *S. succinus* subsp. *succinus* or other *Staphylococcus* species, it was identified on the basis DNA–DNA hybridization and G + C content.

DNA–DNA hybridization studies were carried out among strains, PK-1 and near relatives (Table 2). We found that PK-1 shows 85% and 75% relatedness with *S. succinus* subsp. *succinus* and *S. succinus* subsp. *casei*, respectively. However, PK-1 shows less than 70% relatedness with *S. xylosus* and *S. saprophyticus* subsp. *saprophyticus* (Table S2). A relatedness value of 70% is generally accepted as the limit of species delineation [21]. Hence PK-1 is either closely related to *S. succinus* subsp. *succinus* or with *S. succinus* subsp. *casei*. G + C content of PK-1 strain was 33 mol % of genomic DNA (Table S2). This is less than 3% different from both *S. succinus* subsp. *succinus* and *S. succinus* subsp. *casei* [19,22]. Generally less than 3% difference in G + C content is the accepted range within well-defined species (reviewed by [23]). The G + C content of the DNA of strain PK-1 strain was 33 mol %, in accordance with the overall content of the genus (reviewed by [23]). We then carried out Fatty Acid Methyl Esters (FAMES) assay to determine if PK-1 belongs to *S. succinus* subsp. *succinus* or *S. succinus* subsp. *casei*. Results show that PK-1 strain contains tuberculostearic acid (TBSA 10Me18:0), which is a special characteristic of the coryneform bacteria but not previously described for the genus *Staphylococcus* species other than *S. succinus* subsp. *succinus* and *S. succinus* subsp. *casei* [19,22]. PK-1 strain is much closer to *S.*

Table 2

Cellular fatty acid composition in percentage of strain PK-1 compared with other *Staphylococcus* species. Data from ^a[22] ^b[19]. AMG-D1 is a strain of *S. succinus* subsp. *succinus*, SB 72 is a strain *S. succinus* subsp. *casei*, ND means not determined.

Fatty acid	PK-1	AMG-D1 ^a	SB72 ^b	<i>S. xylosus</i> ^a	<i>S. saprophyticus</i> ^a
11:0 iso	0.49	0.61	ND	0.00	0.00
11:0 anteiso	0.40	0.23	ND	0.00	0.00
12:0 iso	0.45	0.52	ND	0.00	0.00
12:00	ND	0.50	ND	0.00	0.00
13:0 iso	19.48	28.38	1.51	1.44	0.55
13:0 anteiso	17.85	12.73	0.51	0.00	0.00
14:0 iso	ND	1.39	0.51	2.40	1.88
14:00	1.02	1.50	ND	1.13	1.10
15:0 iso	11.57	18.24	17.09	23.73	21.19
15:0 anteiso	28.86	18.12	41.69	39.24	41.55
16:0 iso	0.78	ND	ND	ND	ND
16:00	1.01	0.97	0.47	0.97	1.94
16:1 ω 11c	ND	0.21	ND	0.00	0.00
Iso 17:1 ω 10c	1.10	1.53	0.40	0.00	0.00
17:0 iso	5.40	6.02	15.10	8.69	6.56
17:0 anteiso	7.70	2.29	8.00	4.48	4.66
18:1 ω 9c	ND	0.00	0.70	0.00	0.00
18:0 iso	ND	0.00	ND	0.00	0.00
18:00	ND	1.65	2.63	5.66	5.52
TBSA10Me 18:0	1.10	1.45	1.19	0.00	0.00
19:0 iso	0.62	0.79	5.09	2.75	1.92
19:0 anteiso	ND	0.52	1.67	0.00	0.00
20:1 ω 9c	ND	1.07	0.56	0.00	0.00
20:00	ND	0.49	1.52	5.47	9.12

succinus subsp. *succinus* on the basis of 13:0 iso, 13:0 anteiso, 15:0 anteiso, 16:0, Iso 17:1 ω 10c, 17:0 iso relative to *S. succinus* subsp. *casei* (Table 2). However, in comparison with *S. succinus* subsp. *succinus*, PK-1 strain contains more 11:0

anteiso, 13:0 anteiso, 15:0 anteiso, 17:0 anteiso but less 11:0 iso, 12:0 iso, 13:0 iso, 14:0, 15:0 iso, Iso 17:1 ω 10c, 17:0 iso, TBSA10Me 18:0, 19:0 iso (Table 2). For further subspecies characterization, we looked at *hsp60* gene sequence (accession number KM516055). PK-1 shows 98.5% *hsp60* gene sequence similarity with *S. succinus* subsp. *succinus* and 97.6% sequence similarity with *S. succinus* subsp. *casei*. Subspecies have a sequence similarity range of 91–98% [24]. We found that PK-1 strain shows more than 98% similarity with *S. succinus* subsp. *succinus* and less than 98% with *S. succinus* subsp. *casei*. Thus, our findings strongly suggest that PK-1 belongs to *S. succinus* subsp. *succinus*.

3.3. Characterization of PK-1 as a model pathogen

3.3.1. Effect of initial dosage of PK-1 pathogen on host survivorship post infection

We found that, post infection survivorship of adults male and females varied across several laboratory populations (LH, PJB, BRB and Canton-S and W1118). In case of males we found significant effect of initial dosage of pathogen on post infection survivorship of various populations. In general, with increasing dosage, total death and mortality rate of the males increased in the host populations (Fig. 1, Table 3A). However, among the females, initial dosage effects were much more variable with LH, PJB and Canton-S females showing declining survivorship with increasing dosage while BRB females showed no such effect (Fig. 2, Table 3B). We did not find any death in the sham infected controls.

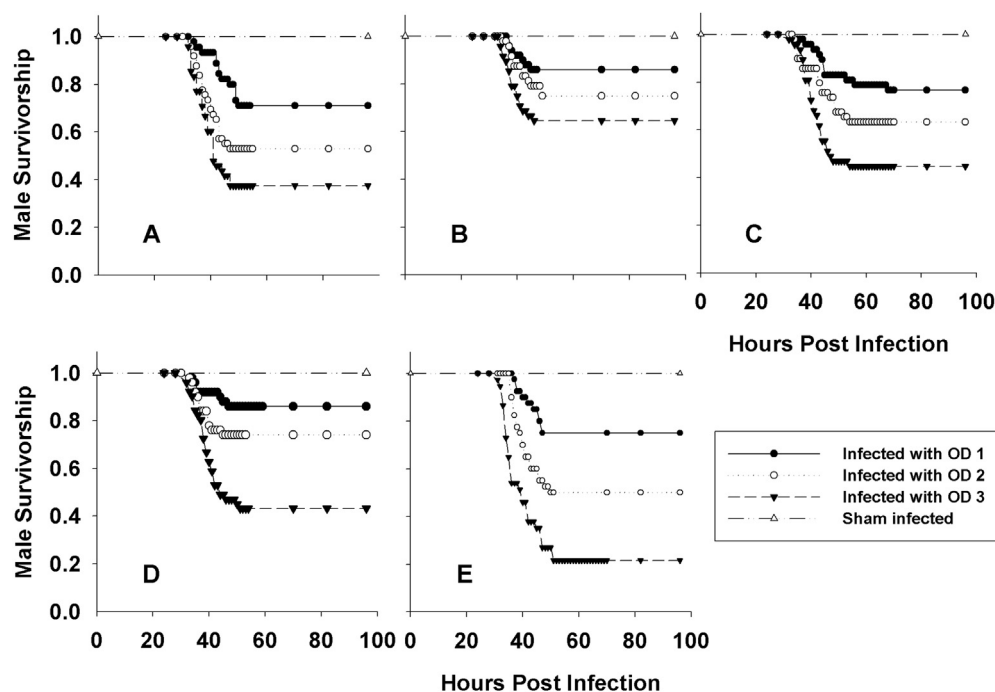


Fig. 1. Effect of initial dosage on male survivorship after infection with PK-1. Kaplan–Meier plots of survivorship. (A) PJB, (B) LH, (C) BRB, (D) Canton-S and (E) W1118 populations. For the experiment, 45–50 male flies were infected for each population and for each OD. Survivorship significantly declines in all population (all $p < 0.05$, Cox proportion regression analysis). We did not find any death in case of sham infected flies over the time of the observation.

Table 3

Effect of bacterial dosage of infection on the survivorship of the males (A) and females (B) host. Summary of Kaplan–Meier analysis of survivorship data of males (A) and females (B) infected with PK-1. *p*-values in bold indicate significant differences. — mortality less than 50%.

Traits	Dosage OD _{600nm}	Host populations	Median time to death (hours)	% of death	<i>p</i>
(A) Males	1	PJB	—	28.889	<0.001
	2		—	46.939	
	3		41	60.417	
	1	LH	—	14	0.017
	2		—	25	
	3		—	37.5	
	1	BRB	—	23.404	0.001
	2		—	36.735	
	3		47	55.319	
	1	Canton-S	—	14	<0.001
	2		—	26	
	3		44	56.863	
	1	W1118	—	25	<0.001
	2		—	51.219	
	3		40	78.378	
(B) Females	1	PJB	—	39.583	0.043
	2		52	55.102	
	3		53	59.574	
	1	LH	—	14.286	0.006
	2		—	30.612	
	3		48	41.667	
	1	BRB	—	37.5	0.899
	2		—	45.833	
	3		—	40.816	
	1	Canton-S	—	26	<0.001
	2		57	58	
	3		47	72.549	
	1	W1118	—	22.5	<0.001
	2		—	37.5	
	3		47	67.5	

3.3.2. Effect of mating status on post infection survivorship

Mating activity does not change the males' and females' survivorship in several populations of the *D. melanogaster* when infected with PK-1 strain (Table S3A, B). The survivorship data of mating status experiment was analysed using Cox proportion regression model and Kaplan–Meier estimator indicated that there was no significant difference between virgin and mated males (Fig. 3A–C, Table S3A) and females (Fig. 3D–F, Table S3B). This result was consistent across the two outbred and one inbred populations (Table S3A, B). We did not find any death in the sham infected controls.

3.3.3. Can flies be infected with PK-1 through oral route?

When PK-1 bacterial suspension was administered orally, we did not find any death in adult flies. Treated flies survived similar to control flies indicating that the bacteria fails to establish a sustained infection if administered orally.

3.3.4. Does PK-1 grow within the flies?

To further confirm whether the PK-1 grows within the fly body post infection, we assayed bacterial load at 0 h and 24 h post infection. We found that males and females received about 330 bacterial cells at 0 h post infection (Fig. S1). At 24 h

post infection, number of colony forming units was significantly higher ($\sim 2.5 \times 10^6$ cells) in both sexes relative to 0 h post infection. We did not find sex $\times$ time interaction indicating that both males and females have similar bacterial load 24 h post infection (Fig. S1, Table S1).

4. Discussion

Drosophila has emerged as an important model in the studies of the evolutionary ecology of immune response. However, there seems to be few bacterial pathogens that are capable of causing mortality in adult flies (*Providencia rettgeri*, *Providencia alcalifaciens*, *Enterococcus faecalis*, *Providencia burhodogranaria*, *Providencia sneebia*) [5,11]. Our major aim in this study was to isolate bacterial strains from wild caught flies that could potentially induce mortality in adult flies. We screened a large number of bacterial isolates from wild caught flies and found one of them was pathogenic to flies. The bacteria belonged to *S. succinus* subsp. *succinus*. The strain was new and was named as PK-1. This strain was pathogenic across different host populations of *D. melanogaster* and the induced mortality in various populations was between 36%–62.5% for males and 40.2–59.8% for females. It is important to note that several of the populations that we have used are maintained as large, outbred populations that are expected to harbour substantial genetic variation and are hence expected to be vigorous. Given that the new bacterial isolate was pathogenic to both outbred and inbred populations it is likely to be a good model pathogen for future studies. This new strain establishes sustained infection in flies by growing from ~ 320 cells to $\sim 2.5 \times 10^6$ cells per fly over a period of 24 h post infection. The new pathogen is more virulent than at least one known pathogen from the same genus, *S. saprophyticus* subsp. *saprophyticus*, described in supplementary information (see SupInfo. S2).

The *Staphylococci* are gram positive bacteria and are ubiquitous in nature (presently 52 species including 28 subspecies are known according to List of Bacterial Names with Standing in Nomenclature LSPN 2015; updated 1 August, 2015). *Staphylococci* have been isolated from diverse environmental sources such as soil, water, plants surfaces, meat and poultry, dairy products, air, skin, skin glands and mucous membrane of various mammals and birds [23,25,26] and human clinical materials [27]. Furthermore, *Staphylococcus* spp. was isolated from *D. melanogaster* pupae [28]. At present only two subspecies of *S. succinus* are known, *S. succinus* subsp. *succinus* [22] and *S. succinus* subsp. *casei* [19]. According to the 16S rDNA gene sequence, our new isolate belongs to genus *Staphylococcus*, sharing 99.70% sequence similarity with *S. succinus* subsp. *succinus* (AMG-D1) and 99.57% sequence similarity with *S. succinus* subsp. *casei* (SB72).

DNA–DNA hybridization assay also indicates that the new isolate belongs to either *S. succinus* subsp. *succinus* or *S. succinus* subsp. *casei*. Whole genome DNA–DNA relatedness shows more than 70% similarity which is generally accepted as a limit of species delineation a species [21]. In FAMES

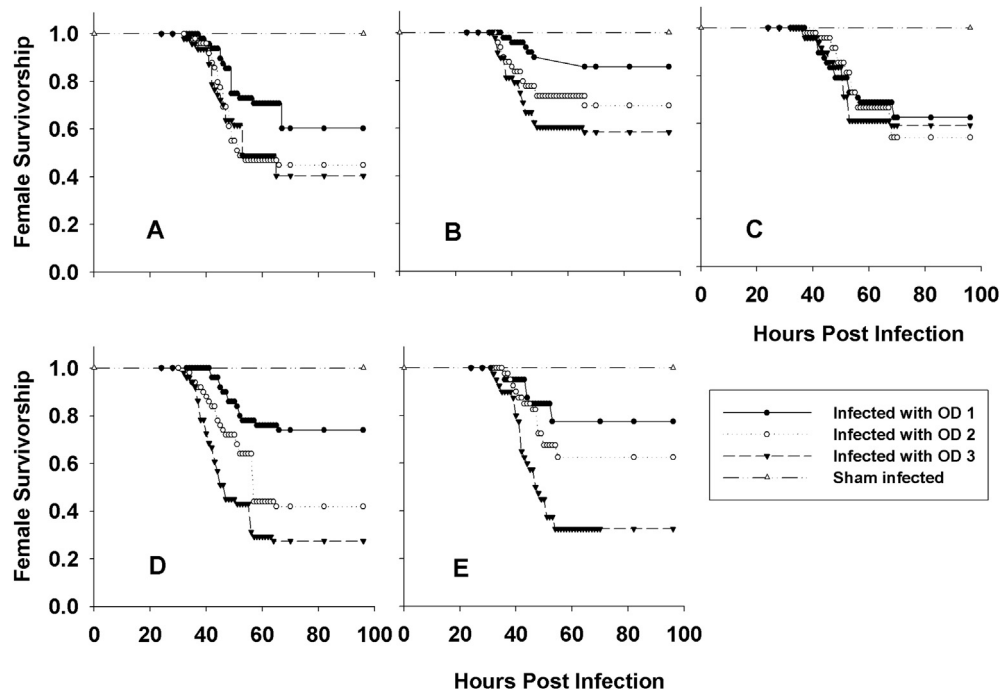


Fig. 2. Effect of initial dosage on female survivorship after infection with PK-1. Kaplan–Meier plots of survivorship. (A) PJB, (B) LH, (C) BRB, (D) Canton-S and (E) W1118 populations. For the experiment, 45–50 female flies were infected for each population and for each OD. Survivorship significantly declines in all populations with increasing OD (all $p < 0.05$, Cox proportion regression analysis) except in BRB population where initial dosage had no significant effect on mortality. We did not find any death in case of sham infected flies over the time of the observation.

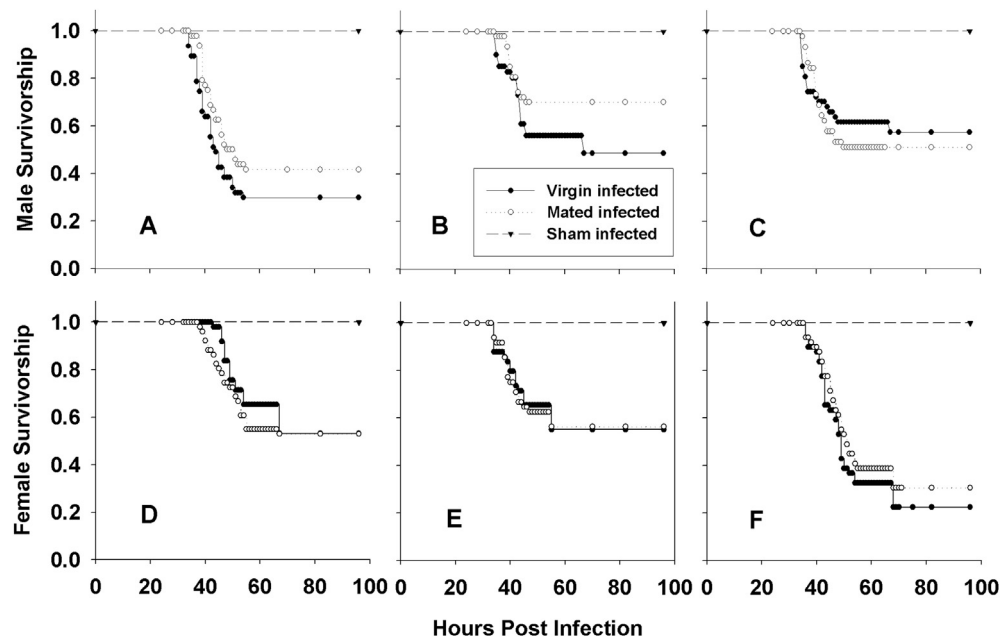


Fig. 3. Effect of mating status on survivorship post infection with PK-1. Kaplan–Meier plots of survivorship of mated males and virgin males from PJB (A), LH (B) and Canton-S (C) populations. Kaplan–Meier plots of survivorship of mated females and virgin females from PJB (D), LH (E) and Canton-S (F) populations. There was no significant difference between sexually active and virgin flies (males and females) (all $p > 0.05$, Cox proportion regression analysis). We did not find any death in the case of sham infected flies over the time of the observation.

analysis, presence of the fatty acid 11:0 iso, 11:0 anteiso, 12:0 iso, 13:0 anteiso, Iso 17:1 ω 10c, TBSA10Me 18:0 (Table 2) strongly suggests that the new isolate belongs to either *S. succinus* subsp. *succinus* or *S. succinus* subsp. *casei* because TBSA10Me 18:0 is reported in only one species

(*Staphylococcus succinus*) in genus *Staphylococcus*. However, *hsp60* gene sequence similarity shows that the new isolate shares 98.6% sequence similarity with *S. succinus* subsp. *succinus* and 97.6% with *S. succinus* subsp. *casei*. These findings strongly suggest that the new isolate is a new strain of

S. succinus subsp. *succinus*. The new isolate, named PK-1 has been deposited in two culture collection centre and assigned collection number LMG 27413 and MTCC 12337 for BCCM/LMG, Belgium and MTCC, Chandigarh, India, respectively.

Our studies also suggest that the mode of infection and initial dosage are important in determining the mortality induced by PK-1. The new isolate is pathogenic only when injected into the thorax and shows no detectable effects on survivorship when fed to the flies. Additionally, the effect of initial dosage was sex and population specific with the males in all the populations showing increased mortality with increased initial dosage while this was true of females from only some of the populations.

Several previous studies have suggested that mating activity can affect the antibacterial immunity of flies [5,29,30]. Using the new isolate, we found no significant effect of mating status on post infection survival in either males or females. Our results are in agreement with previous study from our lab [31] which found no effect of mating status on anti-bacterial immunity of males or females. Thus, our results seem to indicate that the mating cost of immunity, if any, depends on the population and the pathogens used [5].

Conflict of interest

No conflict of interest declared.

Acknowledgements

We thank Prof. Anand Kumar Bachhawat for providing lab facilities to carry out the experiments. We also thank Dr. Gandham S. Prasad (MTCC, IMTECH, Chandigarh), Dr. Stefanie Van Trappen (BCCM/LMG, Belgium) and Dr. Tapan Chakrabarti (MCC, Pune) for their help in identifying the new strain of *Staphylococcus succinus* subsp. *succinus* PK-1. Karan Singh thanks Indian Institute of Science Education and Research Mohali, and Mohammad Zulkifli thanks CSIR, Govt. Of India, for financial assistance in the form of Junior and Senior Research Fellowship, respectively. We thank Manas Samant, Dr. Anil Thakur and Saudamini Venkatesan for proof reading the manuscript. This study was supported by Indian Institute of Science Education and Research Mohali.

Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.micinf.2016.07.008>.

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