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CHARACTERIZATION OF INDIGENOUS *BACILLUS COAGULANS* ISOLATED FROM CATTLE AND BUFFALO MILK

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Abstract- Spore forming and lactic acid producing Bacillus species, because of their heat stability and ability to survive the gastric barrier, are being attractive and gaining recognition for use as probiotics in human diets and animal feeds as well as in registered medicines. In the present study, we attempted to isolate *Bacillus* strains with special reference to *Bacillus coagulans* from milk, soil and tomato sources. Thirty-six representative isolates were characterized according to their morphological, biochemical properties, single strand conformational polymorphism (SSCP) banding patterns and partial 16S rRNA gene sequences. A wide range of polymorphism or heterogeneity within the species was recorded. Thirty-two isolates were identified as *Bacillus coagulans* (n= 32), while each two isolates were identified as *Bacillus pumilus* (n= 2) and *Bacillus subtilis* (n= 2) of which mostly were isolated from cattle and buffalo milk.

Keywords- Single strand conformational polymorphism, *Bacillus coagulans, Bacillus pumilus, Bacillus subtilis*.

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Introduction

Microbes most commonly used as probiotics over few decades include many species of the genera, *Lactobacilli* and *Bifidobacterium,* while less commonly, species of *Enterococcus, Streptococcus* and fungi like *Saccharomyces* have been suggested for probiotic effects [1,2]. However, the probiotic microorganisms commonly used in the market are susceptible to high heat, pressure, low water activity and higher acidity [3]. The stability of probiotic organisms during processing, storage and shipping is a limiting factor, particularly in subtropical countries like India and thus sometimes satisfactory viability may not be achieved with the available probiotic formulations, despite its declarations on the product label [4]. The scientific interest in spore-forming *Bacillus* species as probiotics has only occurred enormously in the last 15 years, because its single and most important character of thermostability that assures its viability in the finished product [5]. Though many probiotic *Bacillus* species like *B. subtilis, B. clausii, B.cereus, B. licheniformis, B. coagulans and B. pumilus* are available in commercial products for human, veterinary use and aquaculture in Italy, France, Germany, UK, USA, China, India etc., few *Bacillus* products have been formally approved so far [6]. In recent past, *Bacillus coagulans* has been reported to function as a probiotic in humans [7,8], in pig [9], in cattle [10] and in poultry birds [11].

On one hand, *Bacillus* probiotic market is in growing trend, because of some attractive properties like survivability at high temperature, high acidity of the stomach and bile acids [12,13]. On the contrary, heterogeneity within the *Bacillus* species [14,15] put forward scope for isolation of *Bacillus* strains from different sources. We thus planned to isolate *Bacillus coagulans* from different sources of milk, soil and tomato.

Materials and Methods

Sample collection: Samples of raw milk (buffalo, cow and mixed), pasteurized milk, skim milk and milk cream were collected from the livestock farm and experimental dairy of National Dairy Research Institute (NDRI), Karnal, India. Besides, samples of raw tomato from local markets and soil samples of NDRI and other areas of Karnal were collected for bacterial isolation.

Reference bacterial strains: The standard strain of *B. coagulans* DSM- 1 (DSM: Deutsche Sammlung von Mikroorganismen) procured from German Culture Collection Centre, Germany and two standard cultures of *B. coagulans*, MTCC-492 and MTCC- 2302 obtained from Microbial Type Culture Collection (MTCC) Centre, Institute of Microbial Technology (IMTECH), Chandigarh, India were used as reference strains in the present study.

Isolation of representative colonies from different sources: The collected samples were processed [16] and plated on *Bacillus coagulans* (BC) growth media [17]. After incubation of all plates for 24- 48 h at 55˚C ± 1˚C, a total of 346 representative colonies were picked up randomly and subjected to catalase test according to the slide method. Based on positive catalase test, 217 isolates were selected for morphological examination under microscope using Gram's staining, spore staining and motility test following hanging drop method [18]. A total of 127 isolates were retrieved as spore forming *Bacillus* organisms.

Biochemical tests: The retrieved isolates were further screened based on various biochemical tests for production ability of β-galactosidase enzyme using O-nitrophenol β-D-galactopyranoside (ONPG) disc, production of acetylmethylcarbinol (AMC) using voges praskaur (VP) borth, hydrolysis of casein, starch, gelatin, reduction of nitrate, utilization of citrate, propionate, tolerance to different concentrations of sodium chloride (2, 5 and 7%), reaction in litmus milk, growth ability under anaerobic condition and growth ability at different temperatures (10, 55 and 65˚C). Based on biochemical properties, 55 isolates were selected for further analysis.

Haemolytic activity: Haemolytic activity of 55 isolates was investigated on blood

agar plates, streaked with colonies from fresh BC agar plates. Readings were taken after incubation at 37˚C for 18 h to detect the appearance of hemolysis, if any.

Sugar fermentation ability: Carbohydrate fermentation test in basal broth medium [19], using 18 sugar discs (Himedia, Mumbai, India) was conducted for the tentative identification of forty seven *Bacillus* isolates along with three standard cultures. The selected *Bacillus* isolates were propagated on BC agar slants and sub-cultured every after 25- 30 d period [20].

Molecular characterization using 16S rRNA sequencing: The selected isolates were identified by partial 16S rRNA sequencing. The genomic DNA of the selected isolates was extracted following the standard protocol [21]. The 16S rRNA sequence of *Bacillus sp*. (Acc. no. AB020199) was used for designing the primer sequence. Forward primer 5/-CTCCTACGGGAGGCAGCACT-3/ (corresponding to 339-358, in *E.coli* numbering) and reverse primer 5/-GTTGCGCTCGTTGCGGGACT 3/ (corresponding to 1112 to 1093 base pair of *E. coli* numbering) were used to amplify a 777 base pair region. The polymerase chain reaction (PCR) was carried out by adding 1 µl of DNA template with 25 µl of master mix containing 25 pM of each of forward and reverse primers, 3 unit/µl Taq DNA polymerase, 200 µM of deoxynucleoside triphosphate (dNTP) and 10x Taq assay buffer containing 15 mM $MgCl_2$. The PCR reaction was performed in a thermal cycler (My Cycler, Bio-Rad, USA) by programming the cycling profile consisting of an initial denaturation at 94˚C for 2 min followed by an amplification for 35 cycles with denaturation at 94˚C for 30 sec, annealing at 50˚C for 30 sec, and extension at 72˚C for 1 min and final extension at 72˚C for 7 min at the end of 35 cycles. In all the reactions, sterile water was used in place of DNA as a control. The amplified 16S rRNA product was subjected to single strand conformation polymorphism (SSCP) analysis in a 7% non-denaturing polyacrylamide gel electrophoresis (PAGE) as per the standard protocol [21], followed by silver staining. The isolates were grouped into four different members based on the pattern of DNA polymorphism. PCR product was purified using Wzard DNA purification kit (Promega, Madison, USA) to remove primers and salts. Four representative purified DNA fragments from each group were subjected to automated sequencing in ABI Prism (Model 3100, Version 3.7) following the Sanger di deoxy chain termination methodology. The sequence data was aligned and analyzed using BLAST server available at http://www.ncbi.nlm.nih.gov/.

Statistical analysis: Forty seven *Bacillus* isolates along with three standard cultures were subjected to cluster analysis on the basis of sugar fermentation profile by Euclidean distance (single linkage) method using SYSTAT 6.0.1 Statistical Software Package, 1996, SPSS, Inc., USA, for exploring their phylogenetic resemblance. The euclidean distance was calculated by the method as described elsewhere [22]. The single linkage or nearest neighbour method measured the proximity between any two profiles and lead to discrete clusters containing one or more than one isolates by smallest possible euclidean distance measure. The hierarchical approaches proceed sequentially such that profiles were joined one by one till one final cluster was obtained. At each step of the hierarchical process, the value of objective function or clustering criterion as distance value was determined. The value represented the squared euclidean distance between two points. It showed which two clusters were to be joined to form a new cluster. The distance value showing the maximum between any two solutions determined the optimum number of clusters of bacterial isolates. Since each step was a cluster solution, the appropriate number of cluster was determined by a second optimality criterion for final classification of the isolates.

Results and Discussion

Isolation of representative colonies from different sources: A total of 346 representatives round shaped colonies with white or creamy coloration, shiny appearance, convex and raised edges were initially recovered from 125 samples. Thereafter, 127 colonies of those isolates were randomly selected as gram positive, catalase positive, rod shaped, motile and spore forming *Bacillus* organisms. Comparing the biochemical properties of 127 isolates with that of three standard cultures of *Bacillus coagulans*, *viz.* DSM- 1, MTCC- 492 and MTCC 2302, we screened 55 closely related isolates showing almost same pattern of biochemical properties with little variations in starch, gelatin and casein hydrolysis, utilization of citrate and nitrate reduction test. Our findings are quite comparable with the previous reports [14,19].

Haemolytic activity: Eight isolates showed haemolysis on blood agar plates after incubation at 37˚C for 18 h and thus they were discarded and rest 47 isolates were selected and subjected to the carbohydrate fermentation tests.

Sugar fermentation profiles: The detail results of sugar fermentation profiles of individual isolate are not shown herein. According to the earlier report [19], the sugar fermentation profiles of 47 *Bacillus* isolates were compared with those of three standard strains of *B. coagulans* DSM- 1, *B. coagulans*, MTCC- 492 and MTCC- 2302. All three standard type strains could ferment glucose, fructose, maltose, galactose, N-acetyl glucosamine (NAG), rhamnose, mellibiose, mannose, cellobiose, lactose (except MTCC- 492) and sucrose (except MTCC- 492 and MTCC- 2302), while they showed inability to produce acid from inulin, xylose, arabinose, raffinose, mannitol (except MTCC- 2302), salicin (except MTCC- 2302) and trehalose (except MTCC- 492 and MTCC- 2302). With rare exceptions, most of the tested isolates were able to ferment glucose, maltose, galactose, NAG, mannose and lactose, while they showed inability to ferment inulin and xylose. As reported by the earlier workers [14,19,23], there was considerable variation in the sugar fermentation profile with the selected isolates in the present study.

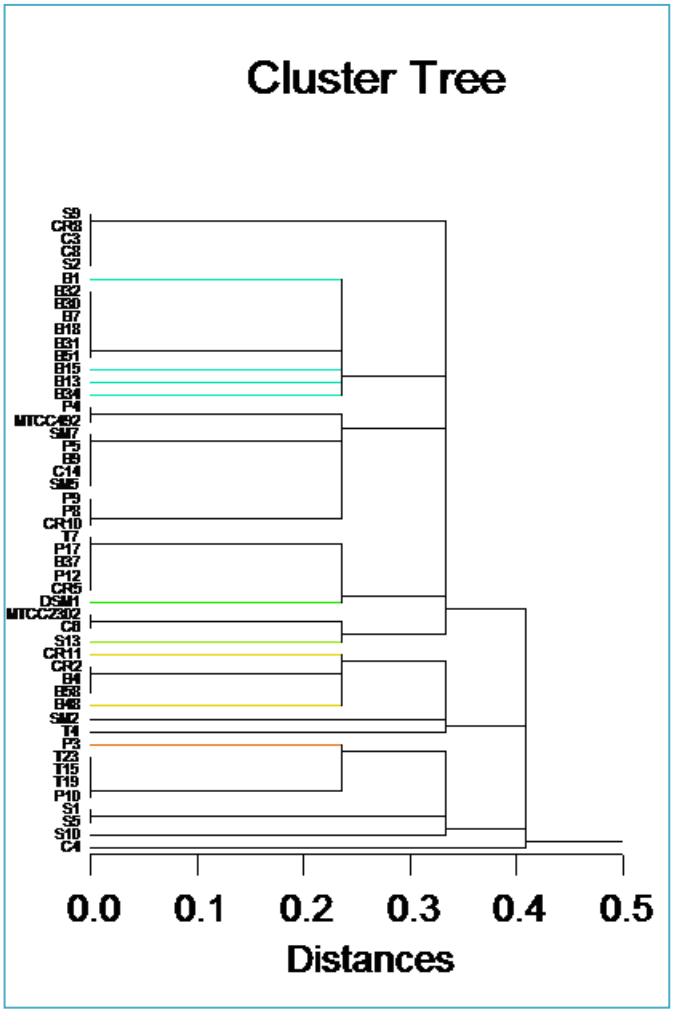

Fig-1 Cluster tree-showing relationship among the selected isolates studied and the standard cultures of *B. coagulans* based on Euclidean distance (Single linkage method).

Table-1 *Euclidean Distance of Different Isolates Using Single Linkage method*

Cluster and containing	Cluster Containing	Criterion Value	No. of members in new cluster
B18	B7	0.0	2
B30	B18	0.0	3
B31	B30	0.0	4
B32	B31	0.0	5
B51	B32	0.0	6
B58	B4	0.0	2
CR2	B58	0.0	3
C14	B9	0.0	2
P5	C14	0.0	3
MTCC492	P4	0.0	2
SM5	P5	0.0	4
P9	P8	0.0	2
CR10	P9	0.0	3
SM7	SM5	0.0	5
T19	T15	0.0	2
T23	T19	0.0	3
P10	T23	0.0	4
MTCC2302	C6	0.0	2
C8	C3	0.0	2
CR8	C8	0.0	3
S2	CR8	0.0	4
S9	S2	0.0	5
P12	B37	0.0	2
P17	P12	0.0	3
CR5	P17	0.0	4
T7	CR5	0.0	5
S1	S5	0.0	2
B51	B1	**0.236**	**7**
B34	B13	0.236	2
B15	B51	0.236	8
B34	B15	0.236	10
CR2	B48	0.236	4
CR2	CR11	0.236	5
SM7	CR10	0.236	8
SM7	MTCC492	0.236	10
P10	P3	0.236	5
MTCC2302	S13	0.236	3
DSM1	T7	0.236	6
B34	SM7	**0.333**	20
S9	B34	0.333	25
SM2	CR2	0.333	6
P10	S1	0.333	7
P10	S10	0.333	8
DSM1	S9	0.333	31
T4	SM2	0.333	7
DSM1	MTCC2302	0.333	34
T4	P10	**0.408**	15
T4	DSM1	0.408	49
C4	T4	0.408	50

The relationship among the selected isolates studied and the standard cultures of *B.coagulans* based on Euclidean distance (Single linkage method) is presented in [Fig-1] and [Table-1]. The cluster analysis by Euclidean distances using single linkage method explored 7 clusters of 44 isolates, at criterion value 0.236, which was the maximum distance between any two solutions. The first cluster, comprising of 5 isolates showed maximum similarity at 0.0 eu distance, indicating the sugar fermentation profile of 5 isolates were exactly similar [Table-1]. The 2nd cluster containing 10 isolates joined at criterion value 0.236, although

Table-2 Classification *and identification of Bacillus isolates*

Group	Total no. of isolates	Name of isolates	Pattern of SSCP bands	Identity
1	20	B18, B30, B32, B34, C4, C6, C8, P5, P8, P9, P10, P12, P17, S5, S9, S13, T19, T23, CR8, SM2, MTCC 492 (Standard culture), **MTCC 2302** (Standard culture)	Single band	***Bacillus coagulans***
2	12	B15, B31, **B37**, B51, B58, P3, T7, T15, CR2, CR5, CR11, SM5, DSM1 (Standard culture)	Double bands, distant	***Bacillus coagulans***
3	2	B4, **B48**	Double bands, close	***Bacillus subtilis***
4	**2**	B9, **SM7**	**Double bands, very close**	***Bacillus pumilus***

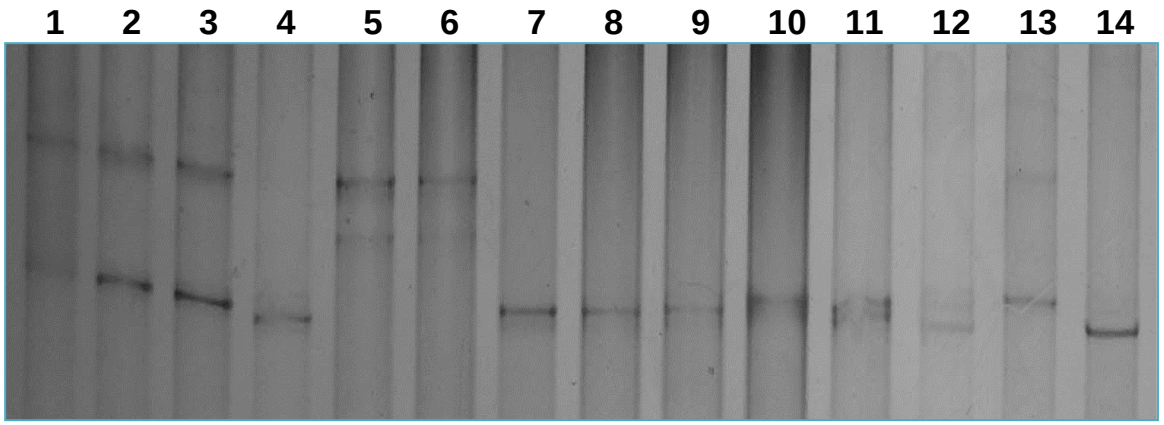

Fig- 2 SSCP pattern of 16S rRNA amplicons in 7% PAGE showing four distinct band patterns of selected *Bacillus* isolates. a) Lane 1- B15, 2- T7, 3- B37, 4- MTCC492, 5- B4, 6- B48, 7- P8, 8- P10, 9- S9,10- S13, 11- SM7, 12- B9, 13- SM5, 14- SM2. Lane 1, 2, 3 and 13 are showing distinct double bands pattern (SSCP type 2), while Lane 4, 7-10 and 14 have similar single band pattern (SSCP type 1). Lane 5 and 6 have distinct close double bands (SSCP type 3) of ssDNA. Lane no. 11 and 12 show a very close double bands pattern (SSCP type 4).

6 of them having complete analogy in sugar fermentation profile showed maximum similarity at 0.0 eu distance, while, rest four had slight differences. Likewise, ten members of 3rd cluster, could be further distributed in three sub-clusters, one consisting of 2 organisms, MTCC 492 and P4, another one having 5 members, SM5, SM7, B9, C14 and P5 and the third sub-cluster comprised of 3 members naming P8, P9 and CR10. Similarly, 5 isolates naming T7, P17, B37, P12 and CR5 having similar sugar fermentation profile resembled the type strain of *B. coagulans* (DSM1) very closely and thus formed a cluster at 0.236 eu distance. Fifth, sixth and seventh cluster included 3, 5 and 5 organisms, respectively. The sugar fermentation pattern of all the isolates of one cluster was found to be identical, while the same differed between the clusters. A cluster analysis based on carbohydrate fermentation profile exhibited a high degree of variations which lead to classify the selected isolates into seven different clusters.

Molecular characterization of Bacillus isolates: 16S rRNA gene was amplified from the genomic DNA of selected isolates by employing primer pairs designed from partial 16S rRNA sequence of *Bacillus sp.* (Acc. no. AB020199). The banding patterns in single stranded DNA are documented in [Fig-2]. SSCP technique was applied to demonstrate the genetic diversity among the suspected isolates of *B. coagulans* selected by morphological and biochemical traits. SSCP analysis of PCR amplified fragments of the 16S rRNA gene has more recently been used as an alternative to genomic sequencing for the identification of bacterial species. SSCP technique was commonly used to search for point mutations in DNA sequences consisting of few hundred base pairs [24] and taxonomical classification of certain bacteria, including *Bacillus* species [25,26]. There were four distinct SSCP band patterns based on SSCP analysis of the amplified 16S rRNA products in PAGE. The banding pattern consisting of only a single ssDNA band (SSCP type 1) was quiet uncommon (lane 4, 7-10 and 14). The possible reason for this uncommon existence might be due to the larger size of the product, which could not unfold the exact banding pattern in 7% acrylamide gel. While migrating the amplicons in 7% acrylamide gel for 26 hours, a slight variation of double stranded DNA could be resolved. In some isolates, two distinct separate double strand bands (SSCP type 2) were obtained (lane 1-3 and 13). Lane 5 and 6 had distinct close double bands (SSCP type 3). Lane 11 and 12 showed a very close double bands pattern (SSCP type 4). This was an indication of slight nucleotide length variation of these amplicons. Different SSCP band patterns clearly revealed a wide range of polymorphism or heterogeneity within the species. A considerable heterogeneity within the species *B. coagulans* was reported previously [14]. Other *Bacillus* species also showed heterogeneities within 16S rRNA gene sequences [27]. On the basis of SSCP band patterns, the isolates were classified into four groups as depicted in [Table-2]. The sequences of partial 16S rRNA of four representative purified DNA fragments from each group, shown in the Clustal- W alignment form in [Fig-3], were compared to those deposited in GenBank. Both the standard type strain MTCC 2302, representing group 1 of 20 isolates and the buffalo milk isolate B37, a member of group 2 consisting of 12 isolates [Table-2], exhibited 98% 16S rRNA sequence identity to database entries corresponding to *Bacillus coagulans*. On the other hand, buffalo milk isolate B48, representative of group 3, displayed 99% sequence identity to *Bacillus subtilis*, while another buffalo milk isolate B9, representing group 4, showed 97% homology with *Bacillus pumilus* [Table-2]. In the present investigation, 32 isolates were recognized as *Bacillus coagulans*, while 2 isolates (B48 and B4) were identified as *Bacillus subtilis* and 2 isolates (B9 and SM7)

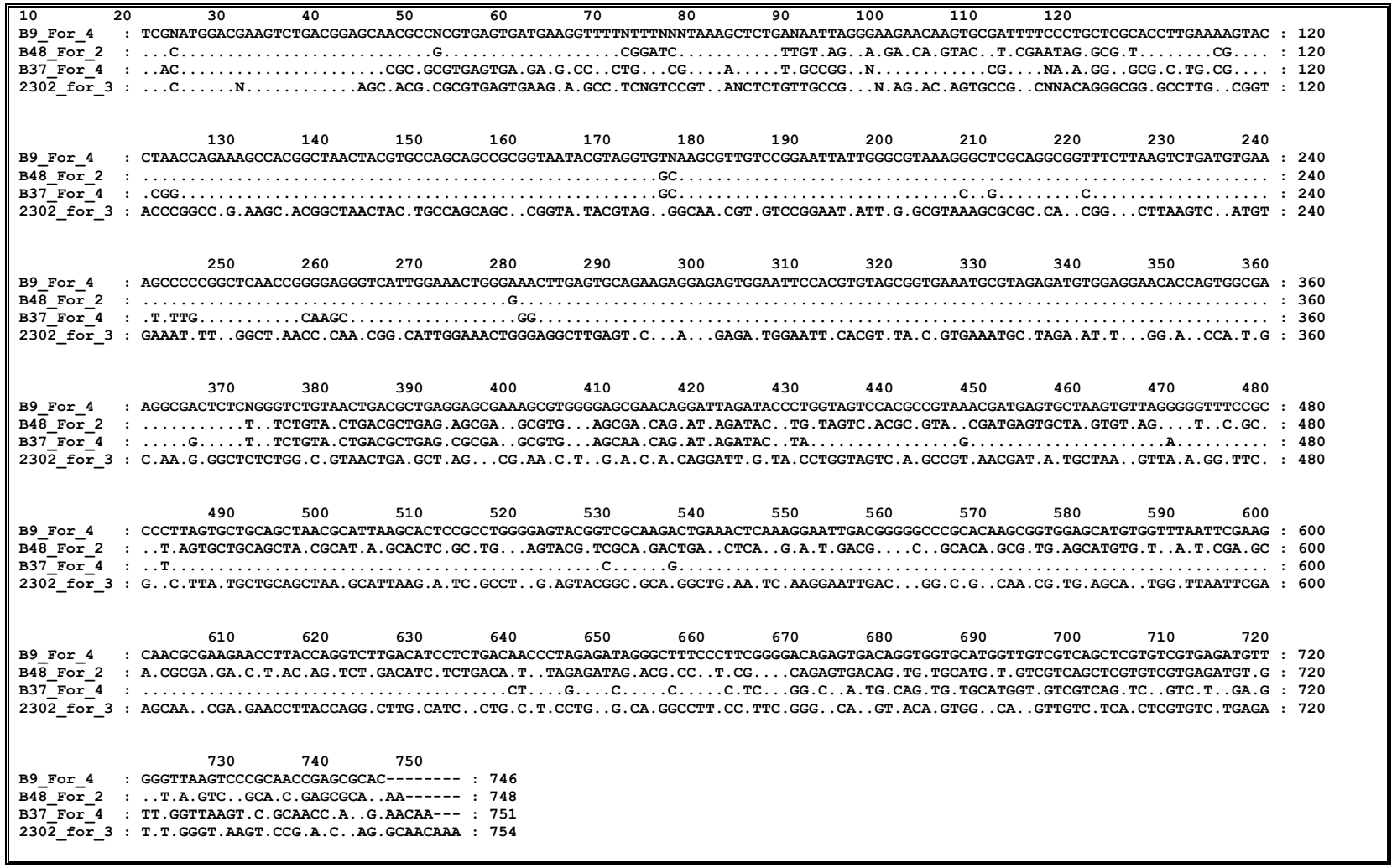

Fig-3 Clustal- W alignment of the sequence

showed resemblance to *Bacillus pumilus.*

Conclusions

The characterization of *Bacillus* strains clearly indicates a wide range of heterogeneity or polymorphism within the species. The heterogeneity both in genetic makeup and in phenotypic traits sometimes creates ambiguity in proper characterization of this organism. The SSCP analysis of partial 16S rRNA amplicons confirms well with the biochemical groupings except in few cases where the isolates belonging to different biochemical groups showed similar SSCP band patterns. Most of the isolates belong to the group of *Bacillus coagulans*, while each two isolates are identified as *Bacillus pumilus and Bacillus subtilis,* which are mostly isolated from cattle and buffalo milk.

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