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***Research Paper***

# IN SILICO 16s rRNA PHYLOGENETIC ANALYSIS OF EXTREMELY TOLERANT AND RESISTANT PLANT GROWTH PROMOTING RHIZOBACTERIA

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**Abstract**

Plant Growth Promoting Rhizobacteria (PGPR) are present in the rhizosphere which can enhance plant growth through a wide variety of mechanisms like phosphate solubilization, siderophore production, biological nitrogen fixation, production of 1-Aminocyclopropane-1-carboxylate deaminase (ACC), quorum sensing (QS) signal, interference and inhibition of biofilm formation, phytohormone production, exhibiting antifungal activity, production of volatile organic compounds (VOCs), induction of systemic resistance, promoting beneficial plant-microbe symbioses, interference with pathogen toxin production etc. The rhizospheric microorganisms have a major influence on plant and guard the health in an eco-friendly manner which helps in overcoming the extensive use of chemical fertilizer and thereby increases soil fertility which in turn helps in heavy tolerance. The study was done to understand the evolutionary relationships among thirty-eight different PGPR species on the basis of their 16s rRNA conserved sequences. Data was collected from NCBI Gen-Bank, multiple sequence alignment was done using ClustalX, nucleotide sequence and conserved sites were seen using BioEdit, and phylogenetic tree was constructed using MEGA 4. The phylogenetic tree revealed homology relations amongst the various PGPR which formed different clades on the basis of their similarity or dissimilarity with each other. Most the PGPR strains formed clades with their own genus. However, two PGPR species formed cluster with different genus of PGPR species rather than its own genus. Inference can be drawn from the phylogenetic characterisation of the various PGPR that a deeper understanding can be developed to figure out the mechanisms underlying the symbiotic association between different PGPR strains and varied plant species.

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Key words: PGPR, Genbank Data collection, Phylogentic Analysis, Conserved Region, Nucleotide composition.

## INTRODUCTION

Growth in plants is influenced by many biotic and abiotic factors. The thin layer of soil surrounding the plant roots is known as rhizosphere which is an active area of root activity and metabolism of plants. The term rhizosphere was first introduced by Hiltner to describe the narrow zone of soil surrounding the roots (Hiltner, 1904). A large number of microorganisms such as bacteria, fungi, protozoa and algae that colonize the rhizosphere can be classified according to their effects on plants and the way they interact with roots, some being pathogens whereas other trigger beneficial effects. Among them, bacteria are the most abundant. Certain specific bacteria are selected by the plants which contribute most to their fitness by releasing helpful organic compound through exudates (Lynch, 1990.). Rhizobacteria inhabit plant roots and exert a positive effect ranging from direct influence mechanisms to an indirect effect. So, the bacteria inhabiting the rhizosphere and beneficial to plants are known as PGPR (Kloepper & Leong, 1980). PGPR in the rhizosphere can enhance plant growth by a wide variety of mechanisms like phosphate solubilization, siderophore production, biological nitrogen fixation, production of 1-Aminocyclopropane-1-carboxylate deaminase (ACC), quorum sensing (QS) signal, interference and inhibition of biofilm formation, phytohormone production, exhibiting antifungal activity, production of volatile organic compounds (VOCs), induction of systemic resistance, promoting beneficial plant-microbe symbioses, interference with pathogen toxin production etc. ( Bhattacharyya *et al*., 2012) .Chemical fertilizer, Heavy metal contamination and Soil salinity are one of the major world environment problems and has a negative impact on human health, agriculture decreases soil fertility and yield loss. To overcome their negative impact on environment as well as human health, PGPR the rhizospheric microorganisms around plant roots have a major influence on plant and guard the health in an eco-friendly manner. In recent years PGPR replaces agrochemical (fertilizer and pesticides) by a variety of mechanism. Certain metal resistance PGPR can seek metal mobility and availability to the plant through releasing chelating agent, acidification, phosphate solubilization and redox changes as well as . PGPR in saline conditions has been used to decrease the harmful effects of salt stress by reducing the ethylene production in the plant through ACC deaminase activities. It also increases plant growth and development

by producing various plant hormones (Sheng *et al*., 2008; Rajkumar, 2007, Nazima *et al*., 2014). PGPR belonging to genera *Acinetobacter, Bacillus, Stenotrophomonas, Brevibacterium, Enterobacter, Pseudomonas, Burkholderia, Gulconacetobacter, Klebsiella* of different strain has been known to show mechanisms like P.solubilization, Siderophore production, biological nitrogen fixation, 1-aminocyclopropane-1-carboxylate deaminase(ACC), phytohormone IAA etc production, quorum sensing (QS) signal interference, anti-fungal activity. The experimental work on the basis of 16s rRna , The 16s rRNA sequence has hypervariable regions which signify that the sequence has diverged over years of evolution. This result in flanking of the conserved region and silencing the variations among them (Soumitesh *et al*., 2007). This attributes leads to an easy analysis, making it lot easier and cheaper to sequence and serves as a handy tool for evolutionary relatedness studies. 16s rRNA in phylogenetics proposed that rRNA is the most conserved sequence in the genome. Phylogenetics is the attempt to reconstruct the evolutionary relationships between species. Historically, this was done using quantitative morphological data, but modern methods rely more heavily on DNA sequence data. In the case of bacterial identification, 16s rRNA sequencing has emerged as the most convenient method which is a stepping stone for phylogenetics using Bioinformatics (Freckleton *et al*., 2002). In this study phylogenetic analysis was performed to understand the relationship among different species of PGPR which help in growth of plant across different environmental conditions such as agricultural fields, heavy metal contaminated soil and coastal area.

## MATERIALS AND METHODS

### *Data Collection*

29 nucleotide sequences of different 16s rRNA plant growth promoting rhizobacteria belonging to agricultural lands, coastal area and heavy metal contaminated soil plant were selected based on literature review. The sequences were downloaded from NCBI-GenBank in Fasta format (*.txt) along with their accession number and the scientific name of the bacteria and saved in notepad.

### *Tools Used*

Nucleotide Sequence Analysis: The range of the length of the sequence downloaded from NCBI was kept between 1300 to 1600 bp. Nucleotide content (%GC and %AT) of all the sequences was found using BioEdit (version 7.0.9.0). BioEdit software was also used to find the different conserved region of multiple sequence alignment file obtains from.

***Sequence Alignment***

The Multiple Sequence Alignment was done using ClustalX version 2.0 which is stand-alone software.

***Phylogenetic analysis:***

The Phylogenetic tree was constructed using MEGA (version 6.1). Neighbour-Joining (NJ) method (Saitou and Nei 1987) was used for the construction of the tree from the aligned file provided by ClustalX (<file_name>.aln). Bootstrapping of the phylogenetics tree was done using Kimura2 model (Bootstrap value = 1000) (Kimura *et al.,* 1980).

## RESULT AND DISCUSSION

All the sequences of PGPR that were downloaded from NCBI Genbank were analyzed using Bioedit software the complete nucleotide composition of all the bacteria along with their total length (A+T)% and (G+C) % content. The results are shown in table 1.

**Table 1:** Different bacteria along with their nucleotide composition, % GC, %AT and length

| BACTERIA | PGP ACTIVITY | G.C % content | A.T % content | Length | A | T | G | C |
|---|---|---|---|---|---|---|---|---|
| *Pseudomonas stutzeri* str PPB1 | Nitrogen fixation suppressed phytophthora crown rot disease | 54.02 | 45.98 | 1392 | 355 | 285 | 437 | 315 |
| *Brevibacterium epidermis* str RS15 (Siddikee *et al*., 2010) *Brevibacterium Idonium* str RS16 (Siddikee *et al*., 2010) | $PO_4$ solubilization, Zn solubilization, Thiosulfate ($S_2O_3$), $NH_3$ production, ACC deaminase | 56.27 | 43.73 | 1372 | 320 | 280 | 453 | 319 |
| | | 56.15 | 43.85 | 1366 | 319 | 280 | 451 | 316 |
| *Bacilus Stratophericus* str RS340 (Siddikee *et al*., 2010) *Bacillus Subtilis* str | $PO_4$ solubilization, IAA Siderophore production Nitrogen fixation, ACC | 54.92 | 45.08 | 1393 | 348 | 280 | 434 | 331 |
| | | 54.96 | 45.04 | 1412 | 350 | 286 | 439 | 337 |
| | | 55.02 | 44.98 | 1405 | 354 | 278 | 440 | 333 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| PPB5(Islam *et al*., 2016) *Bacillus amyloliquefaciens* str PPB4 (Islam *et al*., 2016) *Bacillus cereus* str ANA4(Trivedi *et al*., 2010) | deminase activity. Nitrogen fixation suppressed phytophthora crown rot disease. $PO_4$ solubilization, Zn solubilization, Thiosulfate ($S_2O_3$), $NH_3$ production, ACC deaminase. | 53.62 | 46.38 | 1477 | 370 | 315 | 451 | 341 |
| *Burkholderia phytofirmans* str PsJN (Sessitsch *et al.,* 2005) *Burkholderia carbensis* str SDSA-I10/1(Roy *et al.,* 2013) | 1-aminocyclopropane-1-carboxylate deaminase activity, production of Quorum-Sensing signal compound, 3-hydroxyl –C8-homoserine lactone. Nitrogen fixation | 55.51 | 44.49 | 1488 | 375 | 287 | 480 | 346 |
| | | 56.29 | 43.64 | 1391 | 344 | 263 | 450 | 333 |
| *Stenotrophomonas maltophia* str PPB3(Islam *et al.,* 2016) | Nitrogen fixation, suppressed phytophthora crown rot disease | 55.3 | 44.7 | 1425 | 356 | 281 | 452 | 336 |
| *Stenotrophomonas chelatiphaga* str ATY55(Trivedi *et al*., 2010) | P.solubilization, IAA Siderophore production Nitrogen fixation, ACC deminase activity | 54.97 | 45.03 | 1450 | 360 | 293 | 458 | 339 |
| *Panibacillus polymyxa* str NSY50 (Shi, Lu *et al.,* 2017) | Antagonistic activity against *Fusarium oxysporum* | 54.8 | 45.2 | 1416 | 361 | 279 | 443 | 333 |
| *Arthobacter nicotianae* str RSA68(Siddikee *et al*., 2010) | $PO_4$ solubilization, Zn solubilization, Thiosulfate ($S_2O_3$), $NH_3$ production, ACC deaminase | 57.04 | 42.96 | 1371 | 310 | 279 | 455 | 327 |
| *Micrococcus yumnanesis* str RS222(Siddikee *et al*., 2010) | | 56.92 | 43 | 1365 | 310 | 277 | 451 | 326 |
| *Exignobacterium acetylicum* str Rs343(Siddikee *et al.*, 2010) | | 55.5 | 44.5 | 1409 | 357 | 270 | 447 | 335 |
| *klebsiella oxytoca* str STY38(Siddikee *et al*., 2010) | | 54.5 | 45.5 | 1455 | 373 | 289 | 456 | 337 |
| *Halomonas korlensis* str RS229 (Siddikee *et al*., 2010) | | 57.87 | 42.13 | 1372 | 332 | 246 | 452 | 342 |
| *Halomonas neptunia* str ES11E(Siddikee *et al*., 2010) | | 55.43 | 44.43 | 1382 | 345 | 269 | 441 | 325 |
| *Microbacterium arborescens* str ANA42(Trivedi et al., 2010) | $PO_4$ solubilization, IAA Siderophore production Nitrogen fixation, ACC deminase activity | 56.59 | 43.41 | 1442 | 347 | 279 | 469 | 347 |
| *Panteoa agglomerans* str STY 28 | $PO_4$ Solubilization, nitrogen fixation | 55.69 | 44.31 | 1440 | 358 | 280 | 462 | 340 |
| *Acinetobacter rhizosphaerae* str BHIB723 (Gulati *et al*., 2008) | $PO_4$ solubilizing, ACCdeaminase, Siderophore production | 52.6 | 47.4 | 1500 | 387 | 324 | 461 | 328 |
| *Enterobacter cloacae* str | $PO_4$ solubilization, | 55.15 | 44.85 | 1476 | 368 | 294 | 472 | 342 |

| MSR1(Khalifa *et al*., 2016) | phytohormone production, antibacterial activity against plant pathogen | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Planococcus rifietoensis* str SAL15(Rajput *et al.,* 2016) | IAA production, $PO_4$ solubilization, ACC deaminase actvity | 56.25 | 43.75 | 1513 | 371 | 291 | 483 | 368 |
| *Rahnella aquatilis* str 36(Palmieri, *et al.,* 2016) | Antagonistic activity against *Fusarium spp.* | 54.29 | 45.71 | 1341 | 341 | 272 | 424 | 341 |
| *Gluconacetobacter diazotrophicus* str PAL 15 | Nitrogen fixation | 56.8 | 43.2 | 1486 | 346 | 296 | 498 | 346 |
| *Gluconacetobacter azotocaptans* str CFN-Ca54(Fuentes-Ramirez *et al.,* 2001) | | 56.6 | 43.4 | 1447 | 339 | 289 | 487 | 332 |
| *klebsiella pneumoniae* str K11 (Sachdev *et al.,* 2009) | IAA production | 54.72 | 45.0 | 1451 | 365 | 288 | 461 | 333 |
| *Aeromonas veronii* str RR8 | IAA production | 55.37 | 44.63 | 1535 | 378 | 307 | 497 | 353 |
| *Methylobacterium fujisawaense* str SNA1(Trivedi *et al*., 2010) | $PO_4$ solubilization, IAA Siderophore production Nitrogen fixation, ACC deminase activity | 56.48 | 43.52 | 1443 | 346 | 282 | 469 | 346 |

| **% Average GC Content of all sequences** | | **% Average AT Content of all sequences** | **Average Length of all sequences (b.p.)** | **Range of the sequences (b.p.)** |
|---|---|---|---|---|
| 55.50 | | 44.47 | 1428 | 1341- 1535 |

Analysis of 16s rRNA sequences of different PGPR reveals that the sequences were within the range of 1341 – 1535 bp. The average GC% was around 55.50% and AT% was around 44.47%. The average length was around 1428 b.p (table 1). Multiple sequence alignment was done using ClustalX2. Different regions of conserved sequences of all the PGPR was found using Bioedit software. The results are shown in table 2.

**Table 2:** Conserved regions of the different PGPR species under observation

| Conserved region | Position (b.p.) | Consensus | Segment Length |
|---|---|---|---|
| Region 1 | 343 to 363 | 343 ACTCCTACGGGAGGCAGCAGT 363 | 21 |
| Region 2 | 520 to 543 | 520 CGTGCCAGCAGCCGCGGTAATACG 543 | 24 |
| Region 3 | 788 to 814 | 788 AACAGGATTAGATACCCTGGTAGTCCA 814 | 27 |
| Region 4 | 890 to 906 | 890 CCGCCTGGGGAGTACGG 906 | 17 |
| Region 5 | 1073 to 1088 | 1073 TGTCGTCAGCTCGTGT 1088 | 16 |
| Region 6 | 1095 to 1112 | 1095 ATGTTGGGTTAAGTCCCG 1112 | 18 |
| Region 7 | 1385 to 1403 | 1385 CGGTGAATACGTTCCCGGG 1403 | 19 |

The Phylogenetic tree was constructed using MEGA 6.1 software to study the phylogenetic relationship among the Plant Growth Promoting Rhizobacteria that have been analyzed on basis of 16s rRNA sequence from all 29 species to access the degree and pattern of interspecific difference. The evolutionary history of PGPR was inferred using neighbor- joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test was kept to 1000 replicates and are shown next to branches. The tree was drawn to scale, with branch length in the same units as those of evolutionary distance used to infer in the phylogenetic tree. The evolutionary distance was compute using the Kimura 2-parameter. condon position include were $1^{st}$ + $2^{nd}$ + $3^{rd}$+ non-coding.

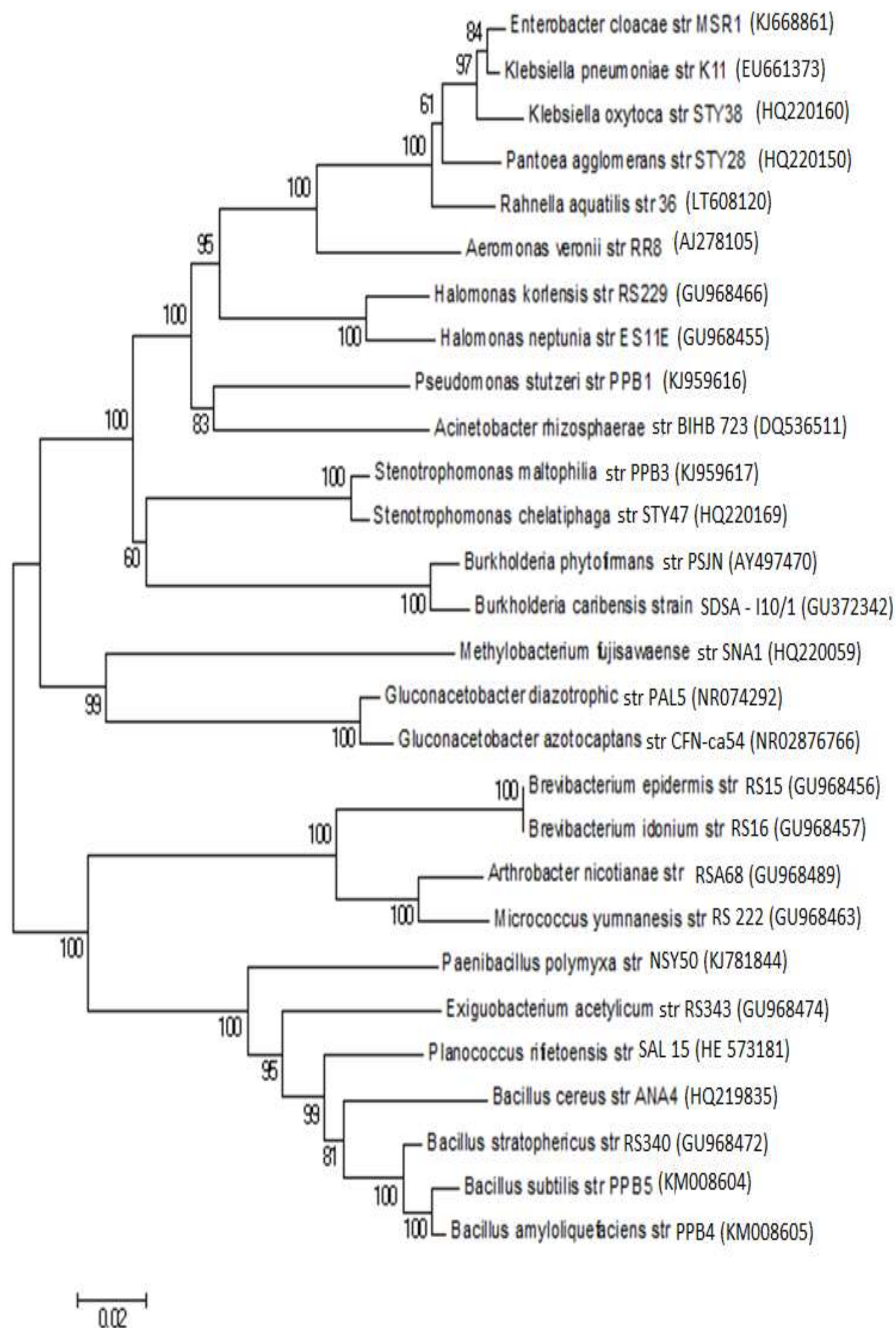


**Figure1:** 16srRNA phylogenetic tree of plant growth promoting bacteria under study

**Tree interpretation:**

According to the 16s rRNA sequences based phylogenetics, it can be interpreted that the tree bifurcated into branches; again the two branches bifurcated into four branches i.*e.*, *Klebsiella sp, Halomonas sp, Pseudomonas sp, Acinetobacter rhizosphearae, Enterobacter*

*cloacae, Rahnella aquatilis, Pantoea agglomerans, Aeromonas veronii, Burkholdreia sp, Stenophomonas sp and Pseudomonas hibiscicola* form first cluster*.* The second cluster was from among *Methylobacterium fujisawaense and Gluconacetobacter sp.* The third cluster was formed among *Micrococcus yumnanesis, Arthrobacter nicotianae, Brevibacterium sp, and panibacillus polyxma, Brevibacillus brevis, Exiguobacterium accetylium, Plancoccus rifietoensis and Bacillus sp.* from the fourth and final cluster.

Four species of *Bacillus* belong to a single clade with a minor bifurcation in the cluster due to minute difference in phylogenetic distance. In first cluster *Klebsiella sp* and *Enterobacter cloacae* feature in the same cluster. Rather, *Enterobacter cloacae* found to sharing high % of homology with *Klebsiella pneumonaie*. Another cluster was found of *Gluconacetobacter sp* having two species because of their high similar due to their same genus they were found the same genus. The same case was observed to belong to the same environment and similar activity and shows high % of homology which was evident from the NJ-tree.

Within the first cluster of the sub branch, it was observed *Enterobacter cloacae* bifurcated the clade of *Klebsiella* sp. (*K. pneumoniae, K. oxytoca*) and got paired with *K.pneumoniae*, Pattern and Ryu et al (2013) stated that indol- 3- pyruvate pathway which regulate the IAA production in plant utilizes indol-3-pyruvate decarboxylate which is encoded by ipdC gene in PGPR *Enterobacter cloacae* UW5. It was found that the sequence of TyrR which regulates the aromatic amino acid transport and metabolism production of tryptophan in *E. coli* was highly similar with promoter region sequence of ipdC in *Enterobacter cloacae* UW5 they also found about 78 % similarity of TyrR gene of *Enterobacter cloacae* with *Klebsiella pneumoniae*. So it can be inferred that TyrR gene is a regulatory protein which help in the production of IAA in plants with the help of PGPR strains such as *Enterobacter cloacae* and *Klebsiella pneumonia*.

Another displacement was observed in which *Pseudomonas hibiscicola* got paired with *Stenotrophomonas maltophilia* rather than pairing with its own *Pseudomonas* clade and showed significant homology with each other. According to the study of Lisellot *et al.*, (2011) gyrB gene and 16s RNA gene of *Stenotrophomonas maltophilia* coincided at same positions which implies that gyrB gene is a functional part of the bacteria. Since, *Pseudomonas hibiscicola* strain shares high percentage of similiraity with

*Stenotrophomonas maltophilia* strain, it can be inferred that they both share the same properties of gyrB gene.

**CONCLUSION**

The current study was a minor effort towards understanding the impact of PGPR activity on plant growth with the help of different bacterial genus collected from diverse fields of agriculture, and diverse environmental sources. 38 nucleotide 16s rRNA partial sequences were studied on the basis of their varying plant growth properties such as phosphate solubilization, IAA Production; ACC deaminases which were some of the most common properties .A phylogentic tree was constructed among the 38 nucleotide sequences of PGPR to study the homologous relationship among them.

From the present study we can conclude that in all the discussed clades, the mismatch seen in every case was due to certain homology and similarity among them.

In *Bacillus* clade, homology is due to plant growth promoting characteristics in agriculture and heavy metal resistance in soil. In *Pseudomonas* clade, similarity is shown due to the fact that they present beneficial properties towards plant-growth. However, *P. hibiscicola* paired with *Stenotrophomonas maltophilia* because of higher homology between them then their respective clade. In case of *Klebsiella sp. Enterobacter cloacae* bifurcated *K. pneumonia* and *K. oxytoca* and shows homology with *Kebsiella pneumonia.*

Inference can be drawn from the phylogenetic characterisation of the various PGPR that a deeper understanding can be developed to figure out the mechanisms underlying the symbiotic association and non symbiotic between different PGPR strains and varied plant species.

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