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Isolation and partial characterization of *Cronobacter sakazakii* by 16S rRNA sequence analysis isolated from milk of dairy cows with Mastitis in Chikmagalur, Karnataka, INDIA

Vineeth B, Priyanka B, Ramesh B S, Makari Hanumanthappa K*, Vinay Suvarna M N and Vivek Chandramohan

Abstract

Mastitis is a chronic inflammation of milk glands which is caused by the bacteriological variations in the milk and other changes in the glandular tissue. Mastitis occurs throughout the world wherever dairy farms are found. *Cronobacter sakazakii* is one of the causative organism and also it is known as *Enterobacter sakazakii. Cronobacter sakazakii* enters the cattle through the udder, where the cattle are reared in the unhygienic condition. The bacteria were isolated from Mastitis infected raw milk around the Chikmagalur District. Bacteria were isolated with the standard protocols and were maintained in Lowry broth (LB) media. All the isolates were subjected to RAPD analysis. Further DNA of the bacteria was isolated by CTAB method and subjected to 16s ribosomal sequence analysis using 16S rRNA FU8 universal primers (16s rRNA F-5'AGAGTTTGATCCTGGCTCAG 3', 16s rRNA R-5'ACG GCTACCTTGTTA3') and phylogenetic analysis were used to molecular relatedness of the isolated animal pathogenic bacteria.16S rRNA sequences were submitted to GenBank, NCBI and accession number was allotted. The obtained 16S rRNA sequences were subjected in-silico analysis by genomics workbench software for Sequence information, atomic composition, nucleic acid distribution, nucleotide distribution, histogram and secondary structure prediction. The findings of this research greatly anticipated for the identification and characterization of the animal pathogen *Cronobacter sakazakii* was first time reported in Chikmagalur, Karnataka, India.

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Key words: Mastitis, *Cronobacter sakazakii*, RAPD analysis, 16S rRNA sequence and phylogenetic analysis**.**

Introduction:

Mastitis is a chronic inflammation of milk glands which is caused by the bacteriological variations in the milk and other changes in the glandular tissue (Radostitis *et al*., 2000)[1]. Mastitis is an endemic disease which makes highly economic loss. Mastitis is characterized by sudden change, swelling, redness and pain in teat of the udder and reduces milk secretion from the affected quarters (M.Z. Khan et al., 2006)[2]. The causative bacteria classification is done as major and minor pathogens (Harmon, 1994)[3]. The major pathogens are further classified into environmental (*E. coli, Streptococcus dysgalactiae and Streptococcus uberis*) or contagious (*S. aureus and S. agalactiae*) based on their pimary reservoir (BramLey *et al*., 1996 and Riffon *et al*.,2001)[4,5]. Enterococci is also known as *Enterobacter*. *E.sakazakii was* defined as a species by Farmer et al in 1980. *Enterobacter sakazakii* species is now named as *Cronobacter sakazakii* (Farmer et al 1980)[6], along with the description of the new species. It is a Gram negative, rod shaped, pathogenic bacteria. The majority

of *Cronobacter* cases are in cattles and additionally it is associated with a rare cause of invasive infection of infants with historically high case fatality rates (40–80%). Mastitis impairs the quality of milk and milk products (Philpot., 2003)[7] In infants it can cause bacteraemia, meningitis and necrotizing enterocolitis. Some neonatal *Cronobacter* (*E.sakazakii*) infections have been associated with the use of powdered infant formula with some strains able to survive in a desiccated state for more than two years.

Diagnosis

Clinical findings like abnormalities of secretions, abnormalities of size, consistency and temperature of mammary gland were examined by visual inspection and palpation. Pain reaction upon palpation, changes in the milk (blood tinged milk, watery secretions, clots, pus), and changes in consistency of udder were considered as indications of the presence of clinical mastitis.

- [**1] *Vineeth B, Department of UG and PG Applied Zoology, IDSG Government College, Chikmagalur-577102, Karnataka, INDIA.*
- [2] *Priyanka B, Department of UG and PG Applied Zoology, IDSG Government College, Chikmagalur-577102, Karnataka, INDIA.*
- [3] *Ramesh B S Department of UG and PG Applied Zoology, IDSG Government College, Chikmagalur-577102, Karnataka, INDIA.*
- [*4] *Makari Hanumanthappa K, Department of Biotechnology, IDSG Government College, Chikmagalur- 577102, Karnataka, INDIA. [*]corresponding author: makari.hk@gmail.com.*
- [5] *Vinay Suvarna M N, Research and Development Centre, Bharathiar University, Coimbatore- 641046, Tamil Nadu, INDIA.*
- [6] *Vivek Chandramohan, Department of Biotechnology, Siddaganga Institute of Technology, Tumkur -572103, Karnataka, INDIA.*

Materials and Methods

A total of 10 dairy cattles at different villages of Chikmaglur District were investigated in the month of January. Among these cattle, 8 were with clinical or subclinical mastitis and 10 raw milk samples were obtained from dairy cattle. Milk samples were taken with the help veterinary practitioner of nearby veterinary hospital. Before sampling, teat ends mastitis infected cows were disinfected with cotton swabs soaked in 70% alcohol and allowed to dry and the first streams of milk were discarded. Sterile tubes were filled with samples about 5 mL by the veterinarian and transported in icebox to the Laboratory of Biotechnology, IDSG Government College, Chikmaglur, Karnataka for further studies.

Isolation and Identification of Microorganism:-

All positive samples were analyzed microbiologically as described previously (Quinn PJ *et al*,. 1994)[8], 0.01 mL milk was plated onto 7% sheep blood agar, as well as on Mac Conkey agar. Strains were maintained in LB broth for further analysis. The plates were incubated at 37ºC for 72 hr under aerobic conditions. The classical characteristics (colony morphology, heamolysis, Gram stain, catalase, coagulase, potassium hydroxide (KOH 3%) and oxidase test) were investigated for the isolated microorganisms.

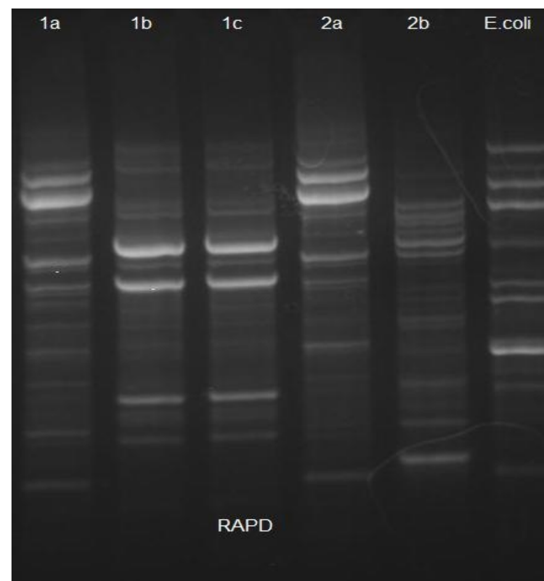
Biochemical test and phenotypic characterization was done by standard protocols. The phenotypic characteristics, based on 18 individual biochemical tests and a commercial identification test correspond to those reported for *cronobacter sakazakii* by other investigators (Devriese et al., 1986 and Soedarmanto et al., 1996)[9, 10]. It is interesting that on primary culture all *C.sakazakii* isolates appeared to be amplicillin and cephalothin negative. Meanwhile, the isolates uniformly gave positive results for amplicillin and cephalothin in after they were sub cultured. This effect could affect the routine diagnosis of mastitis. The probability of identification of all isolates was 99.9%, on the basis of the biochemical reaction implemented with the Bioscience test kit.

Susceptibilities to antimicrobial agents

The antibiotic sensitivity test was performed as follows. All the identical colonies were incubated in LB broth for 72 hrs at 37^0c. Then 0.1mL of bacterial suspension was placed on macconkeys media, followed by addition of antibiotic disks ampicillin, cephalothin, spectinomycin and nalidixic acid.

RAPD analysis of isolated strain

All the isolates were subjected to RAPD is generated by single primer OPA-02 '3-TGCCGAGCTG-5' PCR were used to compare the relatedness of the isolates. For each isolates data record was constructed in which each band of a particular molecular weight, as generated by each primer.



RAPD-PCR profiles obtained (1b, 2b) with primer OPA-02 '3-TGCCGAGCTG-5'

Cronobacter sakazakii isolated from mastitis infected milk

Antibiotic sensitivity test

- Mueller - Hinton (MH) agar plates were prepared and the cultures of bacterial isolates 1b, 2a, 2b and also 1a were spread on respectively labeled plates.
- The antibiotic discs of cephalothin, spectinomycin, nalidixic acid and ampicillin were placed on the spread culture.
- For antibiotics in solution- kanamycin, tetracycline, streptomycin and chloramphenicol wells were punched and antibiotics were added in the wells.
- The plates were incubated for 18 hours and zones formed were observed.

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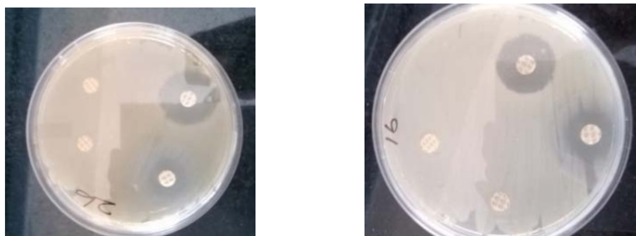

Genomic DNA extraction and PCR based amplification of 16S rRNA sequences

The genomic DNA of *Cronobacter sakazakii* was isolated for PCR amplification was done according to the instructional manual provided by Aristogene Biosciences Pvt Ltd, Bangalore, India, (Aristogene PCR kit, 16S rRNA sequence amplification kit, Aristogene Biosciences Pvt Ltd, Bangalore). The genomic DNA was isolated by CTAB method and subjected to PCR amplification using 16S rRNA F8U universal primers (16s rRNA F-5'AGA GTTTGATCCTGGCTCAG3'; 16S rRNA R-5'ACGGCTACCTTGTTA3') PCR amplification of DNA was performed in Effendorf gradient thermal cycler at the suitable conditions for PCR according to the standard procedure [Makari H K *et al*.,2013, Kumar A, M Anandraj, 2006 and Opina, N *et al*, 1997][11,12,13] and the PCR amplified products were separated in agarose gel electrophoresis. Electrophoreses gel was observed for DNA bands on a UV trans-illuminator. The results were documented in Alpha imager Gel Doc system. Eluted DNA samples were subjected to Sequence analysis using cycle sequence method and generated sequences were analyzed by BLAST at NCBI.

Sequences analysis

The sequence related analysis for the newly identified sequenced was performed using CLC genomics workbench software [CLC Bio]. 16S rRNA sequences were compared with those available in the GenBank databases using the gapped BLASTN.

Phylogenetic analysis of unidentified isolates

For those isolates which were not identified by 16S rRNA sequence analysis, taxonomic relationships were inferred from 16S rRNA sequence comparison. Sequence were obtained from the Genbank database and aligned by using the multisequence alignment program ClustalW [Thompson J D, *et al*, 1994][14] in the CLC genomics workbench. Phylogenetic relationships were inferred from this alignment by using programs in version 3.4 of the PHYLIP [Felsenstein J, 1993][15]. A distance matrix was generated using DNADIST under the assumptions of Jukes and Cantor and Kimura. Phylogenetic trees were derived from these matrices using neighbor joining.

Result and Discussion

The present study was conducted with isolates obtained from the Mastitis milk samples identified at 16S rRNA-based RAPD -PCR sequence analysis. An almost complete 16S rRNA sequence containing fewer than positions was obtained for all of the isolates included in the study; top nine query sequences were available for

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comparison (Figure 3). For this isolates belonging to the organism *Cronobacter sakazakii* (Earlier name was *Enterobacter).* The Phylogenetic tree was produced using PHYLogeny inference package (PHYLIP) with neighbor joining method shows some of the closely related to identified bacteria previously described in dairy environments the result as shown in the (Figure 2). Another interesting observation was that more than one pathogen was found in some mastitis samples reported earlier by others (EL-Khodery S A., et al., 2008)[16]. It is accepted that bacterial, environmental, management and cow factors may affect the occurrence and severity of mastitis, some reports have indicated that mastitis mainly depends on cow factors as shown by cases where cows were infected by the same species (Buvernich C., et al., 2003)[17].The CLC genomics technique with improved workbench software has generated the following information for the input sequence. Sequence information, melting temperature(^{0}C), atomic composition and nucleotide distribution. The results are shown in the (Table 1). Nucleotide Guanine has the maximum number of occurrence (304) and Uracil being the lowest (171).

From the table it is clear that C+G combination is more (514) compared to A+G (395). The RNA structure prediction results are shown in the (Figure 6 and Figure 7). In that result gives the information about stem, multi loop bulge loop and hairpin loop. Nucleotide distribution histogram is shown in (Figure 4). 16S rRNA sequencing is a powerful tool for rapid identification and phylogenetic analysis of bacterial species. This method gives increasingly comprehensive and more precise picture of the bacterial group associated with the mastitis milk sample. The obtained 901bp 16S rRNA nucleotide sequence was compared with available 16S ribosomal sequence in the NCBI database using BLASTN. The submitted nucleotide sequence as depicted in (Figure 1) was provided a genbank accession number KJ415049. Based 16S rRNA sequence, a fast minimum evaluation tree revealed that the isolate shares a same clade with *Cronobacter sakazakii* and occupies a distinct phylogenetic position within the representative members of the genus *Cronobacter* as illustrated.

Fig 1:- The 901 bp 16S rRNA nucleotide sequence of bacterial isolate.

1 cgtgcggcaa ggcctaacac atgcagtcga acggtgacag ggagcagctt gctgctctgc
61 tgacgagtgg cggacgggtg agtaatgtct gggaaactgc ctgatggagg gggataacta
121 ctggaaacgg tagctaatac cgcataacgt cttcggacca aagtggggga ccttcgggcc
181 tcatgccatc agatgtgccc agatgggatt agctagtagg tggggtaacg gctcacctag
241 gcgacgatcc ctagctggtc tgagaggatg accagccaca ctggaactga gacacggtcc
301 agactcctac gggaggcagc agtggggaat attgcacaat gggcgcaagc ctgatgcagc
361 catgccgcgt gtatgaagaa ggccttcggg ttgtaaagta ctttcagcgg ggaggaaggc
421 gttgtggtta ataaccgcag cgattgacgt tacccgcaga agaagcaccg gctaactccg
481 tgccagcagc cgcggtaata cggagggtgc aagcgttaat cggaattact gggcgtaaag
541 cgcacgcagg cggtctgtta agtcagatgt gaaatccccg ggctcaacct gggaactgca
601 tttgaaactg gcaggcttga gtctcgtaga gggggtaga attccaggtg tagcggtgaa
661 atgcgtagag atctggagga ataccggtgg cgaaggcggc ccccctggac gaagactgac
721 gctcacgtgc gaaagcgtgg ggagcaaaca ggattagata ccctggtagt ccacgccgta
781 aacgatgtcg acttggaggg ttgtgcccat tgagcgtggc ttcccgggag ctaacgcgtt
841 taagtcgacc cgccctgagg gagtacggcg gcaatgttaa aactcaaaat

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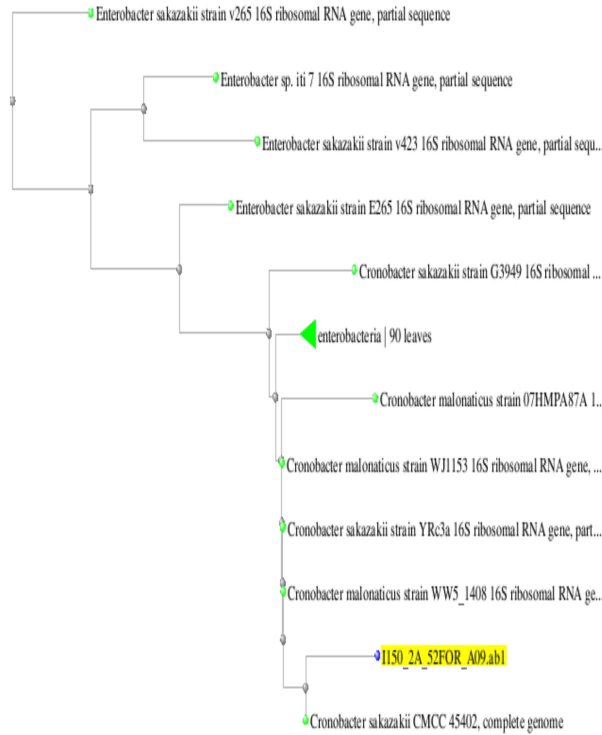

Hit	Description	E-value	Score	%Identity	%Gaps
HQ880385	Cronobacter sakazaki strain G4069 16S ribosomal RNA gene, ...	0.00	784.00	98.00	1.00
JF330153	Cronobacter sakazaki strain crj03 16S ribosomal RNA gene, p...	0.00	780.00	98.00	1.00
HQ880405	Cronobacter sakazaki strain G4083 16S ribosomal RNA gene, ...	0.00	780.00	98.00	1.00
HQ880327	Cronobacter sakazaki strain G4062 16S ribosomal RNA gene, ...	0.00	780.00	98.00	1.00
HQ880318	Cronobacter sakazaki strain G4049 16S ribosomal RNA gene, ...	0.00	780.00	98.00	1.00
HQ880310	Cronobacter sakazaki strain G4037 16S ribosomal RNA gene, ...	0.00	780.00	98.00	1.00
GU122187	Cronobacter sakazaki strain 05CHPL18 16S ribosomal RNA ge...	0.00	780.00	98.00	1.00
GU122192	Cronobacter sakazaki strain 05CHPL37 16S ribosomal RNA ge...	0.00	780.00	98.00	1.00
GQ504715	Cronobacter sakazaki strain M2PFe 16S ribosomal RNA gene,...	0.00	780.00	98.00	1.00

Figure 3: Blast result for newly identified 16S rRNA sequence

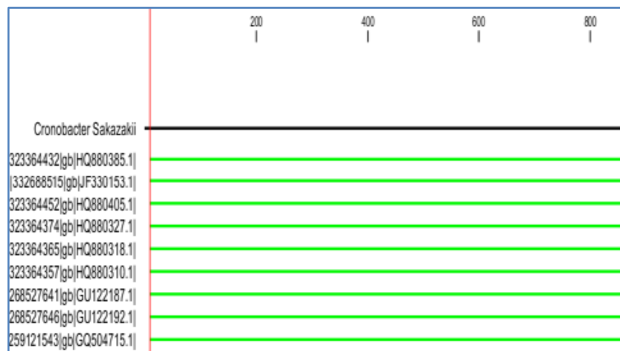

Table 1: 16S rRNA Sequences statistics

<table>
<tr><td colspan="5">Sequence information</td></tr>
<tr><td colspan="2">Sequence type</td><td colspan="3">rRNA</td></tr>
<tr><td colspan="2">Length</td><td colspan="3">909bp</td></tr>
<tr><td colspan="2">Organism</td><td colspan="3">Cronobacter Sakazakii</td></tr>
<tr><td colspan="2">Weight (single-stranded)</td><td colspan="3">283 kDa</td></tr>
<tr><td colspan="2">Weight (double-stranded)</td><td colspan="3">561.761 kDa</td></tr>
<tr><td colspan="5">Melting temperature - degrees Celsius</td></tr>
<tr><td>[salt] = 0.1M</td><td>[salt] = 0.2M</td><td>[salt] = 0.3M</td><td>[salt] = 0.4M</td><td>[salt] = 0.5M</td></tr>
<tr><td>88.08</td><td>93.08</td><td>96.00</td><td>98.08</td><td>99.69</td></tr>
<tr><td colspan="5">Atomic composition</td></tr>
<tr><td colspan="5">As single-stranded</td></tr>
<tr><td>Atom</td><td colspan="2">Count</td><td colspan="2">Frequency</td></tr>
<tr><td>Hydrogen (H)</td><td colspan="2">11,081</td><td colspan="2">0.371</td></tr>
</table>

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Carbon (C)	8,880	0.297
Nitrogen (N)	3,612	0.121
Oxygen(O)	5,402	0.181
Phosphorus(P)	909	0.030
As double-stranded		
Hydrogen (H)	22,215	0.373
Carbon (C)	17,666	0.297
Nitrogen (N)	6,877	0.116
Oxygen(O)	10,910	0.183
Phosphorus(P)	1,818	0.031
Nucleotide distribution		
Nucleotide	**Count**	**Frequency**
Adenine(A)	224	0.246
Cytosine(C)	210	0.231
Guanine(G)	304	0.334
Uracil (U)	171	0.188
C+G	514	0.565
A+U	395	0.435

Fig 4: Nucleotide distribution histogram

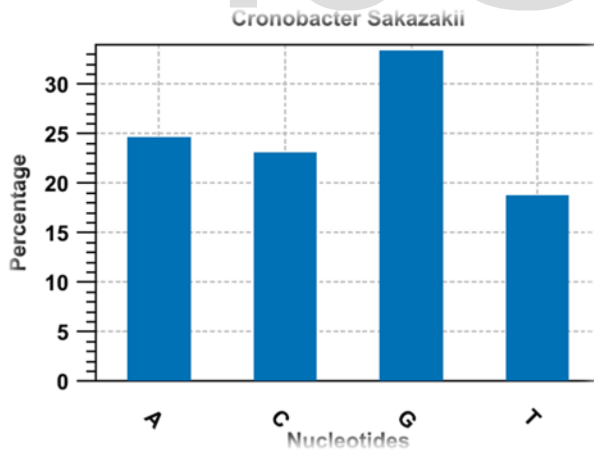
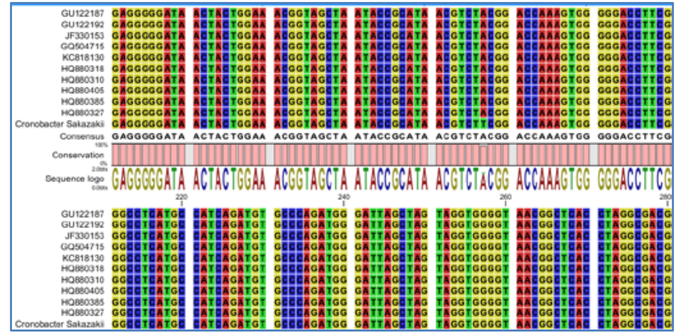
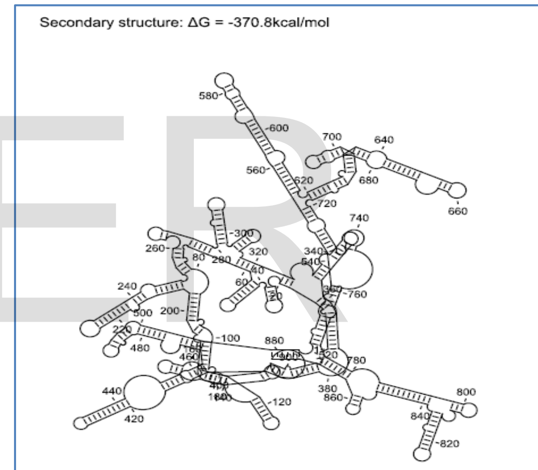


Fig 5: Multiple sequences alignment with 16s rRNA sequences



***Note: Red color = A, Green color = T, Blue color = C, Yellow = G**

Fig 6: Secondary structure 16s RNA sequence of *Cronobacter Sakaszakii*



$$\Delta G = -370.8 \frac{\text{kcal}}{\text{mol}}$$ **The**

ΔG is obtained by using the formula. $\Delta G = \Delta H - T\Delta S$

Figure 7: Secondary structure 16s RNA sequence of *Cronobacter Sakaszakii*

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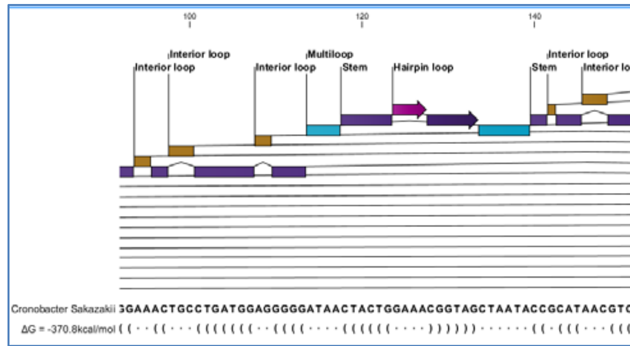

$\Delta G = -370.8\ \text{kcal/mol}$The ΔG **is obtained by using the formula.** $\Delta G = \Delta H - T\Delta S$

Conclusion

The isolation and partial genome characterization of mastitis infected bacterial type in cows was analyzed with advanced molecular tools. This report would serves as first report in India. Mastitis is characterized by multibacterial etiology. The attempts made to identify pathogens within mastitic milk were traditional culture techniques. Identification of *C.sakazakii* by PCR-RFLP analysis of 16S rRNA gene demonstrated that all isolates had species specific restriction profiles compared with the profiles of other *Cronobacter* species. Genomic diversity of *Cronobacter sakazakii* can be analyzed using 16S rRNA sequence analysis. Bioinformatics tools are used for structural analysis and determination of its molecular term. This research would be helpful for the identification of animal pathogen *Cronobacter sakazakii* bacteria with advanced molecular tools.

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