Chapter 5

Molecular systematic study: phylogenetic position of three nickel-resistant strains of River Torsa

5. Molecular systematic study: phylogenetic position of three nickel-resistant strains of River Torsa:

Both numerical taxonomy and molecular genetic characterization of the three nickel resistant Torsa isolates, BB1A, NiVa 51, and NiVas 114, have produced results identifying potential in them to enrich the field of microbial as well as genetic diversity. The remarkable diversity exhibited by them, in terms of conventional biochemical tests and metal resistance phenotype, compelled an active scientific urge to ascertain their proper systematic positions. Depending on the physiological and biochemical properties the isolates were tentatively assigned to the genus *Acinetobacter* sp. (BB1A), *Serratia* sp. (NiVa 51) and *Enterobacter* sp. (NiVas 114). Polyphasic taxonomic approaches were undertaken to ascertain the phylogenetic position of the strains.

5.1. Phylogenetic analysis using 16S rRNA gene sequences:

Besides functional constancy, ubiquitous distribution and size (1.5 KB), genes encoding for 16S rRNA exhibited both evolutionary conserved locus as well as highly variable regions. For these reasons, comparison of 16S rRNA gene sequences of the organisms could be used to calculate evolutionary distances between organisms (Woese *et al.*, 1990). The 16S rRNA gene of bacteria can be amplified and sequenced by using specific primers, designed from conserved regions. To identify the systematic position of an isolate, the 16S rRNA gene was sequenced and the sequences were used to generate phylogenetic trees with nearest relatives.

5.1.1. Materials and Methods:

5.1.1.1. Isolation of total genomic DNA:

The total genomic DNA of the strain BB1A, NiVa 51 and NiVas 114 were isolated following protocols described in section 3.2.2 of Chapter 3.

5.1.1.2. Amplification, cloning and sequencing of 16S rRNA gene of the strains:

PCR amplification was performed using 'PCR Amplification Kit' (GENEI, India), in 50µl reaction volume, following instructions provided by the supplier. Each 50 µl PCR mix contain; 3 µl of 10mM dNTP mix, 5 µl of 10X buffer containing 15 mM $MgCl_2$, 12.5 pmol of each forward (f) and reverse (r) primers [27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1500r (5'-AGAAAGGAGGTGATCCAGGC-3') (Gerhardt *et al.*, 1994) corresponding to the *Escherichia coli* numbering system] (Brousius *et al.*, 1978)], 20 ng target DNA and 3U *Taq* DNA Polymerase. The PCR was done in a GeneAmp PCR system (Applied Biosystems). PCR cycling parameters included an initial denaturation at 94 ^{0}C for 3 min; followed by 30 cycles of denaturation at 94 ^{0}C for 30 sec, annealing at 58 ^{0}C for 30 sec and amplification at 72^{0}C for 1 min and final extension at 72 ^{0}C for 5 min. The 1.5 KB amplicon was purified using PCR purification kit (KT 62, GENEI, India), cloned into pGEM®T–easy vector system II (Promega, USA) and transformed in *E. coli* XL1 Blue following techniques stated in section 4.3.2 of Chapter 4. Plasmid from the resulting clones, extracted by using 'alkaline lysis method' (Brinboim and Doly, 1979), was checked for the presence of 1.5 KB inserts from *Eco*RI digestions prior to sequencing. Sequencing was carried out with an ABI DNA sequenetor model 377a (Applied Biosystems) using Big-Dye Terminator kit (Applied Biosystems).

5.1.1.3. Phylogenetic analysis of the strains:

The 16S rRNA gene sequences of the strains were used as query sequences to search for homologous sequence in the nucleotide sequence databases by using BLASTN program (Altschul *et al.*, 1997). The 16S rDNA sequences showing high similarities were retrieved from the GenBank database and were aligned with 16S rRNA gene sequences of the isolates by using CLUSTAL W software (Thompson, 1997) of the European Bioinformatics Institute website (http://www.ebi.ac.uk/clustalw). Distances were calculated according to Jukes & Cantor (1969) one-parameter, Kimura two-

parameter (1980), Tajima & Nei (1987) three-parameter, and Tamura and Nei (1993) four-parameter methods when software package MEGA 3.0 (Kumar *et al.*, 2004) was used for generation of phylogenetic trees. When PHYLIP v 3.6 software package (Felsenstein, 2002) was used for the same purpose, distances were calculated by using Jukes & Cantor one-parameter and Kimura two-parameter methods.

Phylogenetic trees were inferred by using the neighbour-joining (Saitou & Nei, 1987), Maximum-likelihood (Yang, 1997) and parsimony (Felsenstein, 1983) analysis methods. Bootstrap analysis was based on 100 re-samplings. For neighbour-joining and parsimony analysis both MEGA 3.0 and PHYLIP 3.6c software packages were used; and for Maximum-likelihood analysis only PHYLIP 3.6c was utilized.

5.1.2. Results and discussion:

The 1.5 kb 16S rDNA amplicons derived from, BB1A, NiVa 51 and NiVas 114, were checked in an agarose gel with respect to the molecular size marker (Figure 5.1). The PCR products were purified and cloned in pGEM T-easy vector prior to sequencing. The recombinant plasmids containing 1.5 KB PCR products of BB1A, NiVa 51 and NiVas 114 were named as pVR1A, pVR51 and pVR114 respectively (Figure 5.2). The phylogenetic analyses of the isolates were discussed:

I. Isolate BB1A:

The 16S rRNA gene sequence (Figure 5.3) of the isolate was deposited in EMBL nucleotide database under accession no. AJ786647. The sequence was used as a query to search for homologous sequence in the nucleotide sequence databases by running BLASTN program. The consensus sequence, obtained using three replicate sequence reads, was compared with those in GenBank using the BLAST program. The 16S rRNA gene sequences of 12 species and one strain (ATCC 9957) of *Acinetobacter* showed 97-99% similarities with the 16S rDNA sequence of BB1A. A phylogenetic tree obtained with

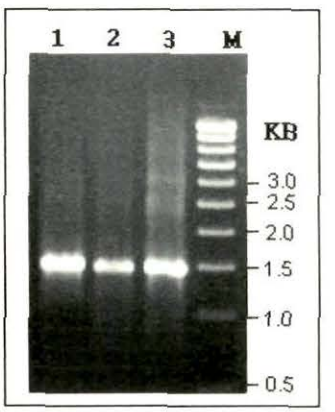

Figure 5.1. Agarose gel electrophoresis of the amplified 16S rRNA gene from isolate BB1A (lane 1), NiVa 51 (lane 2) and NiVas 114 (lane 3) using primers 27f – 1500r (M, marker lane).

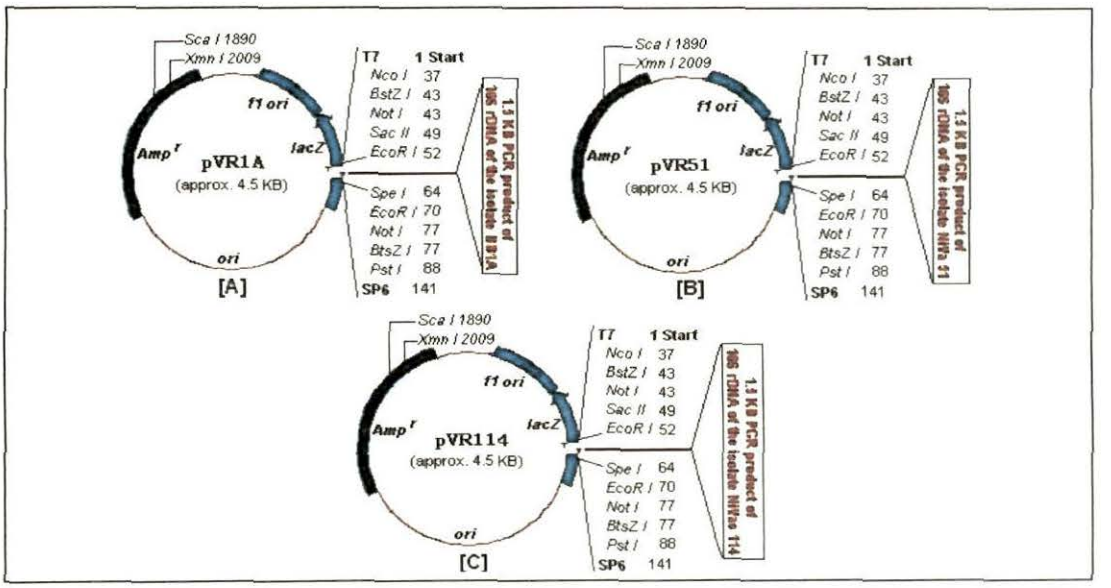

Figure 5.2. Construction of recombinant plasmids pVR1A, pVR51 and pVR114 (not in scale). *Amp*^{r}, gene for ampicillin resistance; *ori*, origin of replication, *lacZ*, gene for the synthesis of ß-galactosidase.

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1    atgagcgctg gcggcaggct taacacatgc aagtcgagcg gagatgaggt gcttgcacct
61   tatcttagcg gcggacgggt gagtaatgct tatgaatctg cctattagtg ggggacaaca
121  ttccgaaagg aatgctaata ccgcatacgt cctacgggag aaagcagggg atcttcggac
181  cttgcgctaa tagatgagcc taagtcggat tagctagttg gtggggtaaa ggcctaccaa
241  ggcgacgatc tgtagcgggt ctgagaggat gatccgccac actgggactg agacacgacc
301  ccacactggt acgggaggca gcagtgggga atattggaca atgggggcaa ccctgatcca
361  gccaggcccg cgatgtgtga agaaggcctt atggttgtaa agcactttaa gcgaggagga
421  ggctactgag actaatactc ttggatagtg gacgttactc gcagaataag caccggctaa
481  ctctgtgcca gcagccgcgg taatacagag ggtgcgagcg ttaatcggat ttactgggcg
541  taaagcgtgc gtagcggctt tttaagtcgg atgtgaaatc cccgagctta acttgggaat
601  tgcattcgat actgggaagc tagagtatgg gagaggatgg tagaattcca ggtgtagcgg
661  tgaaatgcgt agagatctgg aggaataccg atggcgaagg cagccatctg gcctaatact
721  gacgctgagg tacgaaagca tggggagcaa acaggattag ataccctggt agtccatgcc
781  gtaaacgatg tctactagcc gttggggcct ttgaggcttt agtggcgcag ctaacgcgat
841  aagtagaccg cctggggagt acggtcgcaa gactaaaact caaatgaatt gacgggggcc
901  cgcacaagcg gtggagcatg tggtttaatt cgatgcaacg cgaagaacct tacctggcct
961  tgacatacta gaaactttcc agagatggat tggtgccttc gggaatctag atacaggtgc
1021 tgcatggctg tcgtcagctc gtgtcgtgag atgttgggtt aagtcccgca acgagcgcaa
1081 ccctttcct tacttgccag catttcggat gggaacttta aggatactgc cagtgacaaa
1141 ctggaggaag gcggggacga cgtcaagtca tcatggccct tacggccagg gctacacacg
1201 tgctacaatg gtcggtacaa agggttgcta cacagcgatg tgatgctaat ctcaaaaagc
1261 cgatcgtagt ccggattgga gtctgcaact cgactccatg aagtcggaat cgctagtaat
1321 cgcggatcag aatgccgcgg tgaatacgtt cccgggcctt gtacacaccg cccgtcacac
1381 catgggagtt tgttgcacca gaagtaggta gtctaaccgc aaggaggacg cttaccagcg
1441 gtgtggccga tggctgc
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Figure 5.3. 16S rRNA gene sequences of the isolate BB1A (EMBL Accession no. AJ786647).

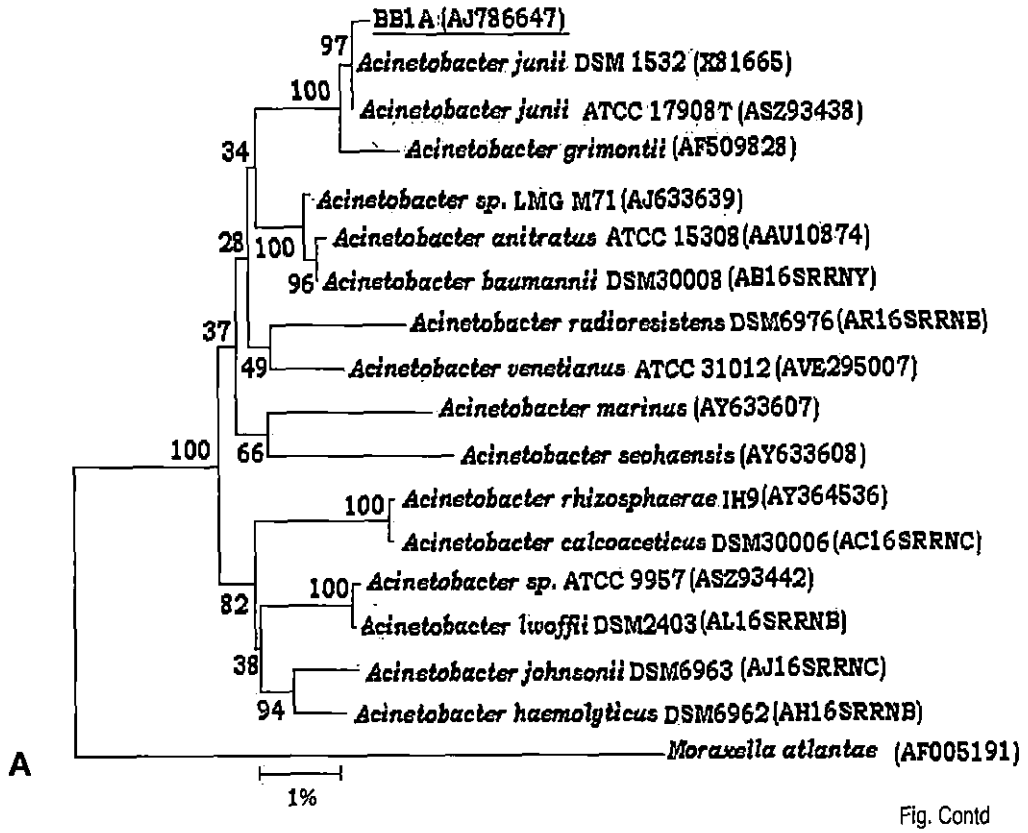

Fig. Contd

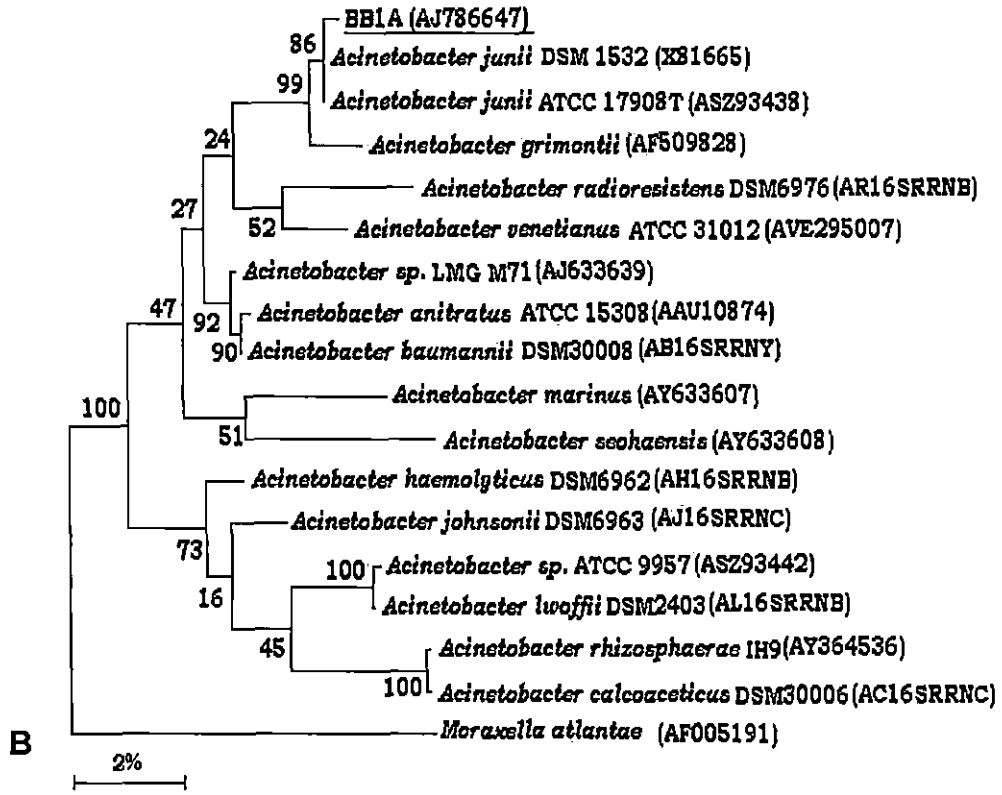

Figure 5.4. Phylogenetic trees of representative members of *Acinetobacter* inferred from 16 S rDNA sequences showing the systematic position of the isolate BB1A. Bootstrap values for 100 replication are also shown at the branch nodes and the bar, indicates number of substitutions per 100 nt. (A), Tree obtained by the neighbour-joining method and evolutionary distances were calculated with using Kimura two-parameter model. (B), Tree obtained by maximum-parsimony analysis method.

Kimura's two-parameter model and the neighbour-joining method (Figure 5.4A), showed that strain BB1A form a separate cluster with *Acinetobacter junii* DSM 1532 (Ibrahim *et al.* 1997), *Acinetobacter junii* ATCC 17908T (Rainey *et al.* 1994), and therefore undoubtedly a member of the genus *Acinetobacter.* The same results were obtained when phylogenetic distances were calculated by using the Jukes-Cantor one-parameter, Tamura-Nei three-parameter and Tajima-Nei four-parameter models. The maximum-parsimony (Figure 5.4B) and maximum-likelihood analysis also showed the same results. Sequence similarity, based on pairwise sequence comparisons, was investigated by using complete 16S rDNA sequences. The data showed that the 16S rDNA sequence of strain BB1A had the maximum similarity of 99.5% with *A. junii* DSM 1532, while 16S rRNA gene *A. baumanii, A. venetianus* and *A. anitratus* showed 97.3 – 97.7% similarities and other species of *Acinetobacter* fell in the range of 95 -96% similarity

II. Isolate NiVa 51:

Almost complete 16S rDNA sequence (1530 nt) of isolate NiVa 51 (EMBL accession no. AJ854062) was obtained (Figure 5.5). The consensus sequence, obtained using three replicate sequence reads, was compared with those in GenBank using the BLAST program. The 16S rDNA sequences of validly published taxa showing 95 -98% similarities with the 16S rDNA sequence of the strain NiVa 51 were retrieved and aligned using CLUSTAL W version 1.8 software. Phylogenetic trees were constructed according to three different methods [neighbor-joining, maximum-parsimony and maximum-likelihood analysis], that included *Pectobacterium carotovorum, Escherichia coli, Enterobacter aerogenes,* seven species of the genus *Serratia* and two sub-species of *S. marcescens*, three species of the genus *Klebsiella* and *Erwinia*, and two species of the genus *Buttiauxella* and *Citrobacter,* using software package PHYLIP v. 3.6c. For neighbor joining,

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   1 atagtttgag atggctcaga ttgaacgctg gcggcaggct tacacatgca agtcgagcgg
  61 tagcacaggg gagcttgctc cctgggtgac gagcggcgga cgggtgagta atgtctggga
 121 aactgcctga tggatgggga taactactgg aaacggtagc taataccgca taacgtcgca
 181 agaccaaaga gggggacctt cgggcctctt gccatcagat gtgcccagat gggattagct
 241 agtaggtggg gtaatggctc acctaggcga cgatccctag ctggtctgag aggatgacca
 301 gccacactgg aactgagaca cggtccagac tcctacggga ggcagcagtg gggaatattg
 361 cacaatgggc gcaagcctga tgcagccatg ccgcgtgtgt gaagaaggcc ttcgggttgt
 421 aaagcacttt cagcgaggag gaaggtggtg agcttaatac gctcatcaat tgacgttact
 481 cgcagaagaa gcaccggcta actccgtgcc agcagccgcg gtaatacgga gggtgcaagc
 541 gttaatcgga attactgggc gtaaagcgca cgcaggcggt ctgtcaagtc ggatgtgaaa
 601 tccccgggct caacctggga actgcattcg aaactggcag gctagagtct tgtagagggg
 661 ggtagaattc caggtgtagc gatgaaatgc gtagagatct ggaggaatac cggtggcgaa
 721 ggcggccccc tggacaaaga ctgacgctca ggtgcgaaag cgtggggagc aaacaggatt
 781 agataccctg gtagtccacg ccgtaaacga tgtcgacttg gaggttgtgc ccttgaggcg
 841 tggcttccgg agctaacgcg ttaagtcgac cgcctgggga gtacggccgc aaggttaaaa
 901 ctcaaatgaa ttgacggggg cccgcacaag cggtggagca tgtggtttaa ttcgatgcaa
 961 cgcgaagaac cttacctact cttgacatcc agagaactta gcagagatgc tttggtgcct
1021 tcgggaactc tgagacaggt gctgcatggc tgtcgtcagc tcgtgttgtg aaatgttggg
1081 ttaagtcccg caacgagcgc aacccttatc ctttgttgcc agcggttagg ccgggaaccc
1141 aaaggagact gccagtgata aactggagga aggtggggat gacgtcaagt catcatggcc
1201 cttacgagta gggctacaca cgtgctacaa tggcgtatac aaagagaagc gacctcgcga
1261 gagcaagcgg acctcataaa gtacgtcgta gtccggattg gagtctgcaa ctcgactcca
1321 tgaagtcgga atcgctagta atcgtagatc agaatgctac ggtgaatacg ttcccgggcc
1381 ttgtacacac cgcccgtcac accatgggag tgggttgcaa aagaagtagg tagcttaacc
1441 ttcgggaggg cgacttacca ctttgtgatt catgactggg gtgaagtcgt aacaaggtaa
1501 ccgta
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Figure 5.5. 16S rRNA gene sequences of the isolate NiVa 51 (EMBL Accession no. AJ854062).

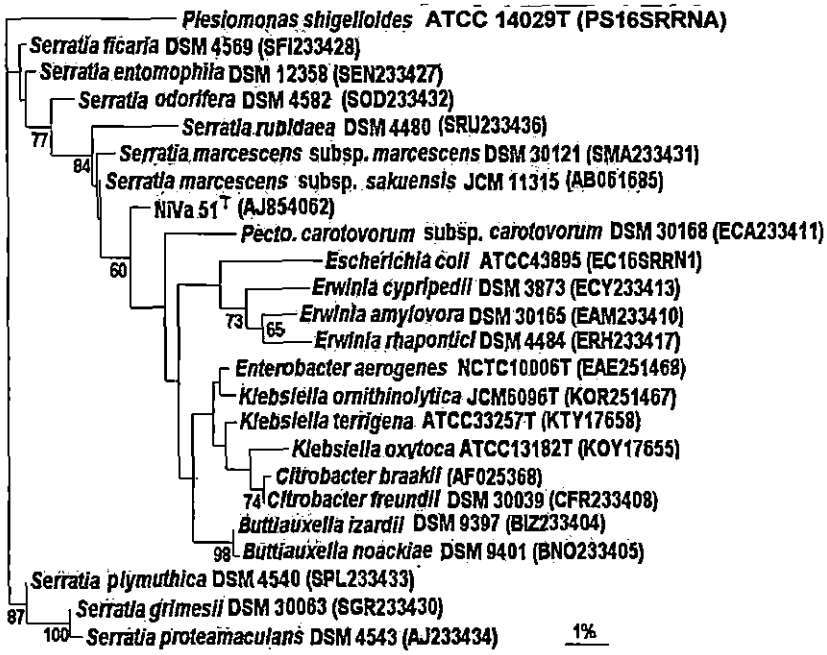

Figure 5.6. Phylogenetic tree derived from 16S rRNA gene sequences (1430 nucleotides) using maximum-likelihood method showing the relationship between isolate NiVa 51 and its phylogeneticaly related taxa. Bootstrap values for 100 replications are given at branch nodes (only values of 60 and above are given). The bar represents single substitutions per 100 nucleotides.

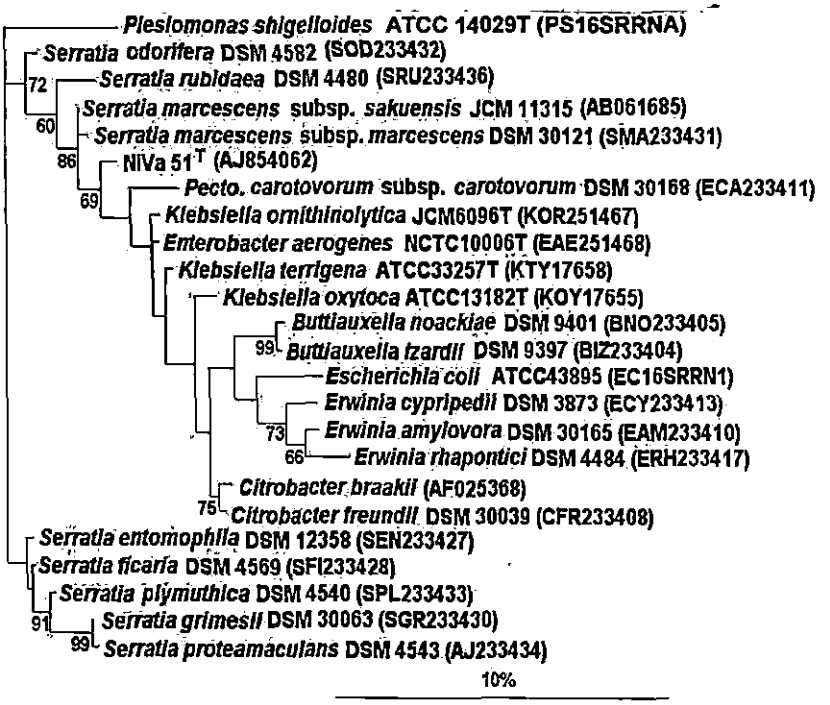

Figure 5.7. Phylogenetic tree derived by maximum-parsimony method showing systematic position of NiVa 51 within members of the family *Enterobacteriaceae*. The bootstrap values were calculated using SEQBOOT program for 100 replications (only values of 60 and above are given). The bar represents one substitution in 10 substitutions per 100 nucleotides.

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   1 agagtttgat cctggctcag attgaacgct ggcggcaggc ttaacacatg caagtcgtac
  61 ggtaacatga atccgcttgc tgcttcgctg acgagtggcg gacgggtgag taatgtctgg
 121 gaaactgcct gatggagggg gataactact ggaaacggta gctaataccg cataacgtcg
 181 caagaccaaa gaggggacc ttcgggcctc ttgccatcgg atgtgcccag atgggattag
 241 ctagtaggtg gggtaacggc tcacctaggc gacgatccct agctggtctg agaggatgac
 301 cagccacact ggaactgaga cacggtccag actcctacgg gaggcagcag tggggaatat
 361 tgcacaatgg gcgcaagcct gatgcagcca tgccgcgtgt atgaagaagg ccttcgggtt
 421 gtaaagtact ttcagcgggg aggaaggtgt tgaggttaat aacctcgtcg attgacgtta
 481 cccgcagaag aagcaccggc taactccgtg ccagcagccg cggtaatacg gagggtgcaa
 541 gcgttatcgg attactggcg taaagcgcac gcagcggtct gtccagtccg atgtgaaatc
 601 cccggctcaa cctgggaact gcattcgaaa ctggcaggct agagtcttgt agaggggggt
 661 agaattccag gtgtagcggt gaaatgcgta gagatctgga ggaataccgg tggcgaaggc
 721 ggccccctgg acaaagactg acgctcaggt gcgaaagcgt ggggagcaaa caggattaga
 781 taccctggta gtccacgccg taaacgatgt cgacttggag gttgtgccct tgaggcgtgg
 841 cttccggagc taacgcgtta agtcgaccgc ctggggagta cggccgcaag gttaaaactc
 901 aaatgaattg acgggggccc gcacaagcgg tggagcatgt ggtttaattc gatgcaacgc
 961 gaagaacctt acctactctt gacatccaga gaacttagca gagatgcttt ggtgccttcg
1021 ggaactctga gacaggtgct gcatggctgc cgtcagctcg tgttgtgaaa tgttgggtta
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1141 ggagactgcc agtgataaac tggaggaagg tggggatgac gtcaagtcat catggccctt
1201 acgagtaggg ctacacacgt gctacaatgg cgcatacaaa gagaagcgac ctcgcgagag
1261 caagcggagg tcataaagtg cgtcgtagtc cggattggag tctgcaactc gactccatga
1321 agtcggaatc gcttagtaat cgtggatcag aatgccacgg tgaatacgtt cccgggcctt
1381 gtacacaccg cccgtcacac catgggagtg ggttgcaaaa gaagtaggta gcttaacctt
1441 cgggagggcg cttaccactt tgtgattcat gactggggtg aagtcgtaca aggacaaggt
1501 agccgtaggg gaacctgcgg ctggatcacc tcctttct
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Figure 5.8. 16S rRNA gene sequences of the isolate NiVas 114 (EMBL Accession no. AJ854063).

KITSCH, and FITCH analysis, distances were calculated by using both Kimura-two parameter (Kimura, 1980) and Jukes-Cantor one parameter (Jukes & Cantor, 1969) model with DNADIST. The 16S rDNA sequence of *Plesiomonas shigelloides* (the most closely related species of the *Enterobacteriaceae* family) was used to root all the trees. To determine the confidence values for individual branches, 100 bootstrap replications were done for each generated tree using SEQBOOT and CONSENSE from the PHYLIP package. Phylogenetic trees obtained with maximum-likelihood method showed that strain NiVa 51 branched deeply with the *Serratia* cluster consisting of *S. marcescens*, *S. rubidaea*, and *S. odorifera* (Figure 5.6). Similar pattern of branching have been noted in trees derived from maximum-parsimony (Figure 5.7), KITSCH and FITCH analysis. Sequence similarity, based on pair-wise sequence comparison, was investigated by using complete 16S rDNA sequences. The 16S rDNA sequence of strain NiVa 51 showed 96 –97 % similarities with many species of the genus *Serratia*, except in the case of *S. marcescens* subsp. *sakuensis* and *S. marcescens* subsp. *marcescens* (to which it shows 98.30 -98.35% similarity).

III. Isolate NiVas 114:

The sequence of the 16S rRNA gene (Figure 5.8) of the isolate NiVas 114 was obtained (1538 bp) and was deposited under EMBL accession no. AJ854063. The sequence obtained was compared with those in GenBank using the BLAST program. The 16S rDNA sequences of validly published taxa showing 96- 98% similarities with the 16S rDNA sequence of the strain NiVas 114 were retrieved and aligned using CLUSTAL W software. Distances were calculated according to the of Jukes & Cantor one parameter, Kimura two parameter, Tajima & Nei three parameter, and Tamura and Nei four parameter methods. Phylogenetic trees were constructed by using the neighbour-joining, Maximum-likelihood and parsimony analysis methods. 16S rDNA sequence of *Plesiomonas shigelloides* (the most closely related species of the *Enterobacteriaceae* family) was used to root all the trees. A phylogenetic tree obtained with Kimura's two-parameter model and the neighbour-joining method that included, twelve species of the genus *Enterobacter*; four species each under the genera *Citrobacter*, *Erwinia* and *Salmonella*; three species of the genus *Klebsiella*; and two species each under the genera *Pectobacterium*, *Pantoea*, *Buttiauxella* and *Kluyvera* (Figure 5.9), showed that strain NiVas 114 is a new member of the *Enterobacter* cluster constituted of *E. cancerogenus*, *E. cloacae* and *E. hormaechei*. The same results were obtained when phylogenetic distances were calculated by using the Jukes-Cantor one-parameter, Kimura two-parameter, Tamura-Nei three-parameter and Tajima-Nei four-parameter models. Maximum- likelihood and maximum- parsimony analysis also showed same results. Sequence similarity, based on pairwise sequence comparisons, was investigated by using complete 16S rDNA sequences. The data obtained showed that the 16S rDNA sequence of strain NiVas 114 depicts more than 98% similarity with *E. cloacae* and *E. hormaechei*. Therefore, strain NiVas 114 form a group within the genus *Enterobacter* that is clearly distinguishable from the other species, based on 16S rRNA gene sequences. The results are in agreement with that of physiological and biochemical tests (table 4.1), based on which isolate NiVas 114 was regarded as a member of the genus *Enterobacter*.

5.1.3. Conclusion:

Phylogenetic analysis based on 16S rRNA gene sequences clearly indicated that the isolate BB1A is a strain of *Acinetobacter junii*. In the phylogenetic trees obtained by the neighbour-joining and maximum-parsimony methods, BB1A branched in a cluster with the type strains of *A. junii*. The isolate also showed some differences in biochemical properties with the type strains (Table 5.1). Based on the biochemical properties and 16S rDNA sequence similarities, the isolate was identified as a novel strain of *Acinetobacter junii*. Other two isolates, NiVa 51 and NiVas 114, showed 97-98% similarities with the type strains of *Serratia* and *Enterobacter* respectively. In the phylogenetic trees, depicted in the Figure 5.6, 5.7 and 5.9, the isolates formed robust clade with *S. marcescens* and *E. hormaechei* respectively, with which the isolates showed highest 16S rDNA sequence similarities.

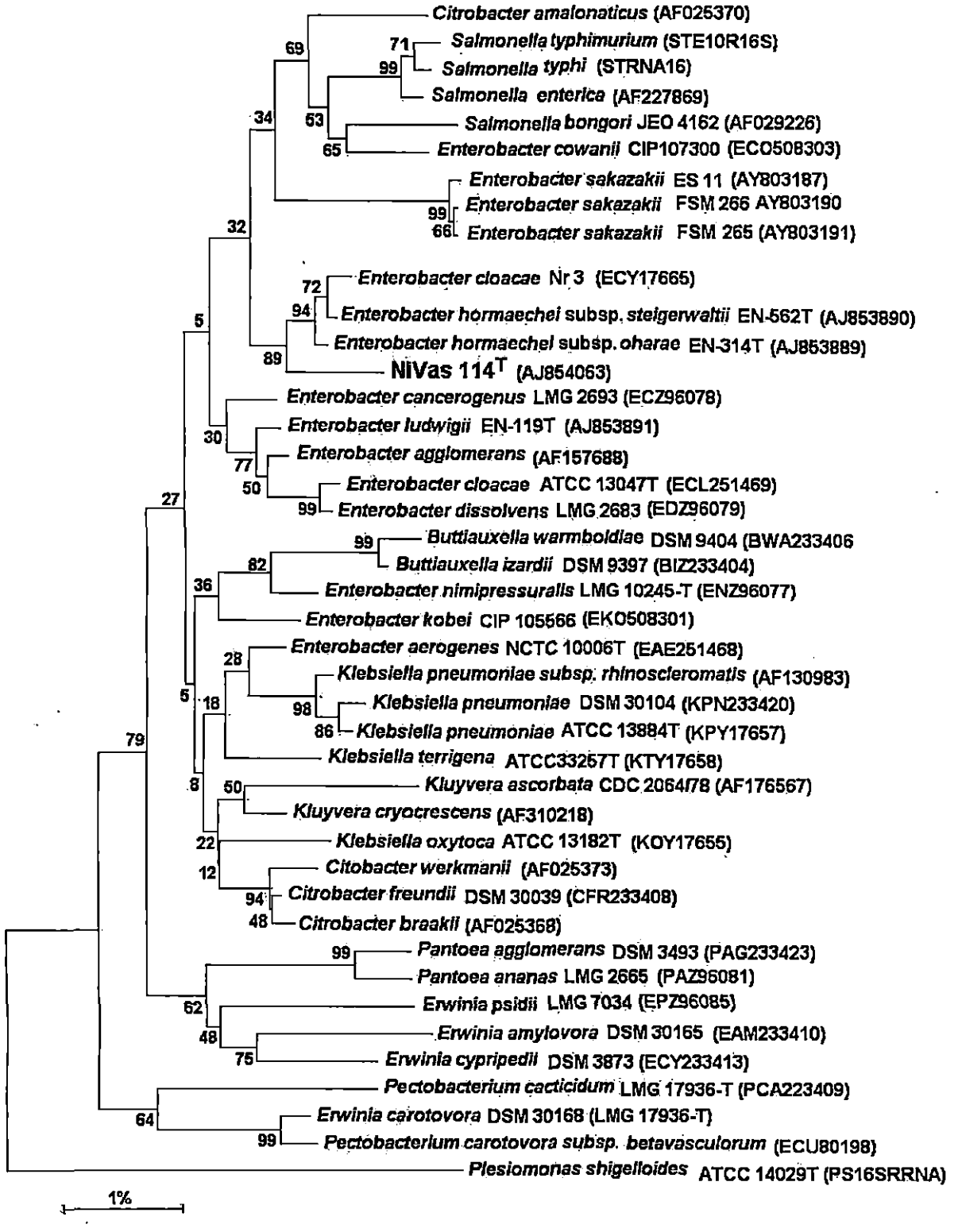

Figure 5.9. Phylogenetic tree of representative members of *Enterobacteriaceae* inferred from 16S rDNA sequences by the neighbour-joining method showing the relationship between isolate NiVas114^T and its relatives. The evolutionary distances were calculated with using Kimura two-parameter model. Bootstrap values for 100 replication sare also shown at the branch nodes. Bar, indicate 1 substitution per 100 nt.

Therefore, further exploration of their genomic DNA relatedness as well as chemotaxonomic features were required for identification of their proper taxonomic positions.

5.2. Determination of fatty acid composition, GC content and DNA-DNA relatedness:

5.2.1. Analysis of cellular fatty acids (FAME):

A popular method of bacterial identification is the characterization of types and proportions of fatty acids present in membrane. The chemistry of fatty acids are so variable, including differences in chain length, the presence or absence of unsaturated groups and rings or branched chains, and hydroxyl groups, the fatty acid profile of a particular bacterium can often be useful diagnostically. For analysis, fatty acids, extracted from bulk lipids of a bacterial culture grown under standardized conditions, are chemically modified to form their corresponding methyl esters. These volatile ester derivatives are then estimated quantitatively and qualitatively by gas chromatography and compared with a database containing the fatty acid profiles of thousands of reference bacteria (that were grown under the same conditions) and the best matches to that of the unknown is selected for identification.

5.2.1.1. Materials and methods:

For determination of CFA compositions cells were grown in Tryptone Soya Broth (HIMEDIA) (g/l; Casein enzymatic hydrolysate, 17.0; peptic digest of soyabean meal, 3.0; NaCl, 5.0; K_2HPO_4, 2.5, Dextrose, 2.5; pH 7.3) at 28 ^{0}C. Fatty acid extraction, methyl-ester preparation and analyses were performed as described by earlier authors (Katayama-Fujimura *et al.*, 1982; Groth *et al.*, 1996; Labrenz *et al.*, 1998). All chemicals used in this experiment are purchased from SRL India Ltd. (India).

5.2.1.1.1. Extraction and analysis of fatty acid methyl esters (FAME):

Freeze dried samples (100 mg) were homogenized in a steel homogenizer for two minutes, with methanol-chloroform mixture (2:1, v/v), filtered and filtrate was collected, residue was further homogenized for two minutes with methanol-chloroform-water mixture (2:1:0.8, v/v/v), filtered and residue was further homogenized for another two minutes with the first solvent mixture and filtered. The combined filtrates diluted with little chloroform and enough water, shaken vigorously and allowed to settle for layer separation in a separation funnels. The bottom chloroform layer containing lipid was removed and dried over anhydrous sodium sulfate. The solvent was evaporated in a rotary evaporator under vacuum of 40 ^{0}C. Finally, the lipids were weighed and stored in a deep freezer at –20 ^{0}C, in redistilled hexane, under nitrogen atmosphere, till used. The lipid sample (500 mg) was hydrolyzed by refluxing it with 1.5 (M) solution of potassium hydroxide in methanol (10 ml) for 2 h in an atmosphere of nitrogen gas. After saponification, methanol was removed under vacuum; the suspension thus obtained was dissolved in water, cooled, transferred to a separation funnel and extracted three times with diethyl ether to remove non-saponifiable material. Whenever necessary, centrifugation was done to break any emulsion that was formed. The solvent extracts were washed several times with water and dried over anhydrous sodium sulfate. The non-saponifiable material was recovered on removal of solvent in a rotary evaporator and then weighed in a microbalance. Water washings were added to the aqueous layer, which was acidified with 4(N) H_2SO_4 and extracted with diethyl ether and hexane for three times. The fatty acids were recovered after washing the extracts with distilled water to make it free from any mineral acid contamination and then dried over anhydrous sodium sulfate, and solvent was removed in a rotary evaporator under reduced pressure at room temperature. Fatty acids were methylated by ethereal solution of diazomethane. The diazomethane gel was generated by treating N-nitrosomethyl urea with methanolic potassium hydroxide, distilled and dissolved in cool diethyl ether. The ethereal solution of diazomethane was added to the ethereal solution of fatty acid containing few drops of dry methanol and was kept overnight at 4 ^{0}C. Solvent was removed in a rotary evaporator.

FA methyl-esters were applied with a syringe to 20 X 20 cm silica gel thin layer plates (thickness 0.25

mm) as a streak (20 mm). The plates were developed in light petroleum ether (40 –60 ^{0}C): diethylether (85:15, v/v) until the mobile phase had nearly reached the top of the plates (19 cm). Chromatograms were then air dried and visualized in I_2 vapor. Normal FAMEs had Rf value of 0.75 – 0.85 in this mobile phase and 2-hydroxy and 3-hydroxy FAMEs had Rf values of 0.22 and 0.16 respectively. The individual fatty acid spots on the thin-layer plates were scraped of by sucking the silica gel into Pasteur pipettes packed with siliconized glass wool. The FAMEs were eluted from the columns with 5 ml diethyl ether. The ether extract was evaporated to dryness and preserved in Hexane for GLC.

5.2.1.1.2. Catalytic reduction of fatty acid methyl esters:

Fatty acid methyl ester samples were hydrogenated in a multiple hydrogenator. In each hydrogenetion flask, 3.0 mg of Adams' platinum oxide catalyst and 3.0 ml of super-dry methanol were taken. Air inside the flask was removed by flushing hydrogen. Stirring of the catalyst in the hydrogen atmosphere was maintained till the catalyst turned clack and coagulated. After activation, 1.0 mg FAMEs of each sample, 2.0 ml of super-dry methanol was added to the flask. Air was removed as before and hydrogenation was continued for 3 h at 30 ^{0}C, maintaining slight positive pressure inside the flasks. The hydrogenated samples were filtered through a Whatman 42 (Whatmanman Int. Ltd., England) filter paper by several transfers with chloroform. Solvent evaporated and the reduced FAMEs were pressured for GLC.

5.2.1.1.3. Gas Liquid Chromatography of the fatty acid methyl esters:

The FAMEs were analyzed by GLC on polar 10% DEGS (Diethylene glycol succinate polyester) liquid phase which was coated on solid support, chromosorb-w(HP), 80-100 mesh in a rotary evaporator. The column material thus obtained was packed into a coiled glass column (1.8 mt. X 2 mm) e.d. by gentle tapping and applying a gentle suction from a vacuum pump. The column was then conditioned at 198 ^{0}C. During the course of analysis, the oven temperature was 196 ^{0}C, whereas detector and infection port temperature were around 250 ^{0}C. Carrier gas was N_2 with a flow rate of 30ml/ min.

Identification of fatty acids was done by; (i) comparison of the relative retention time of their ethyl esters with that of standard fatty acids (14:0, 15:0, 16:0, 17:0, 18:0, 19:0, 16:149, 18:149); (ii) unsaturated FAMEs were identified comparing of the chromatograms of hydrogenated methyl esters samples with those of original samples (hydrogenation process described earlier)); (iii) cyclopropene acids were identified by following elimination procedures: another aliquot (2-3 mg) of the sample was heated in 5 ml of 5.4 % (w/v) methanolic solution of anhydrous HCl under reflux for 3h. After 1ml of water being added to the refluxed solution, fatty acid methylesters were extracted with petroleum ether and analyzed by Gas Liquid Chromatography. Although under the present gas chromatographic conditions cyclopropane acids were eluted with the corresponding straight-chain mono-unsaturated acid, they could thus be differentiated from the later. Positional isomers of mono-unsaturated acids were tentatively identified by the comparison of their retention data in the 10% DEGS column.

5.2.1.2. Results and discussion:

The cellular fatty acid compositions of the isolates, NiVa 51 and NiVas 114 are summarized below:

(I) CFA analysis of strain NiVa 51:

The fatty acid profile of the strain NiVa 51 was compared with the other members of the same *Serratia* cluster (*S. marcescens*, *S. rubidaea* and *S odorifera*) and represented in the Table 5.1. The major cellular fatty acid of the isolate was identified as 16:0, Summed feature 3 (comprising 16:1 w7c/ 15iso2OH) and 18:1 (see Fable 5.1). The fatty acid composition of the isolate, generated the highest of 0.747 SIM-index value with *S. marcescens*, when compared with the fatty acid content of other bacteria of Sherlock TSBA 40 (v 4.5) identification library of MIS (Microbial Identification Library). When compared with the closest phylogenetic relative of the isolate, *S. marcescens*, the fatty acid 17:0 CYCLO and 19:0 CYCLO content of the strain

Table 5.1. Cellular fatty acid composition of the strain NiVa 51 and its phylogenetically related taxa of the same genus.

Fatty acid type	NiVa 51^T	*Serratia marcescens*[a] GC subgroup B	*Serratia marcescens* subsp. *sakuensis*[b]	*Serratia odorifera*[a]	*Serratia rubidaea*[a]
12:0	1.40	1.46	1.25	2.95	4.28
12:0 2OH	0.28	0.28	-	0.52	-
Unknown 13.957	0.97	0.57	-	-	-
14:0	6.28	6.94	-	7.40	6.52
Unknown 14.502	0.85	0.71	-	0.74	-
14:0 2OH	2.21	2.05	2.83	-	-
15:0	0.29	-	0.58	-	1.31
16:0	31.01	31.86	32.2	30.74	17.98
17:0 CYCLO	2.69	11.68	-	4.96	-
17:0	0.35	0.43	0.66	-	0.63
18:1 w7c	17.89	16.88	-	14.40	11.39
18:0	0.46	0.43	1.93	0.47	0.45
19:0CYCLO w8c	0.25	1.24	-	0.47	-
Summed feature 2	8.01	8.44	-	8.98	4.11
Summed feature 3	27.07	16.51	-	28.04	47.83

[a], Data from Sherlock MIS TSBA50 identification library (courtesy, ir. Claudine Vereecke, BCCM™/LMG Bacterial collection, Gent, Belgium); [b], Data from Ajitkumar et al. (2003); -, data not available.

Table 5.2. Cellular fatty acid composition of the isolate NiVas 114^T and its phylogenetically related taxa.

Fatty acid type	NiVas114^T	*Enterobacter cloacae**	*Enterobacter aerogenes**	*Enterobacter cancerogenus**
12:0	1.51	2.86	1.25	3.30
12:0 2OH	0.29	-	-	-
Unknown 13.957	0.86	-	-	-
14:0	6.59	6.76	9.34	7.17
Unknown 14.502	0.72	0.80	0.92	-
14:0 2OH	1.95	-	1.27	-
15:0	0.24	1.68	-	-
16:0 w5c	0.10	-	-	-
16:0	32.18	27.58	31.00	24.77
17:0 CYCLO	3.53	5.94	11.04	15.36
17:0	0.21	1.27	0.69	3.25
18:1 w7c	16.24	22.69	19.27	19.97
18:0	0.32	0.30	-	-
19:0 CYCLO w8c	0.27	-	2.67	0.56
Summed feature 2	8.04	7.970	9.00	7.09
Summed feature 3	26.95	20.890	12.98	15.46

*, Fatty acid data was received from Sherlock MIS TSBA50 identification library; -, data not available.

was recorded to be 5 times higher and 4 times lower respectively. The existing diversity of the CFA content of NiVa 51 indicated towards the novelty of the strain from any other existing taxa so far discovered. The fatty acid profile of the isolate was also kindly confirmed by Deutsche Sammlung von Microorganismen and Zellkulturen GmbH (DSMZ), Germany.

(II) CFA analysis of strain NiVas 114:

The fatty acid profile of the strain NiVas 114 has been presented in the Table 5.2. The major fatty acids of the isolate were, 16:0, Summed feature 3 (comprising 16:1 w7c/ 15iso2OH) and 18:1 (see Table 5.2). The CFA profile of the isolate was found to be distinctly different from *Enterobacter cloacae*, with which it showed highest 16S rDNA sequence similarities. The fatty acid 15:0 and 17:0 content of the isolate was found to the 6-7 times lower than *E. cloacae*. When CFA profile of the isolate was compared with the fatty acid content of other bacterium enlisted in Sherlock TSBA 40 (v. 4.5) identification library of MIS, a Sim-index value of 0.665 and 0.580 was recorded with *E. pyrinus* and *E. entermedius* respectively. The fatty acid profile of the isolate was also kindly confirmed by DSMZ, Germany. The analysis of cellular fatty acid result strongly suggested that the isolate does not belong to any other taxa discovered previously.

5.2.2. Analysis of base composition of DNA:

The analyses of base composition of DNA were made by BCCM™/LMG bacterial collection, Ghent

University, Belgium. The genomic DNA was prepared according to the methods described in section 3.2.2 of Chapter 3. The DNA of the strains were hydrolysed with P1 nuclease and the nucleotides were de-phosphorylated with bovine alkaline phosphatase as described by Mesbah *et al.* (1989). From the resulting deoxyribonucleosides, G+C contents (mol%) were analyzed using HPLC (Mesbah *et.al.*, 1989). The instrument was calibrated with non-methylated lambda DNA (Sigma; G+C content 49.86 mol%). The G+C contents were calculated from the ratio of deoxyguanosine (dG) and deoxyadenosine (dA) as described by Mesbah *et.al.* (1989).

The G+C content of the DNA of the isolate NiVa51 was found to be 60 mole%. The value observed for the isolate was found to be higher than *Serratia marcescens* subsp *sakuensis* [(58 mol%), Ajithkumar *et al.*, 2003], but comparable with that of *Serratia marcescens* [(57.5- 60%), Grimont & Grimont, 1992]. On the other hand the G+C content of the DNA of the isolate NiVas 114 was found to be 59.9 mole%. The value observed for the isolate NiVas 114 was found to be higher than its phylogenetically related taxa, *Enterobacter aerogenes* [(57 % mol), Holt *et al.*, 1994], and *Enterobacter cancerogenes* [(56.4 % mol), Holt *et al.*, 1994], but comparable with that of *Enterobacter cloacae* [(53- 60% mol), Grimont & Grimont, 1992].

5.2.3. Study of DNA-DNA relatedness:

For the study of genomic relatedness with the nearest phylogenetic relatives 'Dot Blot' hybridization technique was used following principles stated by Ezaki *et al.* (1989). The type strains used in the experiment were collected from recognized culture collection centers. DIG-High Prime DNA labeling and detection kit (Roche Diagnostics GmbH, Germany) was used for the experiments.

5.2.3.1. Materials and Methods:

5.2.3.1.1. Preparation of the total genomic DNA:

The total genomic DNA of the test strains and type cultures were prepared following techniques stated in section 3.2.2 of Chapter 3. For quantification and purity checking, 10 µl of genomic DNA preparation were diluted in 995 µl sterile TE (10 mM Tris-Cl: 0.1 mM EDTA, pH 7.6) in a 1 ml quartz cuvette and absorption (A) was measured at 260nm as well as at 280nm. The preparations showed A260nm/ A280 nm ratio of >1.7 which were used for the experiments. The concentration of DNA was calculated considering the A260nm of 1.0 is equivalent to 50 µg of double stranded DNA (Towner, 1991).

5.2.3.1.2. Transfer of DNA to the nylon membrane:

The nylon membrane was cut according to the size of the dot-blot apparatus (with 96 wells, BRL, USA) attached to a vacuum pump. This membrane was soaked in 6 X SSC and was placed on the dot-blot apparatus. DNA samples were denatured by heating for 10 min in a boiling water bath and were immediately chilled. The dot-blot apparatus was continuously run under vacuum during the following steps:

Slots were washed with 6 X SSC and were charged with the DNA samples to be fixed in the membrane. Finally the slots were washed with 6 X SSC, the membrane was removed from the apparatus and DNAs were fixed by UV crosslinker (Stratalinker, Stratagene, USA) as recommended by the manufacturer.

5.2.3.1.3. Preparation of probe DNA:

The genomic DNA preparation of the isolates, NiVa 51 and NiVas 114 were separately digested with *Sau* IIIA restriction enzyme (New England Biolabs Inc.) following procedure discussed in section 4.3.1.1.2 of Chapter 4. According to the random primer DNA labeling technique, approximately 5 µg of the restricted DNA of each isolate was labeled by digoxigenin-dUTP. The detailed procedure of the probe preparation was discussed in section 4.3.1.1.3 of Chapter 4.

5.2.3.1.4. Hybridization and detection of hybridization-intensities:

The hybridization was carried out in a hybridization bag, and the technique used for prehybridization, hybridization, post hybridization; blocking and detection were done following techniques stated in

section 4.3.1.1.3 of chapter 4. Approximately 100-times excess of the probe DNA was used in hybridization against frames of three target DNAs taken in quantities of triplicate (10 ng, 20 ng and 30 ng) at a time in a positively charged nylon membrane. Hybridization was carried out at 65 ^{0}C and the membrane was washed under high stringency condition. All the hybridization blots were analyzed by densitometer scanning device (BIO RAD Inc., model GS- 700 Imaging densitometer and molecular analyst software) and the results were expressed as % of positive control. In all experiments genomic DNA of *E. coli* DH 5α and *Acinetobacter junii* were used as negative control, and NiVa 51 or NiVas 114 were used as positive controls.

5.2.3.2. Results and discussion:

Using genomic DNA of NiVa 51 as a probe, DNA-DNA hybridization was recorded as 43.7% with *Serratia marcescens* subsp. *marcescens*, 29.2% with *Serratia odorifera*, 27% with *Serratia rubidaea*, 18.3% with *Enterobacter aerogenes*, 19% with *Citrobacter freundii*, 20% with *Erwinia amylovora*, and 21.7% with *Pectobacterium carotovorum* subsp. *carotovorum*. When the genomic DNA of NiVas 114 was used as probe, DNA-DNA hybridization was recorded as 35% with *Enterobacter cloacae*, 28.4% with *Enterobacter cancerogenus*, 23.3% with *Enterobacter aerogenes*, 18.2% with *Erwinia carotovora*, 12.3% with *Citrobacter freundii*, 16.3% with *Klebsiella* oxytoca, and 11% with *Serratia marcescens*. When the recommended threshold value of 70% DNA-DNA similarity for species definition was considered (Wayne *et al.*, 1987), the result indicate that strains NiVa 51 and NiVas 114 does not belong to any of the taxa used in DNA-DNA hybridization.

5.3. Conclusion:

Therefore, on the basis of 16S rDNA sequence analysis, fatty acid profile, DNA base composition and DNA-DNA hybridization studies, it can be concluded that the isolates NiVa 51 and NiVas 114 are novel species of *Serratia* and *Enterobacter* respectively. The detailed phenotypic analysis of the isolate NiVa 51 and NiVas 114 were performed following protocols described in section 4.1.1.3, and were compared with there phylogenetically related taxa (Table 5.4 & 5.5).

On the basis of phenotypic tests, NiVa 51 scored similarity coefficient (S_{SM}) values in the range of 0.75- 0.87 with the members of *Serratia* with which it branched most deeply (data not shown). One of the unique characteristics of the isolate NiVa 51 is to utilize urea as a sole source of nitrogen for growth, whereas none of the strains under the genus *Serratia* was reported to have urease activity. In the active culture medium containing 0.5 mM urea as sole nitrogen source and initial cell density of 3 x 10^{8} cells/ ml, the rate of free ammonia 'N' released in the culture medium in the first two hours of incubation was 0.6 ppm/ h and reached to the maximum rate of 1.4 ppm/ h during 6th to 10th hour of incubation (Bhadra *et al.*, 2005b). The maximum S_{SM} value (0.87) observed between NiVa 51 and *S. marcescens* corresponds with the highest SIM index value (0.747) of the same pair (in terms of FAME profiles) obtained from the matches with entries in the Sherlock MIS TSBA 50 Identification library. Also the DNA-DNA hybridization values are typical of those found between species of *Serratia*. The 16S rDNA analysis showed that the strain NiVa 51 is closest to *S. marcescens*. Therefore, isolate NiVa 51 demands its classification as a novel species of *Serratia*, for which the name *Serratia ureilytica* was proposed.

On the other hand, NiVas 114 scored highest S_{SM} values of 0.87 -0.91 that of *Enterobacter cloacae* with which it generated a highest of 35% DNA-DNA similarity. The claim for a novel species of *Enterobacter* for the isolate can thus be justified with its unique fatty acid profile, low DNA-DNA similarities with related taxa, GC content and 16S rRNA phylogeny.The isolate showed high MTC (12 mM) towards nickel chloride, and novelty in nickel resistance genetic determinant of the isolate can be derived from the data obtained from PCR study, Southern hybridization and nucleotide sequencing (discussed in detail at Chapter 3 & 4). We therefore

Table 5.4. Biochemical and physiological characteristics of strain NiVa 51.

Serratia marcescens subsp. *marcescens*, *Serratia marcescens* subsp. *sakuensis* , *Serratia odorifera* and *Serratia rubidaea* were used as reference strains. All the species are oxidase negative; catalase positive; rod shaped; motile by means of peritrichous flagella; can grow in presence of 1.4 M NaCl; does not produce indole, amylase and H_2S; but showed positive tests towards- VP; esculine hydrolysis; utilization of acetate, succinate and citrate (Simmons); lysine and ornithine decarboxylation; nitrate reduction; utilization of L-arginine and L-histidine as sole carbon source; and acid production from glucose and sucrose. The characteristics that distinguished NiVa 51^T from *S. marcescens* subsp. *marcescens* are indicated by asterics (*).

Characters	NiVa51	*Serratia marcescens* subsp. *marcescens* LMG 2792	*Serratia marcescens* subsp. *sakuensis* JCM 11315[a]	*Serratia odorifera* MTCC495	*Serratia rubidaea*[b]
Spore	-	-	+ (Round)	-	-
Pigment*	-	+	+	-	-
Methyl red*	+	-	-	+	-
Caseinase *	+	-	+	-	-
Urease *	+	-	-	-	-
Lipase (Tween 80)	+	+	+	-	+
Arginine dihydrolase*	+	-	-	-	-
Acid production from carbohydrates:					
Adonitol*	+	-	Na	-	+
Lactose	-	-	-	+	+
Sorbitol	+	+	+	+	-
Arabinose	-	-	-	+	+
Raffinose	-	-	-	nd	+
Rhamnose	-	-	-	+	-
Xylose*	+	-	-	+	+
Mellibiose	+	-	Na	+	+
Utilization of organic acid salts:					
Oxalate*	+	-	+	nd	Na
Lactate	+	+	-	+	Na
Amino acid utilization:					
DL-Serine	+	+	+	nd	Na
L-Ornithine	+	+	-	nd	-
DL-Alanine	+	+	+	+	-
L-Proline	+	+	Na	nd	Na
DL-Threonine*	+	-	Na	-	Na
DL-Phenyl alanine	+	+	Na	nd	-
L-Hydroxy proline	+	+	-	+	Na
L-Tryptophan*	-	+	Na	+	Na

Na, data not available; nd, not done; a, data are from Ajithkumar, *et al.* (2003); b, Data from Grimont & Grimont, (1992).

propose the name, *Enterobacter indica,* for the isolate NiVas 114; which could be an important bacterial strain for studying the genomics of nickel resistance in bacteria.

Compared to *H. alvei* 5-5 and *K. oxytoca* CCUG 15788 [require 5 and 0.5 mM Ni^{2+}, respectively, for induction, (Park *et al.*, 2003 & Stoppel *et al.*, 1995)] much lower concentration of nickel ion (0.05-0.1 mM) is required to induce nickel resistance in novel isolates NiVa 51 and NiVas 114. Moreover, pre-incubation in 0.1 mM Zn^{2+} was also found to induce nickel resistance in both the isolates; which was not reported in case of *nir/ ncr* locus. Therefore, the new bacterial strains *Serratia ureilytica* strain NiVa 51^T and *Enterobacter indica* strain NiVas 114^T, could be important bacterial strains for enriching the genomics of metal resistance in *Enterobacteria.*

Serratia ureilytica; **(type strain, NiVa 51^T = LMG 22860 ^{T} = CCUG 50595^T=MTCC6935^T).**

Enterobacter indica; **(type strain, NiVas 114^T = CCUG 50594^T = MTCC7087^T).**

Table 5.5. Biochemical and physiological characteristics of strain NiVas 114.
Enterobacter cancerogenus and *Enterobacter aerogenes* was used as reference strains. All strains were positive for- Simmons Citrate; nitrate reductase; catalase, esculin hydrolysis and ornithine decarboxylase test; produced acid from dextrose, arabinose, rhamnose, maltose, xylose, trehalose, cellibiose and mellibiose; acetate, succinate, gluconate, L-arginine, DL-alanine and DL-serine was utilized as carbon source. All taxa showed negative tests for iodole and H_2S Production; enzymes like caseinase, amylase, gelatinase , oxidase and lipase are not produced; oxalate, DL-aminobutyrate, glycine, DL-valine, DL-phenyl alanine are not utilized as sole carbon source. +, posotive; -, negative; na, data not available; [d], 25-75% are positive; [+], 76- 89% positive; [-], 11-25% positive.

Biochemical Characters	NiVas114	*Enterobacter cloacae**	*Enterobacter cancerogenus* LMG 2693	*Enterobacter aerogenes* MTCC 111
Methyl red	+	-	+	-
Voges-Proskauer	+	+	-	+
Lysine decarboxylase	-	-	+	+
Urease	+	[d]	-	-
Acid production from carbohydrates:				
Sucrose	+	+	-	+
Mannitol	+	+	-	+
Dulcitol	-	[-]	+	-
Salicin	+	[+]	-	+
Adonitol	-	[-]	-	+
Inositol	+	+	-	+
Sorbitol	+	+	-	+
Raffinose	+	+	-	+
Utilization of organic acids:				
Tartarate	-	-	+	-
Lactate	-	na	+	+
Malonate	-	na	+	-
Amino acid utilization:				
L-Ornithine	+	na	-	-
L-Histidine	+	+	-	+
L-Proline	+	na	-	+
L-Tryptophan	+	na	+	-
L-Leucine	+	na	+	-
DL-Threonine	-	+	+	+
L-Hydroxy proline	+	na	+	-
DL-Methionine	-	+	+	+

*, The characteristics of *Enterobacter cloacae* were taken from Grimont & Grimont (1992) and Holt *et al.* (1994) for comparison.

Description of *Serratia ureilytica* sp.nov.

Serratia ureilytica [N.L.n.urea –ae, urea; N.L.adj. lyticus –a –um (from Gr.adj. lutikos), dissolving; N.L. fem. adj. *ureilytica*, urea dissolving].

Gram-negative, motile, with peritrichous flagella, non-fluorescent, non-pigmented, catalase positive and oxidase negative. Cells are straight rods, 0.7-1.0 µm long and 0.8-1 µm wide and can grow between 8-43 °C over a pH range of 5-11 in nutrient broth media and can tolerate NaCl concentration up to 1.4 M. Freshly grown colonies are white, round, with convex surfaces and smooth edges. Gives negative tests in indole and H_2S production and positive tests towards, Voges-Proskauer, lysine and ornithine decarboxylation, methyl red, citrate (Simmons') utilization, arginine dehydrolase, and esculine hydrolysis. Enzymes such as urease, casinase, lipase and nitrate reductase are present but amylase is absent. Produces acids from glucose, sucrose, mannitol, salicin, adonitol, inositol, sorbitol, maltose, xylose, trehalose, mellibiose and glycerol. Organic acid salts such as tartarate, oxalate, acetate, lactate, succinate and gluconate is used as a carbon source for growth in inorganic media. Amino acid such as, L-arginine, DL-alanine, DL-serine, L-ornithine, L-histidine, L-proline, DL-threonine, DL-phenyl alanine, L-hydroxy proline and DL-aminobutyrate are utilized and L-tryptophan, L-leucine, glycine, DL-valine and DL-methionine are not utilized. The isolate has a plasmid of 50 kb of unknown function and can grow in nutrient agar media containing 40 µg/ml ampicillin, 15 µg /ml tetracycline and 20 µg/ml chloramphenicol.

Description of *Enterobacter indica* sp.nov.

Enterobacter indica (In di'.ca. L. fem. adj. *indica* pertaining the geographical source of the organism, of country India).

Gram-negative, motile, with peritrichous flagella, non-fluorescent, catalase positive and oxidase negative. The strain showed negative Voges-Proskauer and positive methyl red tests; can grow between 8-43 ^{0}C over a pH range of 5-11 in nutrient broth media and can tolerate NaCl concentration up to 7%. Freshly grown colonies are white, round, with convex surfaces and smooth edges. Gives a positive ornithine decarboxylase and negative lysine decarboxylase test. Enzymes such as urease and nitrate reductase is present but amylase, lipase and gelatinase is absent.

Produce acid from glucose, sucrose, mannitol, salicin, adonitol, inositol, sorbitol, arabinose, raffinose, rhamnose, maltose, xylose, trehalose, cellibiose and melibiose. Organic acid salts such as malonate, tartarate, oxalate and lactate are not used and acetate, succinate and gluconate are used as a carbon source for growth in inorganic media. Amino acid such as, L-arginine, DL-alanine, DL-serine, L-ornithine, L-histidine, L-proline, L-tryptophan, L-leucine and L-hydroxy proline are utilized and glycine, DL-valine, DL-threonine, DL-phenylalanine, DL-methionine and DL-aminobutyrate are not utilized. The isolate has a plasmid of 70 kb of unknown function and can grow in nutrient agar media containing 40 µg/ml ampicillin and 15 µg /ml tetracycline.

5.4. Summary of the chapter 5:

The taxonomic positions of three nickel-resistant bacteria, isolated from Torsa River of India, were studied using polyphasic approach. Phylogenetic analysis with 16S rRNA gene sequences indicated that strains BB1A, NiVa 51 and NiVas 114 are *Acinetobacter junii*, a novel species of *Serratia* and a novel species of *Enterobacter* respectively. Cellular fatty acid composition of the isolates NiVa 51 and NiVas 114 showed similarities with *Serratia marcescens* and *Enterobacter cloacae*. DNA-DNA hybridization analyses, on the other hand, showed relatively low level of homologies (34- 43%) with their closest phylogenetically related species. The G+C %mol content of the isolate NiVa 51 and NiVas 114 are 60 and 59 respectively. Therefore, on the basis of DNA-DNA relatedness, G+C content, cellular fatty acid profile and 16 rDNA similarities, the name *Serratia ureilytica* sp. nov. (type strain, NiVa 51^{T} = LMG 22860 ^{T} = CCUG 50595^{T}=MTCC6935^{T}) and *Enterobacter indica* sp. nov. (type strain, NiVas 114^{T} = CCUG 50594^{T} = MTCC7087^{T}) was proposed for isolate NiVa 51 and NiVas 114 respectively.