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Characterization of Bacterial Isolates as Natural Biocontrol Agents of Bollworm from an Epizootic Pest (*Heliothis armigera*)

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ABSTRACT

In an attempt to screen biocontrol agents, nine bacterial strains were isolated from a dead lepidopteron pest *Heliothis* sp. (bollworm) of a heavily infested pea field of the village Khalad, Pune, Maharastra, India. The collected epizootic *Heliothis* sp. specimens were brown in color with a blackish alimentary canal, clearly visible from the ventral side. While testing their pathogenicity, isolates AB_2, AB_4, AB_7, and AB_8 were found to be pathogenic to *Heliothis armigera* following Koch's postulate. Through a biochemical assays and 16S rRNA gene sequencing the isolates were identified as *Serratia entomophila* (AB_2), *Cronobacter sakazakii* (AB_4), and *Salmonella choleraesuis* (AB_7 and AB_8) belonging to the family Enterobacteriaceae. The isolates are the first report as bacterial entomo-pathogens of an insect epizootic from India.

Keywords: bacteria, biocontrol, entomopathogen, *Heliothis armigera*

INTRODUCTION

Insects and other pests cost billions of dollars annually to farmers resulted from crop loss (in terms of quantity and quality) which are added to the cost of production (Ignacimuthu and Jayaraj 2003). Every year millions of dollars of chemical pesticides are invested for chemical pesticides to control lepidopteron pests like *Heliothis armigera* (bollworm), *Spodoptera littoralis* (cutworm) and *Plutella xylostella* (diamondback moth) (Khan and Law 2005). During the last couple of decades, the use of such synthetic chemicals has raised a number of environmental issues causing health hazards (Nauen and Denholm 2005). Thus, it needs scientists to comply with the upcoming adverse situation by exploring potential microbes (Lacey and Shapiro-Ilan 2008).

Insect pests, the entomopathogens, are susceptible to different pathogens, like other living organisms. Common entomopathogens include viruses (granulosis virus, GV; nuclear polyhedrosis virus, NPV), bacteria (*Bacillus thuringiensis*, *Serratia entomophila*), and fungi (*Beauveria bassiana*, *Aspergillus nomius*). Many bacterial entomopathogens have been developed to utilize as commercial controlling agents, including Gram-positive (*Bacillus thuringiensis*, *B. cereus*, *B. subtilis*, *Burkholderia cepacia*) and Gram-negative (*Pseudomonas fluorescens*, *Serratia entomophila*) microorganisms (Howell and Stipanovic 1979; Johnson *et al.* 2001; Roh *et al.* 2007; Jeong *et al.* 2010). The use of such biocontrol agents is gaining momentum, since they are easy to deliver, less prone to pest resistance and improve plant growth (Ignacimuthu *et al.* 2000; Nauen and Denholm 2005; Young *et al.* 2009). Among biocontrol agents, bacteria are being increasingly recognized as plant protection agents as some bacteria inhibit the growth of pathogenic fungi, nematodes and insects (Whipps 2001; Siddiqui *et al.* 2007). The epizootics in nature provide a rare chance to isolate and to identify natural pathogens of pests. The present paper describes the isolation and characterization of bacterial entomopathogens of epizootic Lepidopteran pests from a pea field in a district of Pune in Maharashtra, India.

MATERIALS AND METHODS

Collection of pests

Dead epizootic *Heliothis* were collected from a heavily infested pea field of the village Khalad, Purandar, Pune district of Maharashtra, India following the methods of Krieg (1987). The field had no history of application of biopesticides and no report of using chemical pesticides, at least six months prior to the collection. The collected specimens were brown with a blackish alimentary canal clearly visible from the ventral side.

Isolation of gut colonizing bacteria

Homogenized suspension (in 5% sterile saline) of alimentary tract was used as source sample to inoculate into brain heart infusion agar (BHI-agar, Hi-media, India) plates and incubated at 30°C for 48 h. Well separated and distinctly different bacterial colonies were detected and transferred to the slants of same medium. Purity of the isolates was checked based on differential colony morphology and maintained at 4°C in a refrigerator.

Rearing of bollworm

Heliothis armigera larvae were obtained from the stock of the R&D facility of M/S Ajay Biotech (I) Ltd, India. Larvae were maintained on a commercial diet separately (IM002, Hi-Media, Mumbai, India) in vials at constant ambient temperature (Sheikh *et al.* 1990; Chenchaiah and Bhattacharya 2005). In order to obtain a homogeneous mass of the test insects, the larvae were reared till emergence of adults. After rearing for two generations, the 5-day-old larvae were used for bioassay tests (Rahman and Talukder 2006).

Screening of pathogens

The experimental larvae were fed with a commercial diet (Hi-Media) mixed with test culture (1.5×10^6 to 1.5×10^{11} cfu ml^{-1}), separately for different doses for 24 h. Thereafter, the larvae were transferred to a fresh set of vials and maintained on a commercial

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Research Note

Table 1 Colony morphology and cell type of the bacterial strains isolated from epizootic *Heliothis armigera*.

Bacterial isolate	Colony morphology on nutrient agar plates at 24 h of growth	Cell type	Gram nature
AB_1	Round, smooth, brown, 3.5 mm	Rods	- ve
AB_2	Round, smooth, cream-white, slimy, 2 mm	Cocci	- ve
AB_3	Round, rough, blackish, slimy, pin-headed	Cocci	- ve
AB_4	Round, smooth, bluish, pin-head colony	Rods	- ve
AB_5	Round, smooth, semi transparent, slimy, 2.5-30 mm	Cocci	- ve
AB_6	Round, rough, purple, slimy, 2.5-3.0 mm	Cocci	- ve
AB_7	Ovoid, rough, semi transparent, slimy, 1-2 mm	Rods	- ve
AB_8	Round, smooth, dirty white, pin headed	Rods	- ve
AB_9	Round, rough, brown, 2.5-3.0 mm	Rods	- ve

diet only. Each experimental batch contained 30 larvae which were examined regularly and mortality rate was recorded every 12 h. From the gut of dead bollworms, bacteria were again isolated and verified for their virulence (Falkow 1988).

Biochemical characterization

To characterize the working isolates, morphological, cultural, staining and biochemical properties were ascertained (Son *et al.* 2003). Test kits (Hi-Media) were used for biochemical (KB002 Hi-AssortedTM), carbohydrate fermentation (KB009 Hi-carbohydrate™) and paper octa-disc for antibiotic sensitivity tests. After detailed characterization, the generic identifications were made following Bergey's Manual of Systematic Bacteriology (Williams *et al.* 2003).

Characterization by 16s rDNA

For their species level identification, 16S rRNA gene sequencing was performed. 16S rRNA genes were amplified with specific primers f27 and r1492 from DNA samples from boiled cell extracts with a thermo cycler (Applied Biosystems, US) using a high fidelity PCR master kit (Roche Applied Science, US) following the manufacturer's instructions (Gerhardt *et al.* 1994). 16S rRNA gene sequences from PCR products were determined using universal primers and fluorescent labeled dideoxynucleotide terminators, in an ABI PRISM 377 automated DNA sequencer in accordance with the manufacturer's protocol for *Taq* DNA polymerase initiated cycle sequencing reactions (Gerhardt *et al.* 1994). Using FASTA version 3.4, 16S rRNA gene sequences of the isolates were compared against those in the EMBL, GenBank and DDBJ databases (Pearson and Lipman 1988) and similarities of the closest related species were determined. Using CLUSTAL W multiple alignments of sequences were executed (Thompson *et al.* 1994) to find a consensus neighbour-joining tree (Saitou and Nei 1987) out of 1000 phylogenetic trees produced through *MEGA* version 4 programme (Tamura *et al.* 2007). Bootstrap values (1000 replicates) were calculated to validate the reproducibility of the branching pattern (Felsenstein 1985).

RESULTS AND DISCUSSION

Selection of pathogens

Based on colony morphology and bacterial cell types nine purified isolates were designated as AB_1 - AB_9 (**Table 1**). When *Heliothis* larvae were fed isolates AB_2, AB_4, AB_7, and AB_8 under a challenged dose, the consumption of feed stopped within 8-10 h (**Fig. 1**). Progress of the disease was very distinct as the color of the insects gradually turned brown and was quite comparable with *Serratia entomophila* (Gatehouse *et al.* 2009). The darkened alimentary canal became clearly visible from the ventral side and succumbed to death by leaching of body fluid. Only isolate AB_8 turned the pest black, instead of browning with disease progression. Therefore, only four isolates out of nine successfully passed through cross checking for entomopathogens. The pathogens were also successfully re-isolated from the pest and identified. These cross checking tests were performed three times for each isolate.

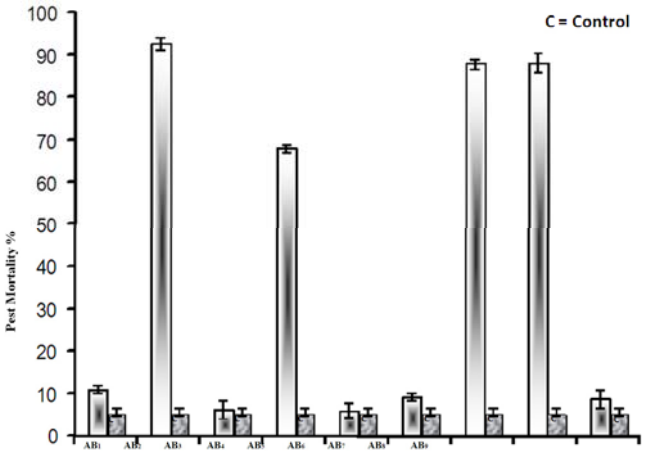

Fig. 1 Mortality percentage of *Heliothis armigera* against nine isolates (AB_1 - AB_9) (control was designated as C inside the column). In each experiment 1.5×10^8 cfu/ml cell concentration was used. Standard errors (SE) were calculated based on three replicates.

Biochemical characterization

The isolates were found to utilize citrate and malonate as the sole carbon source, reduce nitrate and were carbohydrate fermentation tests (CFT) positive for fructose, dextrose, galactose, raffinose, trehalose, melibiose, mannose, mannitol, and ribose, but negative for adonitol, α-methyl-D-glucoside, and 2-nitrophenyl β-D-galactopyranoside (ONPG), indicating the close relationship among the isolates and belonging to the family Enterobacteriaceae (**Table 2**). Isolate AB_2 was identified as a member of the genus *Serratia* because of its inability to utilize ornithine as the sole carbon source and was CFT-negative to lactose, L-arabinose, dulcitol, inositol, sorbitol, and esculin, but not salicin (**Table 3**). Isolate AB_4 was identified as *Cronobacter* (*Enterobacter*) being positive to Tryptophan de-aminase (TDA) and CFT-negative to dulcitol, sorbitol, except inositol and esculin (Williams *et al.* 2003). Isolates AB_7 and AB_8 were members of *Salmonella* being positive to lysine and ornithine and CFT-negative to sucrose, salicin, and esculin, except L-arabinose (Williams *et al.* 2003). The only difference between

Table 2 Identification index for the selected isolates.

Tests	AB_2	AB_4	AB_7	AB_8
Citrate utilization	+	+	+	+
Lysine	-	-	+	+
Ornithine	-	+	+	+
Urease	-	-	-	-
TDA	-	+	-	-
Nitrate reduction	+	+	+	+
H_2S production	-	-	+	+
Glucose	+	+	+	+
Adinitiol	-	-	-	-
Lactose	-	+	-	+
Arabinose	-	+	+	+
Sorbitol	-	-	+	+

Table 3 Carbohydrate fermentation test (CFT) index of the selected isolates.

Tests/ Isolates	AB_2	AB_4	AB_7	AB_8
Lactose	-	+	-	+
Xylose	+	+	+	+
Maltose	+	+	+	+
Fructose	+	+	+	+
Dextrose	+	+	+	+
Galactose	+	+	+	+
Raffinose	+	+	+	+
Trehalose	+	+	+	+
Melibiose	+	+	+	+
Sucrose	+	+	-	-
L-Arabinose	-	+	+	+
Mannose	+	+	+	+
Inuline	-	+	+	+
Sodium gluconate	+	+	+	-
Glycerol	+	+	+	-
Salicin	+	+	-	-
Glucosamine	V	+	+	V
Dulcitol	-	-	V	+
Inositol	-	+	-	V
Sorbitol	-	-	+	V
Mannitol	+	+	+	+
Adonitol	-	-	-	-
α-Methyl-D-glucoside	-	-	-	-
Ribose	+	+	+	+
Rhamnose	+	+	-	-
Cellobiose	V	V	+	+
Melezitose	V	V	-	-
α-Methyl-D-mannoside	V	-	-	-
Xylitol	V	-	-	-
ONPG	-	-	-	-
Esculin	-	+	-	-
D-Arabinose	-	V	-	-
Citrate	+	+	+	+
Malonate	+	+	+	+
Sorbose	+	+	-	-

(*N.B.: Based on % of color reactions and presented as + = >90%; - = <10%; V = 11-89%).

Table 4 Antibiotic sensitivity profile of the selected isolates.

Antibiotics	Conc.	Inhibition Zone Diameter (mm)			
	(µg)	AB_2	AB_4	AB_7	AB_8
Ampicillin (A)	10	13	12	13	11
Carbenicillin (Cb)	100	16	13	16	18
Gentamicin (G)	10	15	15	13	15
Clindamycin (Cd)	2	-	-	-	-
Cephalothin (Ch)	30	13	26	-	-
Cephalexin (Cp)	30	21	15	21	14
Chloramphenicol (C)	30	11	19	19	19
Sulphamethoxazole (Sx)	25	18	-	14	-
Tetracyclin (T)	30	13	14	12	11
Co-Trimazine (Cm)	25	20	11	21	21

these two isolates (AB_7 and AB_8) was in the CFT pattern, particularly of lactose, sodium gluconate, glycerol, and inositol. All four isolates showed resistance to ampicillin and tetracycline, but were sensitive to clindamycin; otherwise they showed a varied response (**Table 4**).

Identification of the bacterial pathogens

16S rRNA gene sequences of the isolates were compared against those in the EMBL, GenBank and DDBJ databases and similarities of the closest related species were determined: the strain AB_2 (accession no. GU370899) as *Serratia entomophila* (100%), AB_4 (accession no. GU370900) as *Cronobacter sakazakii* (100%), AB_7 (accession no. GU370901) as *Salmonella enterica* subsp. *Entericalow* (100%), and AB_8 (accession no. GU370902) as *Salmonella enterica* subsp. *Diarizonae* (100%) (**Fig. 2**).

Phylogenetic analysis on the basis of 16S rRNA gene sequences clearly suggest that the isolates belong to the classical branch of the family Enterobacteriaceae comprising the genera *Escherichia*, *Salmonella*, *Shigella*, *Klebsiella*, and *Serratia* (**Fig. 3**). From the high values of 16S rRNA gene sequence similarity (> 99%) observed between the new isolates and related species, together with their unequivocally high similarity to species of all the closest genera, it can be concluded that AB_2, AB_4, AB_7 and AB_8 form a taxonomically coherent assemblage and represent the same phylogenetic lineage. The stability of the cluster is also reflected in its bootstrap value of 98-100%, which signifies that the four isolates form a consolidated and homogeneous phylogenetic group.

Strains of *S. entomophila* and *S. proteamaculans* were reported as a natural biocontrol agent for the grass grub *Costelytra zealandica*, a major pasture pest of New Zealand (Trought *et al.* 1982; Stucki *et al.* 1984; Jackson *et al.* 1992; Young *et al.* 2009). Recently, the insecticidal activity of a bacterial strain, *Serratia* sp. EML-SE1, was evaluated against diamondback moth (Jeong *et al.* 2010). Pathogenicity determinants of *S. entomophila* and *P. luminescens* also showed similarity (Hurst *et al.* 2000).

CONCLUSIONS

Amongst the worked out isolates, *Serratia* normally shows an association involving several insect genera and species of the orders Orthoptera (crickets and grasshoppers), Isoptera (termites), Coleoptera (beetles and weevils), Lepidoptera (moths), Hymenoptera (bees and wasps), and Diptera (flies). Strains of *S. entomophila* and *S. proteamaculans* are natural biocontrol agents of the major pasture pest of New Zealand grass grub *Costelytra zealandica. Serratia* also carries an extensive history of commercial exploitation as a biopesticide. The present study suggests that the insect epizootics could be exploited as a lucrative source for natural biopesticides. All the entomopathogens could not be commercially exploited but would, at least, provide a potential source of a gene pool for high efficiency pest management.

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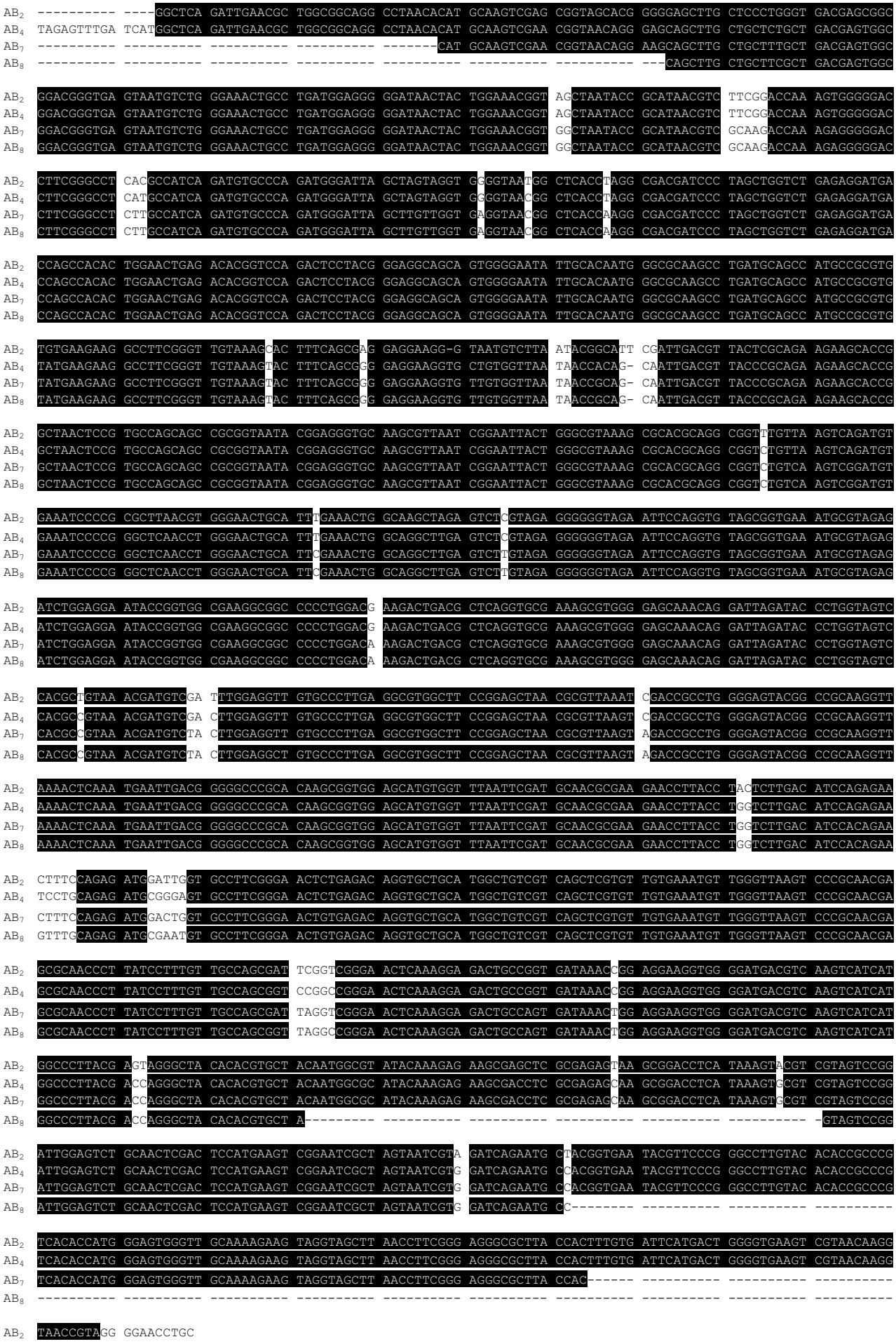
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AB2 ATTGGAGTCT GCAACTCGAC TCCATGAAGT CGGAATCGCT AGTAATCGTA GATCAGAATG CTACGGTGAA TACGTTCCCG GGCCTTGTAC ACACCGCCCG
AB4 ATTGGAGTCT GCAACTCGAC TCCATGAAGT CGGAATCGCT AGTAATCGTG GATCAGAATG CCACGGTGAA TACGTTCCCG GGCCTTGTAC ACACCGCCCG
AB7 ATTGGAGTCT GCAACTCGAC TCCATGAAGT CGGAATCGCT AGTAATCGTG GATCAGAATG GCACGGTGAA TACGTTCCCG GGCCTTGTAC ACACCGCCCG
AB8 ATTGGAGTCT GCAACTCGAC TCCATGAAGT CGGAATCGCT AGTAATCGTG GATCAGAATG GC--------- ---------- ---------- ----------
AB2 TCACACCATG GGAGTGGGTT GCAAAAGAAG TAGGTAGCTT AACCTTCGGG AGGGCGCTTA CCACTTTGTG ATTCATGACT GGGGTGAAGT CGTAACAAGG
AB4 TCACACCATG GGAGTGGGTT GCAAAAGAAG TAGGTAGCTT AACCTTCGGG AGGGCGCTTA CCACTTTGTG ATTCATGACT GGGGTGAAGT CGTAACAAGG
AB7 TCACACCATG GGAGTGGGTT GCAAAAGAAG TAGGTAGCTT AACCTTCGGG AGGGCGCTTA CCAC------ ---------- ---------- ----------
AB8 ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ---------- ----------
AB2 TAACCGTAGG GGAACCTGC
AB4 TAACCGTA-- ----------
AB7 ---------- ----------
AB8 ---------- ----------

Fig. 2 (previous page) Partial 16S rRNA gene sequences were shown for the isolates AB_2, AB_4 (A) and AB_7, AB_8 (B). Sequencing were carried out at least three times each and thereafter compared within and the gray zones were removed.

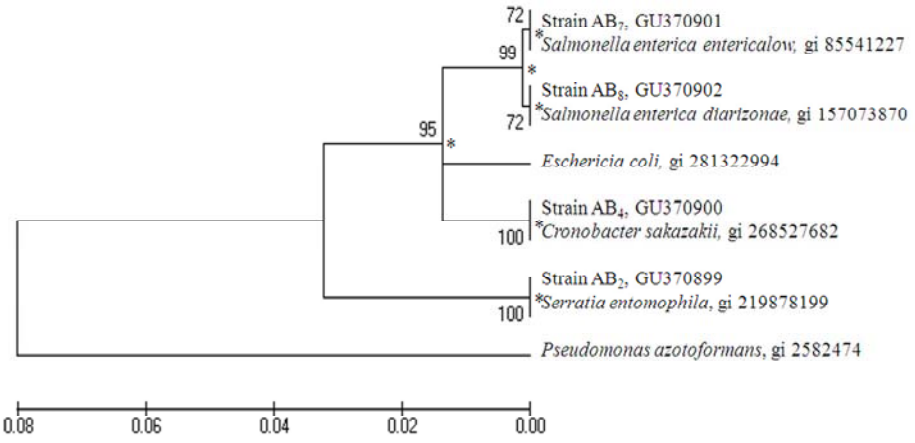

Fig. 3 NJ tree showing the phylogenetic status among the isolates showing a close relation being members of the family Enterbacteriaceae based on analysis of aligned completed 16S rDNA sequence. *Pseudomonas azotoformans* was used as an outgroup. Asterisks indicate branches that were recovered in the maximum-likelihood tree. The scale bar unit represents 0.01 substitutions per nucleotide position. Bootstrap support values greater than 50% for 1,000 replications were shown at the nodes.

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