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Full Length Research Paper

Phenotyping and molecular characterization of *Lysinibacillus* sp. P-011 (GU288531) and their role in the development of *Drosophila melanogaster*

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The bacterial strain *Lysinibacillus* sp. (P-011) was isolated from the midgut of the *Drosophila melanogaster* larvae. The bacteria were gram positive, spore forming, rod shaped ranging from 1.86 to 2.5 µm in length and 0.50 to 0.67 µm in diameter, positive for catalase, indole, oxidase, nitrate reduction, starch and gelatin hydrolysis, sensitive to tetracycline, chloramphenicol, doxycycline hydrochloride, gatifloxacin, ofloxacin, vancomycin, rifampicin, levofloxacin, ciprofloxacin, nalidixic acid, but resistant to ampicillin, streptomycin, gentamycin and kanamycin. The phylogenetic tree showed that the strain *Lysinibacillus* sp. P-011 (GU288531) branched with *Lysinibacillus boronitolerans* with 89% bootstrap support. *Lysinibacillus* sp. P-011 (×10^5 cfu/ml) played an important role on larval development of *D. melanogaster* under controlled environmental condition. Wild larvae when fed on normal food as well as normal food mixed with ineffective antibiotics, developed puparium within seven days whereas took more than 10 days when fed on normal food mixed with anti P-011 antibiotics and sterile food mixed with bacterial suspension and anti P-011 antibiotics. 94 to 98% cured larvae developed puparium within seven days when fed on only sterile food mixed with bacterial suspension (P-011) or sterile food mixed with bacterial suspension (P-011) and ineffective antibiotics.

Key words: *Drosophila melanogaster*, gut-bacteria, larval development, *Lysinibacillus* sp. P-011 (GU288531), 16S rRNA gene sequence, phylogenetic tree.

INTRODUCTION

Insect guts act as reservoirs and fermentation vessel for a large variety of microorganisms. The enormous microbial diversity of insect gut may be originated from their different feeding habits, different gut structures and functions of different groups of insects promoting the establishment of different group of microbes (Dillon and Dillon, 2004). These gut microbes play important roles in various types of interactions ranging from pathogenesis to obligate mutualism (Dillon and Dillon, 2004). In various organisms, gut microbiota act as vital resource of novel bioactive compounds (Chernysh et al., 2002), enzymes (Zhang and Brune, 2004) and novel metabolites (Wilkinson, 2001). Proper scientific exploration of symbiotic gut microbes may be an alternative and effective strategy for controlling the spread of pathogens which utilize insects as hosts (Mickes and Ferguson, 1961; Lehane et al., 1997; Beard et al., 2002; Dillon et al., 2005). The presence and diversity of insect gut bacteria are influenced by the gut pH, redox conditions, digestive enzymes of insect gut and types of food ingested. The optimum pH for the growth of most bacteria ranges from 6 to 7, but some bacteria can grow at acidic pH. Anaerobic bacteria show their growth only at negative redox potentials whereas aerobic bacteria survive at positive redox potentials. Intestinal micro-organisms help in digestion of food material and also produce essential vitamins for the host. Several experimental evidences revealed that the symbiotic gut bacteria of some beetles can provide vitamin B to their

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host (Blewett and Fraenkel, 1944). The role of symbiotic gut bacteria on the survival of fruit fly refers to obligate symbiotic relationships between insect larvae and their gut microbes with respect to larval nutrition, growth and development (Brummel et al., 2004). *Drosophila melanogaster* breeds in decaying organic matter or necrotic plant material in the presence of various micro-organism and they have to interact with micro-organisms during all stages of their life cycle. Symbiotic micro-organisms may be found in the gut (Douglas et al., 2011), gonad (Mateos et al., 2006) and some other parts of the fly body. It has been proposed that several fruit-feeding *Drosophila* species are nutritionally dependent on bacteria (Mateos et al., 2006). Laboratory experiments have revealed that sugar, essential amino acid, fat, cholesterol and some salts are important nutrients for the development of the *D. melanogaster* (Sang, 2006; Sang and King, 1961; Sang, 1956). Riboflavin, nicotinic acid and pyridoxin are the essential substances for the normal growth of *Drosophila* larvae which are known to be supplied by some micro-organism (Tatum, 1939). Symbiotic bacteria have different influences on different aspects of fly life-cycle such as contribution on host nutrition (Douglas, 1998), immunity (Hedges et al., 2008; Osborne et al., 2009; Teixeira et al., 2008) and reproduction (Serbus et al., 2008). Some bacteria can enhance the life-span of the *Drosophila* sp. (Brummel et al., 2004).Scanty information is available on the resident bacterial flora of the larval midgut of *Drosophila* sp. We used *Drosophila* sp. and their gut microbiota as an experimental model of insect microbial symbiosis. Present work was designed to study the phenotypic and molecular characterization of the gut bacteria in *Drosophila* sp. and to determine their effects on larval development.

MATERIALS AND METHODS

Wild type *D. melanogaster* flies were cultured in biochemical oxygen demand (B.O.D) incubator at 24 ± 1°C using standard fly-food medium containing maize powder, sucrose, agar, yeast, sterile distilled water in the Department of Zoology of The University of Burdwan, Burdwan.

Bacteria isolation from the midgut of *Drosophila*

The third instar larvae of *D. melanogaster* were selected for the experiment. The larvae were sterilized with 70% ethanol for 3 min, washed thoroughly with sterile distilled water and their midguts were dissected out under the binocular microscope in laminar air flow. Each midgut was crushed separately on a sterile slide, gut extract was aspirated and diluted with 250 ml sterile distilled water and mixed with 100 ml nutrient agar (NA) medium (peptone–beef extract–NaCl–agar at 5:3:3:18 g/l) at pH 7.4, plated on five Petri plates and incubated in a biochemical oxygen demand incubator at 30 ± 0.1°C for 24 h (Roy et al., 2010). The most prevalent colonies developed from the gut triturate of *Drosophila* sp. were then maintained on nutrient agar slants at 4 ± 0.1ºC in refrigerator.

Morphological and biochemical characterization

The bacteria P-011 was obtained throughout the year from various larval stages of *D. melanogaster*. Colony characteristics (shape, size, colour, margin and opacity of the colonies on NA plates), morphology of the strains (shape and size of vegetative cells and spores, if any) and motility of the strain were recorded under 100X objective of a phase-contrast microscope following standard methods (Smibert and Krieg, 1995; Lacey, 1997). Physiological and biochemical properties of the bacteria were studied following standard methods (Pelczar et al., 1957; Sneath, 1986; Collee and Miles, 1989; Lacey, 1997). Gram staining, NaCl (1 to 10%) tolerance and hydrolysis of different substrates (starch, protein and lipid) were observed. Antibiotic sensitivity was tested using the following antibiotic discs: ampicillin (10 µg/disc), tetracycline (30 µg/disc), chloramphenicol (30 µg/disc), doxycycline hydrochloride (30 µg/disc), gatifloxacin (5 µg/disc), streptomycin (10 µg/disc), kanamycin (30 µg/disc), ofloxacin (5 µg/disc), vancomycin (30 µg/disc), rifampicin (5 µg/disc), gentamycin (10 µg/disc), levofloxacin (5 µg/disc), ciprofloxacin (5 µg/disc), nalidixic acid (30 µg/disc) (Brown, 2007) and sensitivity to antibiotics was judged by inhibition zone formation.

Scanning electron microscopy (SEM) of bacterial isolates

Bacterial smears were prepared on cover glasses, heat fixed over a flame for 1 to 2 s followed by 2.5% glutaraldehyde (aqueous) for 45 min. The slides were then dehydrated passing through 50, 70, 90% ethanol and finally with absolute alcohol for 10 min each. The specimens were gold coated and finally scanned and photographed under Scanning Electron Microscope (Model Hitachi S-530).

Molecular characterization and phylogenetic analysis of gut bacteria

Genomic DNA was isolated from the pure culture pellet using genomic DNA isolation kit. The ~1.5 kb rDNA fragment was amplified using high-fidelity PCR polymerase. The PCR product was sequenced bi-directionally through a genetic analyzer using the forward primer and reverse primer. The nucleotide sequence of the bacterial isolate P-011 has been submitted to the NCBI GenBank database and assigned accession number GU288531. Most similar strain sequences were retrieved from EzTaxon-e, a prokaryotic 16S rRNA Gene sequence database taking *Lysinibacillus* sp. P-011(GU288531) as a reference sequence. Alignment view and distance matrix table was constructed following Kim et al. (2012). Sequence was analyzed and restriction map was prepared with enzymes available in New England Biolab. The sequence data were aligned using the ClustalW submission form (http://www.ebi.ac.uk/clustalw) and analyzed by ClustalW software (Thompson et al., 1994). Evolutionary distances were calculated using the method of Jukes and Cantor (1969) and phylogenetic tree was constructed according to Tamura et al. (2007)'s research.

Evaluation of the role of gut bacteria on larval development

In order to observe the effect of the symbiotic bacteria on host body, we recorded the duration of larval development and formation of puparium in the presence and absence of the gut bacteria. For each test, 50 1st instar larvae and three replications were used. All the tests were conducted in culture bottles holding standard *Drosophila* food medium, autoclaved at 121°C at 15 lb pressure. Third instar larvae were cultured for 24 h on food containing 100 µl mixture of antibiotics (chloramphenicol (10 µg/ml), tetracyclin (10 µg/ml), and doxycyclin (10 µg/ml) to which the bacterial isolate

Table 1. Phenotypic and biochemical characterization of the *Lysinibacillus* sp. P-011.

Character	Observation	Character	Observation
Colony character	Spherical, cream, opaque, elevated, smooth, entire rods, Gram (+) ve	Urease production test	–
		Oxidase	+
		H_2S Production test	–
		Starch hydrolysis	+
Bacterium (l x w, µm)	(1.86 - 2.5 × 0.50 - 0.67) µm	Gelatin hydrolysis	+
		Caesin hydrolysis	–
		Chitin hydrolysis	–
NaCl tolerance	Up to 6%	**Acid and gas production**	
Temperature tolerance	Up to 60ºC	Glucose	+
pH tolerance (up to 8)	+	Sucrose	+
Catalase	+	Lactose	–
Indole production	+	Dextrose	+
Methyl red test	–	Maltose	+
Vogues-Proskauer test	–	Mannitol	+
Citrate Test	–		
Nitrate reduction test	+	**Antibiotic sensitive (µg/disc)**	
		Doxycycline hydrochloride (30)	
Antibiotic resistant (µg/disc)		Tetracycline (30)	
Ampicillin (10)		Chloramphenicol (30)	
Streptomycin (10)		Gatifloxacin (5)	
Gentamycin (10)		Ofloxacin (5)	
Kanamycin (30)		Vancomycin (30)	
		Rifampicin (5)	
		Levofloxacin (5)	
		Ciprofloxacin (5)	
		Nalidixic acid (30)	

showed sensitivity. These axenically cultured *D. melanogaster* flies were transferred to each experimental culture bottle containing normal or sterile food medium. To assess the role of the bacteria P-011, on *D. melanogaster* larvae, 100 µl bacterial solution (10^5 cfu/plate) were mixed separately with food medium except the bottle containing only normal food and only sterile food. Duration of larval development to form puparium was recorded to show whether presence of bacteria have played any role in the development of *D. melanogaster*. Identical experiments were done with untreated *D. melanogaster* flies separately at 24 ± 1°C and were observed daily for the first 10 days and every other day thereafter, developmental duration of each stage being noted.

RESULTS AND DISCUSSION

The colonies of the bacteria (P-011) were spherical, cream colour, opaque and elevated (Table 1). The bacteria were rod shaped. Length of the organisms ranged from 1.86 to 2.5 µm and 0.50 to 0.67 µm in diameter (Plate 1). The bacteria were positive for Gram staining, spore forming and could tolerate up to 60°C and up to 6% NaCl (Table 1). The organism was positive for catalase, indole, oxidase, nitrate reduction, starch and gelatin hydrolysis but negative for citrate utilization, methyl red, vogues-Proskauer test, casein and chitin hydrolysis. Response of the organisms to the recommended doses of different antibiotics showed that all of them were sensitive to tetracycline (30 µg/ml), chloramphenicol (30 µg/ml), doxycycline hydrochloride (30 µg/ml), gatifloxacin (5 µg/ml), ofloxacin (5 µg/ml), vancomycin (30 µg/ml), rifampicin (5 µg/ml), levofloxacin (5 µg/ml), ciprofloxacin (5 µg/ml), nalidixic acid (30 µg/ml), but resistant to ampicillin (10 µg/ml), streptomycin (10 µg/ml), gentamycin (10 µg/ml), kanamycin (30 µg/ml) (Table 1). The nucleotide composition is shown in Figure 1. AT and GC content were 46.55 and 53.45%, respectively. Restriction map has been displayed by Figure 2. Phylogenetic affiliation of the bacterium (P-011) was done by 16S rRNA gene sequence analysis. Alignment view and distance matrix table (Table 2) depicted that *Lysinibacillus* sp. (P-011) showed 96.30% similarity with *Lysinibacillus macroides* (AJ628749) and 95.92% with *Lysinibacillus boronitolerans* (AB199591). To assign the taxonomical affiliation of this bacterium, the phylogenetic tree was constructed through multiple sequence alignments followed by a neighbor-joining analysis (Saitou and Nei, 1987) (Figure 3). The phylogenetic

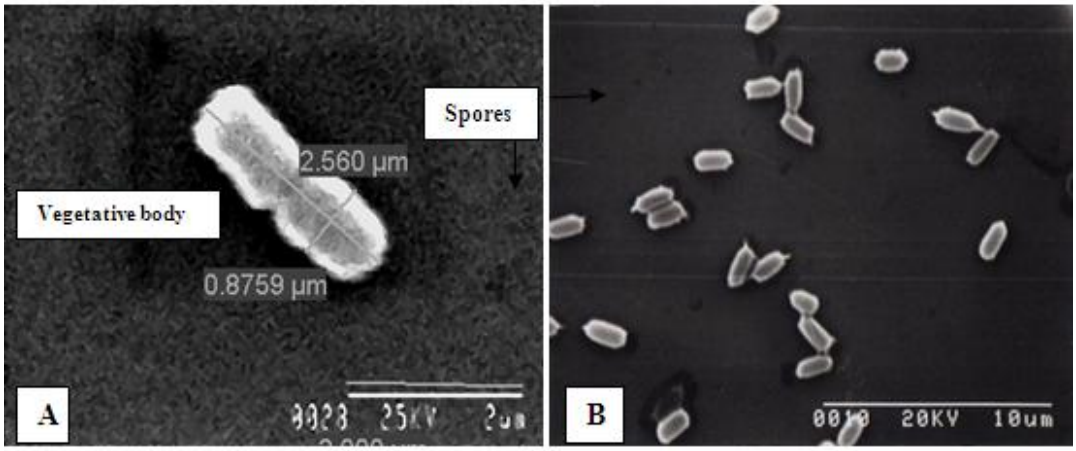

Plate 1. Vegetative body (A) and spores (B) of *Lysinibacillus* sp. P-011.

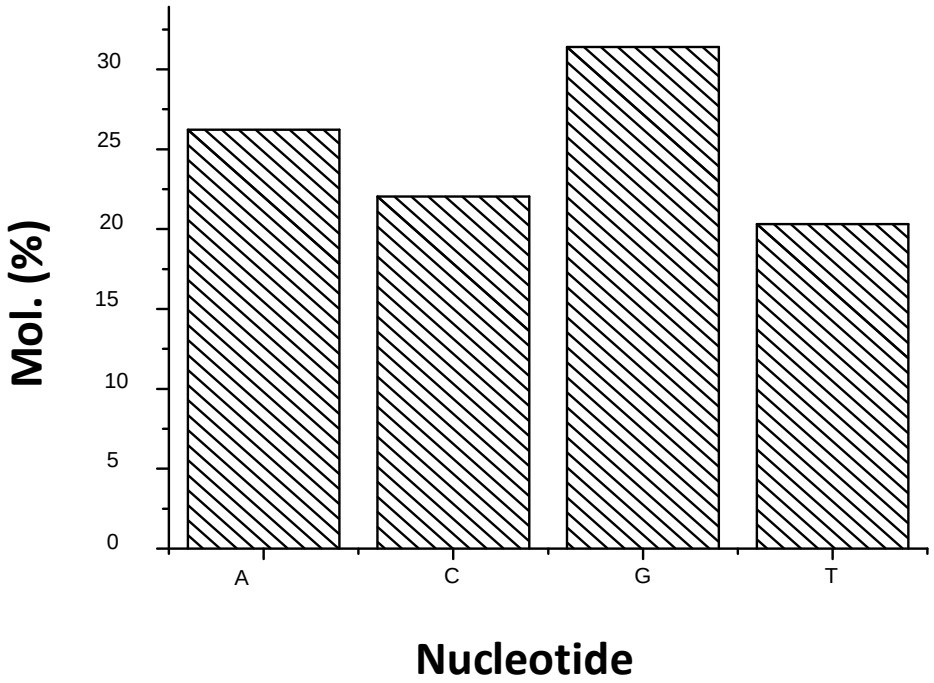

Figure 1. Nucleotide composition of 16s rRNA gene sequence of *Lysinibacillus* sp. (GU 288531).

tree showed that the strain *Lysinibacillus* sp. P-011 (GU288531) branched with *L. boronitolerans* (AB199591) with 89% bootstrap support. The cluster containing *Lysinibacillus* sp. (GU288531) and *L. boronitolerans* (AB199591) branched with *L. marcoides* (AJ628749) with 70% bootstrap support. To observe the effect of bacteria *Lysinibacillus* sp. (P-011) on duration of larval development of *D. melanogaster*, several experiments were done (Table 3). When the wild type larvae were fed on normal food, it developed puparium within seven days in the B.O.D incubator at controlled environmental condition. Similar result was found when the wild type larvae were fed on normal food with ineffective antibiotics. Wild type and cured larvae took more than 10 days to develop puparium when fed on normal food mixed with anti P-011 antibiotics and sterile food mixed with bacterial suspension and anti P-011 antibiotics, respectively (Table 3). Previous published works show that *Lysinobacillus* sp. can promote plant growth (Vendan et al., 2010) and nitrogen fixation (Vendan et al., 2010;

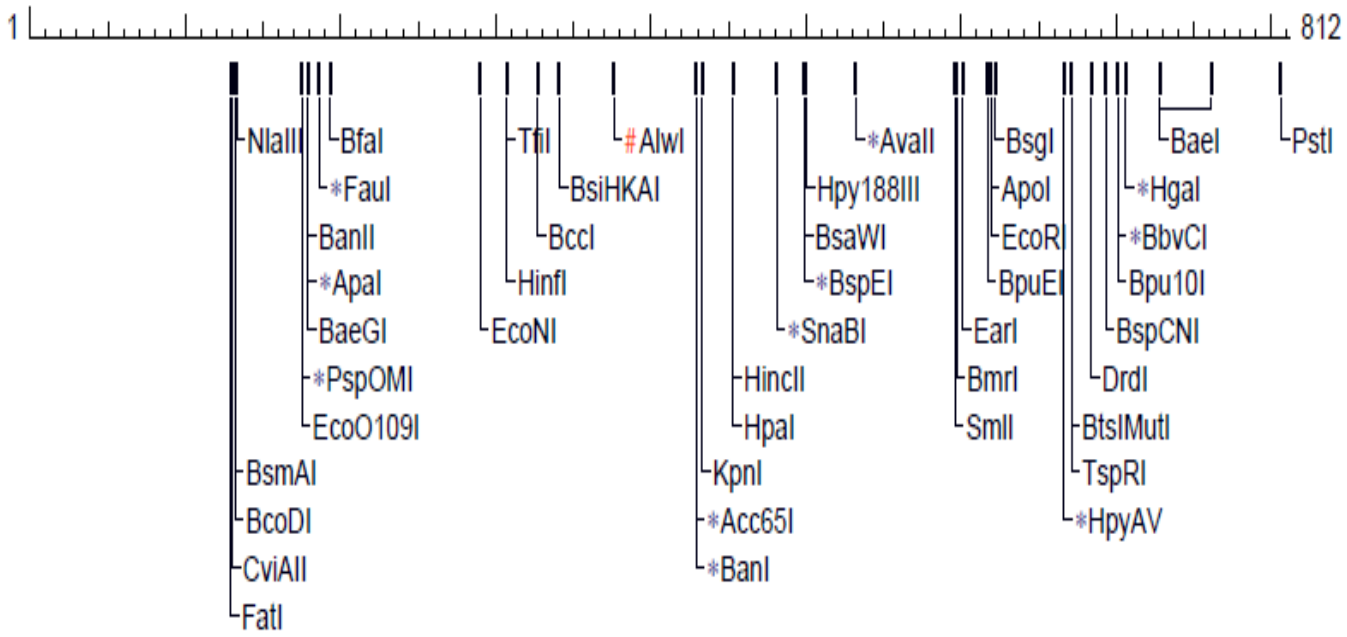

Figure 2. Restriction map of the nucleotide sequence of *Lysinibacillus* sp. P-011 (GU288531).

Table 2. Alignment view and distance matrix table taking *Lysinibacillus sp*. (GU288531) as reference sequence.

Rank	Name	Strain	Accession	Pairwise similarity (%)	Completeness (%)
1	*Lysinibacillus macroides*	LMG 18474(T)	AJ628749	96.3	100
2	*Lysinibacillus boronitolerans*	T-10a(T)	AB199591	95.92	98.1
3	*Lysinibacillus xylanilyticus*	XDB9(T)	FJ477040	95.8	91.5
4	*Bacillus decisifrondis*	E5HC-32(T)	DQ465405	94.72	88.4
5	*Lysinibacillus mangiferahumi*	M-GX18(T)	JF731238	94.7	98.4
6	*Lysinibacillus sp*	NBRC 15717(T)	AB271743	94.45	100
7	*Lysinibacillus sphaericus*	C3-41	CP000817	94.45	100
8	*Lysinibacillus sphaericus*	ATCC 14577(T)	L14010	93.88	100
9	*Lysinibacillus massiliensis*	4400831(T)	AY677116	93.32	100
10	*Lysinibacillus parviboronicapiens*	BAM-582(T)	AB300598	93.05	100
11	*Lysinibacillus odysseyi*	34hs-1(T)	AF526913	92.85	100
12	*Paenisporosarcina quisquiliarum*	SK 55(T)	DQ333897	92.36	98.7
13	*Chryseomicrobium imtechense*	MW 10(T)	GQ927308	92.24	96.8
14	*Sporosarcina antarctica*	N-05(T)	EF154512	91.95	98.3
15	*Sporosarcina soli*	I80(T)	DQ073394	91.84	100
16	*Psychrobacillus psychrodurans*	DSM 11713(T)	AJ277984	91.63	100
17	*Paenisporosarcina macmurdoensis*	CMS 21w(T)	AJ514408	91.62	99.3
18	*Sporosarcina ureae*	DSM 2281(T)	AF202057	91.47	100
19	*Psychrobacillus psychrotolerans*	DSM 11706(T)	AJ277983	91.38	99.4
20	*Psychrobacillus insolitus*	DSM 5(T)	AM980508	91.37	100
21	*Sporosarcina saromensis*	HG645(T)	AB243859	91.34	100
22	*Sporosarcina newyorkensis*	6062(T)	GU994085	91.27	100
23	*Sporosarcina contaminans*	CCUG 53915(T)	FN298444	91.22	99.2
24	*Filibacter limicola*	DSM 13886(T)	AJ292316	91.22	100
25	*Bacillus seohaeanensis*	BH724(T)	AY667495	91.19	95.1
26	*Viridibacillus arenosi*	LMG 22166(T)	AJ627212	91.12	100

Table 2. Continued.

27	*Planococcus rifietoensis*	M8(T)	AJ493659	91.09	100
28	*Planomicrobium koreense*	JG07(T)	AF144750	91.09	100
29	*Planococcus plakortidis*	AS/ASP6(II)(T)	JF775504	91.09	97.8
30	*Caryophanon tenue*	DSM 14152(T)	AJ491303	91	100
31	*Caryophanon latum*	DSM 14151(T)	AJ491302	91	99.5
32	*Sporosarcina globispora*	DSM 4(T)	X68415	90.98	100
33	*Planomicrobium psychrophilum*	CMS 53or(T)	AJ314746	90.98	100
34	*Planomicrobium alkanoclasticum*	MAE2(T)	AF029364	90.95	97
35	*Planomicrobium okeanokoites*	IFO 12536(T)	D55729	90.92	100
36	*Planomicrobium flavidum*	ISL-41(T)	FJ265708	90.92	100
37	*Viridibacillus arvi*	LMG 22165(T)	AJ627211	90.88	100
38	*Sporosarcina aquimarina*	SW28(T)	AF202056	90.86	100
39	*Sporosarcina psychrophila*	IAM 12468(T)	D16277	90.85	100
40	*Bacillus circulans*	ATCC 4513(T)	AY724690	90.82	100
41	*Bacillus cecembensis*	PN5(T)	AM773821	90.75	100
42	*Rummeliibacillus pycnus*	NBRC 101231(T)	AB271739	90.73	100
43	*Bacillus nealsonii*	DSM 15077(T)	EU656111	90.72	100
44	*Planococcus maitriensis*	S1(T)	AJ544622	90.61	95.8
45	*Bacillus kochii*	WCC 4582(T)	FN995265	90.56	100
46	*Planococcus maritimus*	TF-9(T)	AF500007	90.48	100
47	*Planomicrobium mcmeekinii*	S23F2(T)	AF041791	90.46	99.7
48	*Planomicrobium chinense*	DX3-12(T)	AJ697862	90.45	100
49	*Jeotgalibacillus salarius*	ASL-1(T)	EU874389	90.26	100
50	*Kurthia gibsonii*	NCIMB 9758(T)	X70320	90.21	97.8
51	*Planococcus salinarum*	ISL-16(T)	FJ765415	90.11	100
52	*Kurthia sibirica*	DSM 4747(T)	AJ605774	90.11	100
53	*Rummeliibacillus stabekisii*	KSC-SF6g(T)	DQ870754	90.09	98.5
54	*Planomicrobium glaciei*	423(T)	EU036220	89.73	98.7
55	*Bacillus aquimaris*	TF-12(T)	AF483625	89.38	100
56	*Ureibacillus composti*	HC 145(T)	DQ348071	89.01	100
57	*Bacillus vietnamensis*	15-1(T)	AB099708	88.88	94.1
58	*Ureibacillus thermosphaericus*	DSM 10633(T)	AB101594	88.52	100
59	*Bacillus horikoshii*	DSM 8719(T)	X76443	88.5	100
60	*Ureibacillus thermophilus*	HC148(T)	DQ348072	88.38	100
61	*Bacillus coahuilensis*	m4-4(T)	ABFU01000135	88.04	100
62	*Bacillus clausii*	DSM 8716(T)	X76440	87.14	100

Table 3. Effect of *Lysinibacillus* sp. (P-011) on the duration of larval development of the *D. melanogaster* *.

S/N	Treatment	Larvae	Number of larvae develop to puparium	
			< 7days	>10 days
1	Normal food (Control)	Wild	50.0 ± 00	0
2	Sterile food	Wild	45.0 ± 0.33	0
3	Normal food + anti P-011 antibiotics	Wild	0	50±0
4	Normal food + ineffective antibiotics	Wild	50±0	0
5	Sterile food +P-011	Cured	49 ±0.57	0
6	Sterile food +P-011+ anti P-011 antibiotics	Cured	0	50±00
7	Sterile food +P-011+ ineffective antibiotics	Cured	47 ±0.33	0

*****For each test, 50 1st instar larvae and three replications were used. Data are means of three replications ± SE.

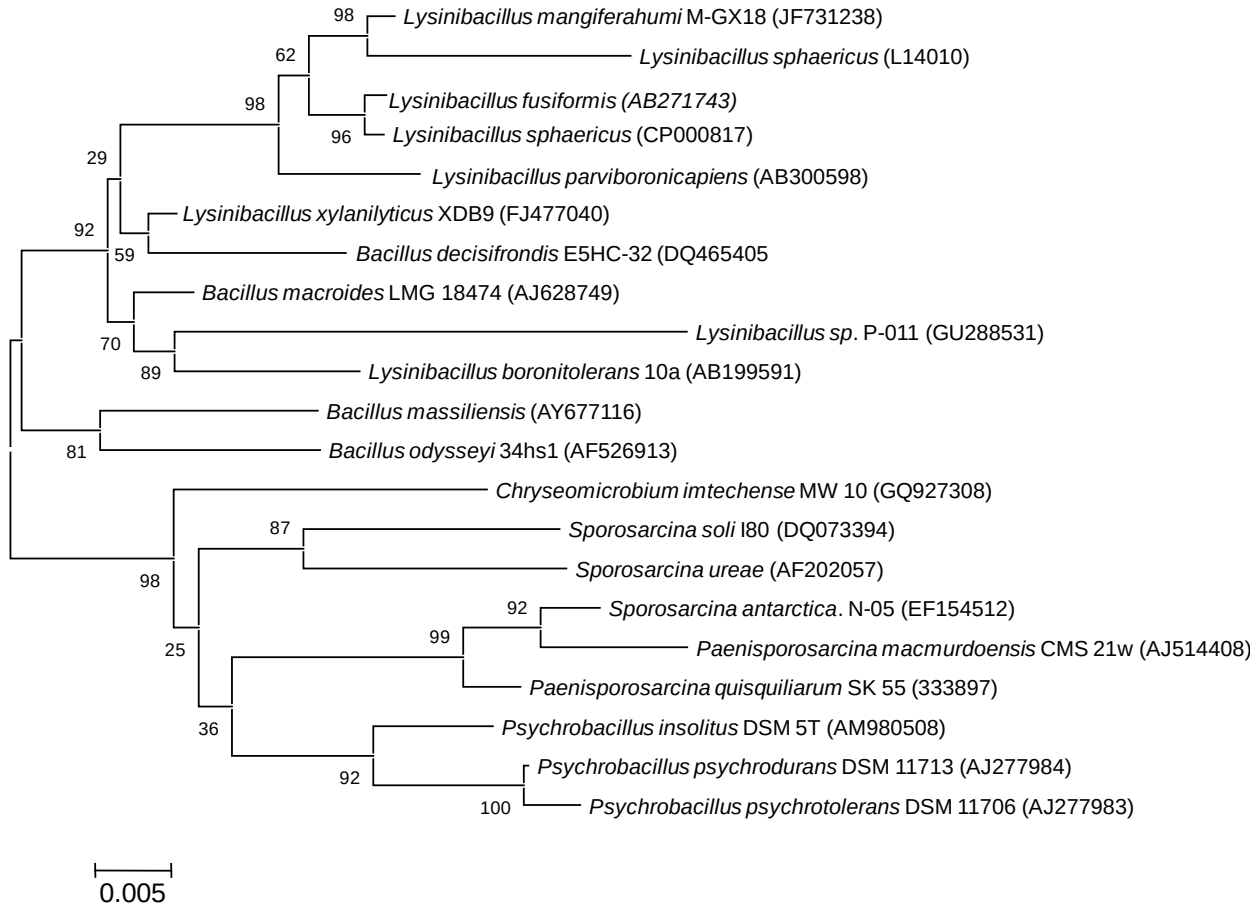

Figure 3. Neighbor-joining tree constructed based on 16S rRNA genes sequence of *Lysinibacillus* sp. *P*-011 (GU288531) along with other 16S rRNA genes.

Sgroy et al., 2009), which supports the growth of the insects (Rajagopal, 2009). It has also been reported that several midgut bacteria like *Acetobacter pomorum*, *Gluconobacter morbifer*, *Lactobacillus plantarum, Lactobacillus brevis* and *Commensalibacter intestine,* have beneficial role on larval development. Absence of these bacteria has been shown to lengthen time duration to reach puparium formation in *D. melanogaster* larvae (Ryu et al., 2011; Douglas et al., 2011). The results clearly indicate that the time to puparium formation is delayed due to the elimination of *Lysinibacillus* sp. (P-011) from larval midgut. *Lysinibacillus* sp. (P-011) has been isolated from all the larval stages in all the seasons throughout the year. So, it is proved that it is not a mere transient flora inhabiting the midgut rather an important resident symbiotic flora of *D. melanogaster* playing an important physiological role in larval development.

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