Identification of *nif*D and *nif*H Genes of Methanotrophic Bacteria from Rice Field

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Abstract

Metanotrophic bacteria have ability to oxidize methane and fix atmospheric nitrogen, hence the bacteria has an important role as a nitrogen source provider on wetland area like rice fields. Nitrogen fixation process is catalyzed by the nitrogenase enzyme complex, encoded by *nif*D and *nif*H genes. However, characteristic of these genes from indigenous-methanotrophic bacteria still poorly understood. Hence, *nif*D and *nif*H genes of methanotrophic bacteria isolated from rice fields in Indonesia (BGM3, BGM9, SS1, SS3, SS10, ST18, SP3 and INP4) were identified and characterized. Detection of *nif*H and *nif*D genes was conducted by polymerase chain reaction (PCR) amplification. *nif*H and *nif*D gene sequences were analyzed using BLAST-X and phylogenetic trees were constructed using Neighbour Joining method. Based on *nif*H sequences analysis, SS1 closely related to *Beijerinckia mobilis* and SS3, SS10, ST 18 closely related to *Beijerinckia indica* subsp. *indica* ATCC 9039, while, BGM3, INP4, and BGM9 related to *nif*H of uncultured nitrogen-fixing bacterium. In other hand, sequence analysis of *nif*D gene showed that SS1, SS3, SS10, ST 18 closely related to *B. indica* subsp. *indica* ATCC 9039 and BGM3, BGM9, INP4 closely related to *Xanthobacter autotrophicus* Py2. Identification by 16 S rRNA indicated that SS1, SS3, SS10, and ST18 had closeness to *Beijerinckia* sp. P310-1, while INP4 closely related to *Xanthobacter* sp. M5C24.

Keywords: methanotrophic bacteria, nitrogen fixation, nitrogenase, *nif*H, *nif*D

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Introduction

Methane oxidation by methanotrophic bacteria in rice fields can reach 80% of total methane (CH_4) that produced by methanogenic bacteria (Conrad & Rothfus, 1991). Therefore, methanotrophic bacteria have a potential role to reduce methane gases in the atmosphere, especially in the rice field. The methanotrophic bacteria were grouped into two types based on their physiological and biochemical features, they are family Methylococcaceae (type I) including the genera *Methylomonas*, *Methylobacter*, *Methylosphaera*, *Methylosarcina*, and *Methylomicrobium*; family Methylocystaceae (type II) including the genera *Methylosinus* and *Methylocystis*; and a new group that classified into type X contain the genera *Methylococcus* and *Methylocaldum* (Hanson & Hanson, 1996). Auman *et al*. (2001) and Fedorov *et al*. (2008) reported that methanotrophic bacteria type II and X of genus *Methylococcus*, have ability to fix atmospheric nitrogen into ammonium. Recent study showed that a species of thermoacidophilic-methanotrophic bacteria, genus *Methylacidiphilum*, phylum *Verrucomicrobia* namely *M. fumariolicum* SolV also capable to fix nitrogen (Khadem *et al*., 2010). Sugitha & Kumar (2009) said that free living nitrogen fixers are a potentially important role of nitrogen fixation in the rice field. Nitrogenase is an important enzyme that catalyze nitrogen fixation proceess, and this complex enzyme consisted of Fe protein (dinitrogenase reductase) and Fe-Mo protein (dinitrogenase) encoded by *nif*H and *nif*DK respectively (Sugitha & Kumar, 2009). *nif*H and *nif*DK genes are the main component of nitrogense-complex enzyme (Choo *et al*., 2003; Dedysh *et al*., 2004) and both of the genes are very

conserved, hence those genes are an ideal molecular tool for advance studies such as nitrogen fixation ability, diversity, and phylogenetic relationship of nitrogen-fixing bacteria (diazotroph) (Boulygina *et al*., 2002).

Nitrogen-fixing bacteria were separated into four group, Group I consist of the 'conventional' nitrogenase or molibdenum-dependent nitrogenase (*nif*H) and some vanadium nitrogenase (*vnf*H) (Zehr *et al*., 2003). Group II or molibdenum-independent nitrogenase including the second alternative of molibdenum or vanadium, that using iron (*anf*H) and also nitrogenase of some Archaea (Raymond *et al*., 2004). Group III contain the *nif*H sequences of obligate anaerobic diazotrophs, Gram positive, low GC content such as *Clostridium* and sulfate-reducing bacteria. Meanwhile, Group IV including the *nif*H-like sequences from some Archaea like methanogenic bacteria and bacteria with chlorophyllide reductase encoding genes (Zehr *et al*., 2003; Raymond *et al*., 2004).

Several studies have been conducted to determine the nitrogen fixation ability of methanotrophic bacteria. Fedorov *et al*. (2008) has been detected the *nif* gene of *Methylobacter*. Characterization of *nif*H and *nif*D genes of acidophylic-methanotrophs, genus *Methylocella* and *Methylocapsa* and their phylogenetic relation with *Beijerinckia* were carried out by Dedysh *et al*. (2004). Auman *et al*. (2001) has been characterized the *nif*H and *nif*D genes in order to examine the ability of *Methylosinus trichosporium* OB3b, *Methylobacter marinus* A45, *Methylomonas methanica* S1, *Methylomonas rubra*, and *Methylomicrobium albus* BG8 to fix nitrogen.

Previous studies were conducted by Hapsary (2008) and Reginaldi (2010) have been successfully isolated the methanotrophic bacteria from rice fields in Indonesia. The selected strains were able to accumulate ammonium in Nitrate-free Mineral Salt (NFMS) medium (Reginaldi, 2010; Bintarti, 2011), but the *nif* genes of those strains have not identified yet. Thus, this research aimed to identify and characterize *nif*H and *nif*D genes of the strains. Information obtained from this study might support the application of methanotrophic bateria, not only as methane oxidizer but also as nitrogen fixer in rice field.

Materials and Methods

Bacterial strains. Several strains from previous studies were used in this research (Table 1). Growth medium used was Nitrate-free Mineral Salts (NFMS) (Auman *et al*., 2001).

Table 1. Strains used in this study and their source

Code of isolates	Sources	Identified as by 16 S rRNA gene	Reference
BGM 3	Rice field in Bogor	*Methylocystis parvus*	Hapsary (2008); Astuti (2009)
BGM 9	Rice field in Bogor	*Methylococcus capsulatus*	Hapsary (2008); Astuti (2009)
INP 4	Rice field in Indramayu	Nd	Reginaldi (2010)
SS 1	Rice field in Subang	Nd	Reginaldi (2010)
SS 3	Rice field in Subang	Nd	Reginaldi (2010)
SS 10	Rice field in Subang	Nd	Reginaldi (2010)
ST 18	Rice field in Subang	Nd	Reginaldi (2010)
SP 3	Rice field in Subang	nd	Reginaldi (2010)

Nd: not determined

Extraction of genomic DNA. Extraction of genomic DNA was conducted using the modified method of Lazo *et al*. (1987). Genomic DNA was subsequently visualized using 1% agarose gel electrophoresis.

Amplification of *nif* H and *nif*D. *nif*H and *nif*D genes were amplified by PCR using specific primers. Specific primers for *nif*H gene were nH17K-F: (TAYGGNAASGGCGGTAT CGGYAA) and nH139P-R: (TGGCATSGCRA ARCCRCCGCAMACMACGTC) (Elbetagy & Ando, 2008). While, the specific primers for *nif*D gene were nifHD-F(CAGGAAATCTAC ATCGTCATGTC) and nifD-R(TCCCANGAR TGCATCTGRCGGA) (Dedysh *et al*., 2004). The PCR conditions for *nif*H gene amplification were as follows: initial denaturation at 94°C for 30 s; 35 cycles of denaturation at 94°C for 1 min, annealing at

55°C for 1 min, elongation at 72°C for 1 min; and a final elongation step at 72°C for 10 min (Elbetagy & Ando, 2008). The PCR conditions for *nif*D gene amplification were as follows: initial denaturation at 94°C for 30 s; 35 cycles of denaturation at 92°C for 30 s, annealing at 63°C for 30 s, elongation at 72°C for 90 s; and final elongation at 72°C for 5 min (Dedysh *et al*., 2004). Electrophoresis was performed using 1% agarose, 70V, for 45 min. Amplicons were visualized using Ethidium Bromide (EtBr) and observed using UV transluminator.

Amplification of 16S rRNA gene. Five unidentified strains of INP4, SS1, SS3, SS10, and ST18 were identified by PCR using specific primers to amplify 16S rRNA genes. The primers used in this study were 63f (5'-CAGGCCTAACACATGCAAGTC-3') and 1387r (5'-GGGCGGWGTGTACAAGGC-3') (Marchesi *et al*., 1998).

DNA sequencing and bioinformatics analysis. DNA sequencing was performed by sequencing services company, 1st Base, Singapore. Sequences were aligned with data obtained from GenBank using BLAST-N and BLAST-X from the NCBI website (National Center for Biotechnology Information) through http://www.ncbi.nlm.nih.gov to determine the similarity of nif gene. Deduction of DNA sequences into proteins was performed using Expasy translate tools. Three-dimensional (3-D) structures of NifH and NifD was predicted using Cn3D 4.1 program. Construction of phylogenetic trees were calculated using Neighbor Joining (NJ) (Tamura *et al*., 2007) with bootstrap 1,000×.

Results and Discussion

Amplification and sequencing of *nif*H and *nif*D

*nif*H genes of eight isolates were successfully amplified using nH17K-F/nH139P-R primer. Gel electroforesis of the amplicons showed the DNA bands size approximately 393 bp (Figure 1A). The nH17K-F/nH139P-R primer was designed to amplify endophytic nitrogen-fixing bacteria of rice roots and resulted approximately 393 bp of amplicons (Elbetagy & Ando, 2008). *nif*D gene from seven isolates were successfully amplified using primers nifHD-F/nifD-R and showed bands size around 1900 bp (Figure 1B). Dedysh *et al*. (2004) designed this primer to amplify *nif*D gene fragments and yielded approximately 1900 bp that started at position 436 of *Bradyrhizobium japonicum* USDA 110 *nif*H gene. The amplicons included the 3 'of the *nif*H and 1380 of the *nif*D genes, confirmed that the primer only amplify the *nif*H and *nif*D genes that cluster contiguously within the same operon. The results confirmed that the *nif*H and *nif*D genes of BGM3, BGM9, SS1, SS3, SS10, ST18, and INP4 contiguosly located within the same operon. Zehr *et al*. (2003) also reported that *nif* genes in most diazotrophic Alphaproteobacteria and Gammaproteobacteria are located in the same operon (nifHDK).

In other hand, the nifHD-F/nifD-R primer can not amplify the *nif*D gene of SP3. That means, the *nif*H and *nif*D gene of SP3 does not locate in a single operon. In some Alphaproteobacteria, the genes encoding nitrogenase separated in two operons, this means the genes encoding NifDK and NifH transcribed from different promoters (Dedysh *et al*., 2004). NifH protein analysis showed that NifH of SS1 had closeness to NifH of *Beijerinckia mobilis* (CAD91829) and SS3, SS10, ST18 had closeness to NifH of *B. indica* subsp. *indica* ATCC 9039 (YP 001831615), SP3 had closeness to BchX (bacteriochlorophyllide reductase) of *Bradyrhizobium* sp BTAi1 (YP 001242253). While, NifH of INP4, BGM3, and BGM9 had closeness to NifH of uncultured nitrogen-fixing bacterium (Table 2). Analysis of NifD protein indicated that NifD of SS1, SS3, SS10, ST18 also homologous with NifD of *Beijerinckia indica* subsp. *indica* ATCC 9039 (YP 001831616), while INP4, BGM3, and BGM9 homologous with NifD of *Xanthobacter autotrophicus* Py2 (YP 001415005) (Table 3).

Homology between NifH of INP4 and BGM3 with NifH of uncultured nitrogen-fixing bacteria (BAE45579), BGM9 with NifH of uncultured nitrogen-fixing bacteria (ACO36748) due to most of NifH sequences database in GenBank belong to unculturable or uncultivated nitrogen-fixing bacteria. Zehr *et al*. (2003) said that NifH protein sequences database in GenBank is directly derived from various environmental samples of terestrial (Mårtensson *et al*., 2009), ocean (Farnelid *et al*., 2001), even marine-hot springs (hydrothermal vent) (Mehta *et al*., 2003).

Conserved Domain (CDD) analysis of NifD showed that NifD belong to Ras-like GTPase superfamily. Three-dimensional (3-D) structure of Fe protein or NifH showed that there were two identical subunits, each subunit consisted of β-sheets that flanked by α-helices, [4Fe-4S] cluster in the centre which link the two subunits and also there were two Mg-ATP binding sites (Figure 2A). [4Fe-4S] cluster has a role as an obligate electron donor to Fe-Mo protein or NifD (Lahiri *et al*., 2008). NifH has structural similarities with other transduction signalling molecules like G-proteins and ras and three main similarities include: (1) parallel β-sheets flanked by α-helices, (2) a phospate binding loop (P-loop) or Walker A motif, and (3) two switch region, switch I dan II (Lahiri *et al*., 2008).

Based on CDD analysis, NifD belong to nitrogenase oxidoreductase superfamily and nitrogenase FeMo alpha family. The alpha subunit contain FeMo-co metallo-cluster [1Mo-7Fe-9S] in the center and P-cluster [8Fe-7S] in the outside which link between α and β-subunit (Figure 2B).

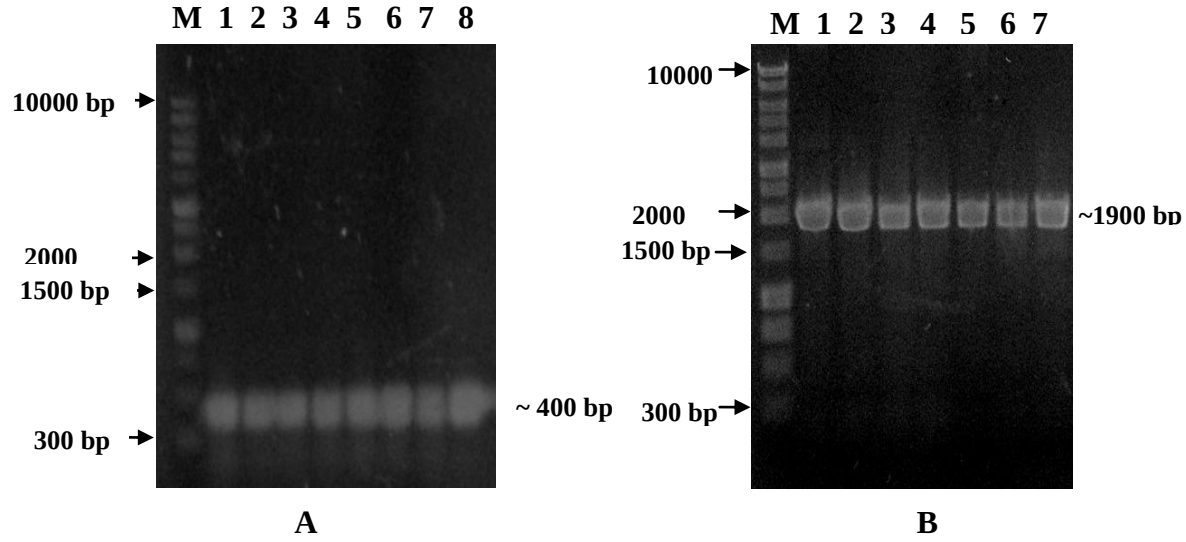


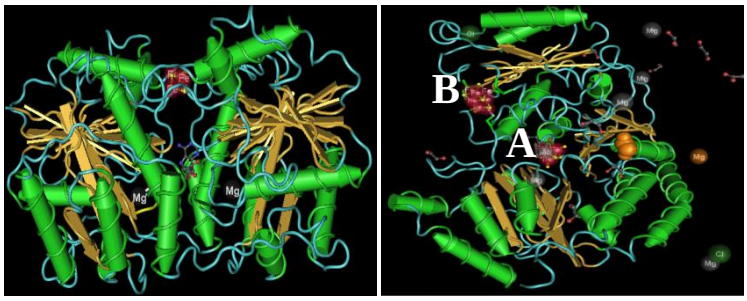
Figure 1. Amplification of *nif*H gene resulted band size of 393 bp using nH17K-F/nH139P-R primer (A) and *nif*D gene with band size of 1900 bp using nifHD-F/nifD-R primer (B) (wells from left to right: M= 1 kb *ladder*, 1= BGM3; 2= BGM9; 3= SS1; 4= SS3; 5= SS10; 6= ST18; 7= INP4; 8= SP3).

Table 2. Analysis of *nif*H gene sequences of nitrogen-fixing methanotrophs isolated from rice fields using BLAST-X program

Code of Isolate	Homology of NifH Sequences	Identity value	e-value	Acession Number
SS1	Nitrogenase iron protein (NifH) of *Beijerinckia mobilis*	99 %	4e-63	CAD91829
SS3	Nitrogenase reductase (NifH) of *Beijerinckia indica* subsp. *indica* ATCC 9039	98 %	5e-63	YP_001831615
SS10	Nitrogenase reductase (NifH) of *Beijerinckia indica* subsp. *indica* ATCC 9039	98 %	7e-68	YP_001831615
ST18	Nitrogenase reductase (NifH) of *Beijerinckia indica* subsp. *indica* ATCC 9039	98%	6e-69	YP_001831615
INP4	*Dinitrogenase reductase (NifH) of uncultured nitrogen-fixing bacterium*	94 %	5e-64	BAE45579
BGM3	*Dinitrogenase reductase (NifH) of uncultured nitrogen-fixing bacterium*	97 %	5e-56	BAE45579
BGM9	Dinitrogenase reductase (NifH) of uncultured bacterium	93 %	8e-53	ACO36748
SP3	Bacteriochlorophyllide reductase subunit of *Bradyrhizobium* sp. BTAi1(BchX)	88 %	4e-50	YP_001242253

Table 3. Analysis of *nif*D gene sequences of nitrogen-fixing methanotrophs isolated from rice fields using BLAST-X program

Code of Isolate	Homology of NifD Sequences	Identity value	e-value	Acession Number
SS1	Nitrogenase molybdenum-iron protein alpha chain (NifD) of *Beijerinckia indica* subsp. *indica* ATCC 9039	97 %	0.0	YP 001831616
SS3	Nitrogenase molybdenum-iron protein alpha chain (NifD) of *Beijerinckia indica* subsp. *indica* ATCC 9039	84 %	0.0	YP 001831616
ST18	Nitrogenase molybdenum-iron protein alpha chain (NifD) of *Beijerinckia indica* subsp. *indica* ATCC 9039	99 %	0.0	YP 001831616
SS10	Nitrogenase molybdenum-iron protein alpha chain (NifD) of *Beijerinckia indica* subsp. *indica* ATCC 9039	73 %	1e-40	YP 001831616
INP4	Nitrogenase molybdenum-iron protein alpha chain (NifD) of *Xanthobacter autotrophicus Py2*	97 %	0.0	YP 001415005
BGM3	Nitrogenase molybdenum-iron protein alpha chain (NifD) of *Xanthobacter autotrophicus Py2*	95 %	0.0	YP 001415005
BGM9	Nitrogenase molybdenum-iron protein alpha chain (NifD) of *Xanthobacter autotrophicus Py2*	94 %	0.0	YP 001415005



A B

Figure 2. Prediction of 3-D structures of (A) NifH (yellow: β-sheets, green: α-helices, red: [4Fe-4S] cluster) and (B) NifD (yellow: β-sheets, green: α-helices, A: FeMo-co cluster, B: P cluster).

Alignment of NifD and NifH sequences

Alignment of NifH sequences of SS1, SS3, SS10, ST18, BGM3, BGM9, INP4, SP3 and NifH sequences of reference species that derived from GenBank included genus *Beijerinckia*, *Methylocapsa*, *Methylocystis*, *Bradyrhizobium* and *Azospirillum* showed high similarities. There were three conserved motif regions that important in the function of the enzyme (Schlessman *et al*., 1998). Those conserved motif regions were Mg-ATP binding site (P-loop) or walker A (GKXXXXKS) motif, switch I (CDPKAD) motif, and switch II or Walker B (DXXG) motif (Figure 3). Both of switch motif regions have a role on the conformational changes of nitrogenase when ATP hydrolisis (Schlessman *et al*., 1998). Amino acid residues, Cys97 and Cys132 were binding sites of [4Fe-4S] cluster, whereas Arg100 involved in the inhibition of nitrogenase activity in response to the presence of ammonium through post-transcriptional modification (Lahiri *et al*., 2008). NifH sequence of SP3 also showed homology with BchX sequence of *Rhodobacter capsulatus*. Lahiri *et al*. (2008) and Yan *et al*. (2010) said that NifH and BchX sequences were highly homologous with the percentage of 63.8%, thus the researchers suspected that those genes were originated from a common ancestor or from one of the protein that evolved in the function.

Meanwhile, alignment of NifD sequences of SS1, SS3, SS10, ST18, BGM3, BGM9 with NifD sequences of reference species included genus of *Beijerinckia*, *Methylocella*, and *Xanthobacter* also showed conserved regions, including the FeMo-co binding sites and P cluster. The important residues of NifD sequences were αCys275 and αHis442 which is FeMo-co cluster binding site, αCys62, αCys88, αCys154, βCys70, βCys95, βCys153 which is P cluster binding sites (Figure 4). The FeMo-co cluster was an active site of substrate reduction, while the P cluster play a role in electron transfer from the [4Fe-4S] cluster to FeMo-co cluster (Raymond *et al*., 2004).

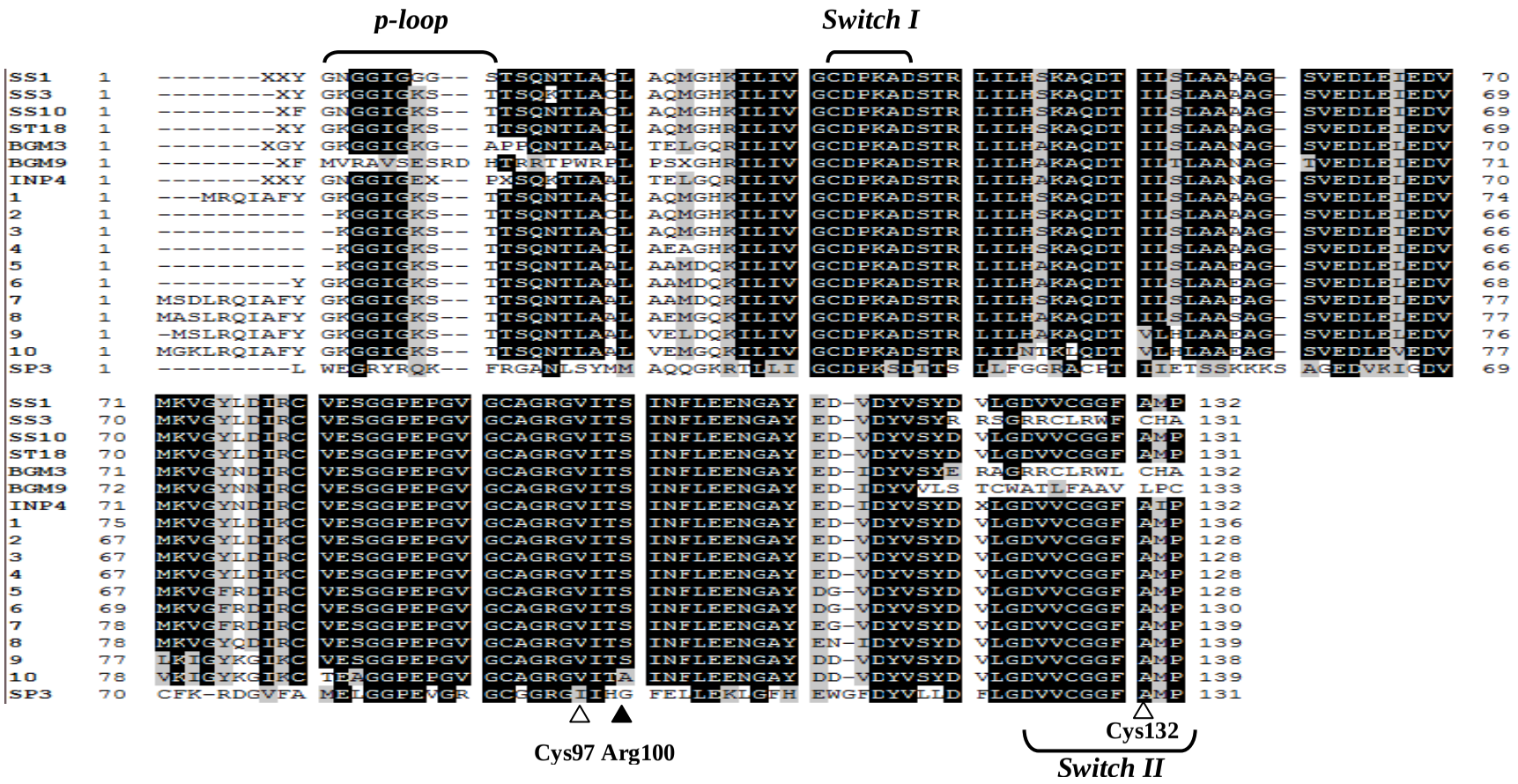
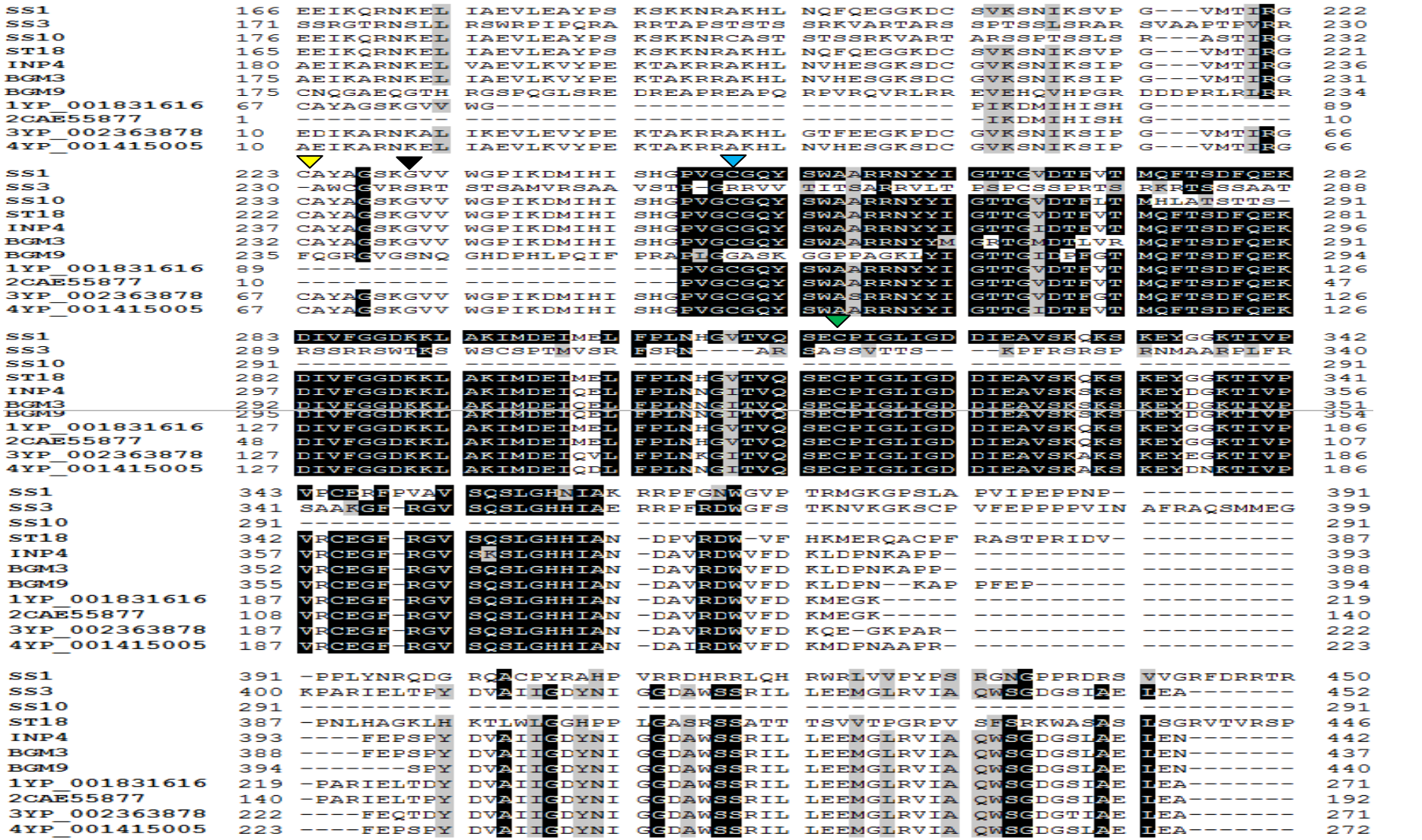


Figure 3. Alignment of deduced amino acid sequences of the approximately 393 bp partial *nif*H genes from BGM3, BGM9, SS1, SS3, SS10, ST18, INP4, SP3 and NifH sequences of reference species derived from GenBank: 1. *Beijerinckia indica* subsp. *indica* ATCC 9039, 2. *B. indica* subsp. *lacticogenes*, 3. *B. mobilis*, 4. *Methylocapsa acidiphila* B2, 5. *Methylocella tundrae*, 6. *M. palustris*, 7. *M. silvestris* BL2, 8. *Bradyrhizobium japonicum*, 9. *Azospirillum brasilense*, BchX sequence of 10. *Rhodobacter capsulatus* (open triangles: Cys97 and Cys132 were [4Fe-4S] cluster binding sites, close triangle: Arg100 residue that involved in nitrogenase activity inhibition in the presence of ammonium). Identical residues are in black boxes, and similar residues are in gray boxes.


SS1 166 EEIKQRNKEL IAEVLEAYPS KSKKNRAKHL NQFQEGGKDC SVKSNIKSVP G---VMTIRG 222
SS3 171 SSRGTRNSLL RSWRPIPQRA RRTAPSTSTS SRKVARTARS SPTSSLSRAR SVAAPTPVRR 230
SS10 176 EEIKQRNKEL IAEVLEAYPS KSKKNRCAST STSSRKVART ARSSPTSSLS R---ASTIRG 232
ST18 165 EEIKQRNKEL IAEVLEAYPS KSKKNRAKHL NQFQEGGKDC SVKSNIKSVP G---VMTIRG 221
INP4 180 AEIKARNKEL VAEVLKVYPE KTAKRRAKHL NVHESGKSDC GVKSNIKSIP G---VMTIRG 236
BGM3 175 AEIKARNKEL IAEVLKVYPE KTAKRRAKHL NVHESGKSDC GVKSNIKSIP G---VMTIRG 231
BGM9 175 CNQGAEQGTH RGSPQGLSRE DREAPREAPQ RPVRQVRLRR EVEHQVHPGR DDDPRLRLRR 234
1YP_001831616 67 CAYAGSKGVV WG--------- ---------- ---------- PIKDMIHISH G---------- 89
2CAE55877 1 ---------- ---------- ---------- ---------- IKDMIHISH G---------- 10
3YP_002363878 10 EDIKARNKAL IKEVLEVYPE KTAKRRAKHL GTFEEGKPDC GVKSNIKSIP G---VMTIRG 66
4YP_001415005 10 AEIKARNKEL IAEVLKVYPE KTAKRRAKHL NVHESGKSDC GVKSNIKSIP G---VMTIRG 66
SS1 223 CAYAGSKGVV WGPIKDMIHI SHGPVGCGQY SWAARRNYYI GTTGVDTFVT MQFTSDFQEK 282
SS3 230 -AWCGVRSRT STSAMVRSAA VSTP-GRRVV TITSARRVLT PSPCSSPRTS RKRTSSSAAT 288
SS10 233 CAYAGSKGVV WGPIKDMIHI SHGPVGCGQY SWAARRNYYI GTTGVDTFLT MHLATSTTS- 291
ST18 222 CAYAGSKGVV WGPIKDMIHI SHGPVGCGQY SWAARRNYYI GTTGVDTFVT MQFTSDFQEK 281
INP4 237 CAYAGSKGVV WGPIKDMIHI SHGPVGCGQY SWAARRNYYI GTTGIDTFVT MQFTSDFQEK 296
BGM3 232 CAYAGSKGVV WGPIKDMIHI SHGPVGCGQY SWAARRNYYM GRTGMDTLVR MQFTSDFQEK 291
BGM9 235 FQGRGVGSNQ GHDPHLPQIF PRAPIGGASK GGPPAGKIYI GTTGIDEFGT MQFTSDFQEK 294
1YP_001831616 89 ---------- ---------- ---PVGCGQY SWAARRNYYI GTTGVDTFVT MQFTSDFQEK 126
2CAE55877 10 ---------- ---------- ---PVGCGQY SWAARRNYYI GTTGVDTFVT MQFTSDFQEK 47
3YP_002363878 67 CAYAGSKGVV WGPIKDMIHI SHGPVGCGQY SWASRRNYYI GTTGVDTFGT MQFTSDFQEK 126
4YP_001415005 67 CAYAGSKGVV WGPIKDMIHI SHGPVGCGQY SWAARRNYYI GTTGIDTFVT MQFTSDFQEK 126
SS1 283 DIVFGGDKKL AKIMDEIMEL FPLNHGVTVQ SECPIGLIGD DIEAVSKQKS KEYGGKTIVP 342
SS3 289 RSSRRSWTKS WSCSPTMVSR FSRN-----AR SASSVTTS-- --KPFRSRSP RNMAARPLFR 340
SS10 291 ---------- ---------- ---------- ---------- ---------- 291
ST18 282 DIVFGGDKKL AKIMDEIMEL FPLNHGVTVQ SECPIGLIGD DIEAVSKQKS KEYGGKTIVP 341
INP4 297 DIVFGGDKKL AKIMDEIQEL FPLNNGITVQ SECPIGLIGD DIEAVSKSKS KEYDGKTIVP 356
BGM3 292 DIVFGGDKKL AKIMDEIQEL FPLNNGITVQ SECPIGLIGD DIEAVSKSKS KEYDGKTIVP 351
BGM9 295 DIVFGGDKKL AKIMDEIQEL FPLNNGITVQ SECPIGLIGD DIEAVSKSKS KEYDGKTIVP 354
1YP_001831616 127 DIVFGGDKKL AKIMDEIMEL FPLNHGVTVQ SECPIGLIGD DIEAVSKQKS KEYGGKTIVP 186
2CAE55877 48 DIVFGGDKKL AKIMDEIMEL FPLNHGVTVQ SECPIGLIGD DIEAVSKQKS KEYGGKTIVP 107
3YP_002363878 127 DIVFGGDKKL AKIMDEIQVL FPLNKGITVQ SECPIGLIGD DIEAVSKAKS KEYEGKTIVP 186
4YP_001415005 127 DIVFGGDKKL AKIMDEIQDL FPLNNGITVQ SECPIGLIGD DIEAVSKAKS KEYDNKTIVP 186
SS1 343 VPCERFPVAV SQSLGHNIAK RRPFGNWGVP TRMGKGPSLA PVIPEPPNP- ---------- 391
SS3 341 SAAKGF-RGV SQSLGHHIAE RRPFRDWGFS TKNVKGKSCP VFEPPPPVIN AFRAQSMMEG 399
SS10 291 ---------- ---------- ---------- ---------- ---------- 291
ST18 342 VRCEGF-RGV SQSLGHHIAN -DPVRDW-VF HKMERQACPF RASTPRIDV- ---------- 387
INP4 357 VRCEGF-RGV SKSLGHHIAN -DAVRDWVFD KLDPNKAPP- ---------- ---------- 393
BGM3 352 VRCEGF-RGV SQSLGHHIAN -DAVRDWVFD KLDPNKAPP- ---------- ---------- 388
BGM9 355 VRCEGF-RGV SQSLGHHIAN -DAVRDWVFD KLDPN--KAP PFEP------ ---------- 394
1YP_001831616 187 VRCEGF-RGV SQSLGHHIAN -DAVRDWVFD KMEGK----- ---------- ---------- 219
2CAE55877 108 VRCEGF-RGV SQSLGHHIAN -DAVRDWVFD KMEGK----- ---------- ---------- 140
3YP_002363878 187 VRCEGF-RGV SQSLGHHIAN -DAVRDWVFD KQE-GKPAR- ---------- ---------- 222
4YP_001415005 187 VRCEGF-RGV SQSLGHHIAN -DAIRDWVFD KMDPNAAPR- ---------- ---------- 223
SS1 391 -PPLYNRQDG RQACPYRAHP VRRDHRRLQH RWRLVVPYPS RGNCPPRDRS VVGRFDRRTR 450
SS3 400 KPARIELTPY DVAIIGDYNI GGDAWSSRIL LEEMGLRVIA QWSGDGSIAE IEA------- 452
SS10 291 ---------- ---------- ---------- ---------- ---------- 291
ST18 387 -PNLHAGKLH KTLWIGGHPP LGASRSSATT TSVVTPGRPV SPSRKWASAS ISGRVTVRSP 446
INP4 393 ----FEPSPY DVAIIGDYNI GGDAWSSRIL LEEMGLRVIA QWSGDGSIAE IEN------- 442
BGM3 388 ----FEPSPY DVAIIGDYNI GGDAWSSRIL LEEMGLRVIA QWSGDGSIAE IEN------- 437
BGM9 394 -------SPY DVAIIGDYNI GGDAWSSRIL LEEMGLRVIA QWSGDGSIAE IEN------- 440
1YP_001831616 219 -PARIELTDY DVAIIGDYNI GGDAWSSRIL LEEMGLRVIA QWSGDGSIAE IEA------- 271
2CAE55877 140 -PARIELTPY DVAIIGDYNI GGDAWSSRIL LEEMGLRVIA QWSGDGSIAE IEA------- 192
3YP_002363878 222 ----FEQTDY DVAIIGDYNI GGDAWSSRIL LEEMGLRVIA QWSGDGTIAE IEA------- 271
4YP_001415005 223 ----FEPSPY DVAIIGDYNI GGDAWSSRIL LEEMGLRVIA QWSGDGSIAE IEA------- 272

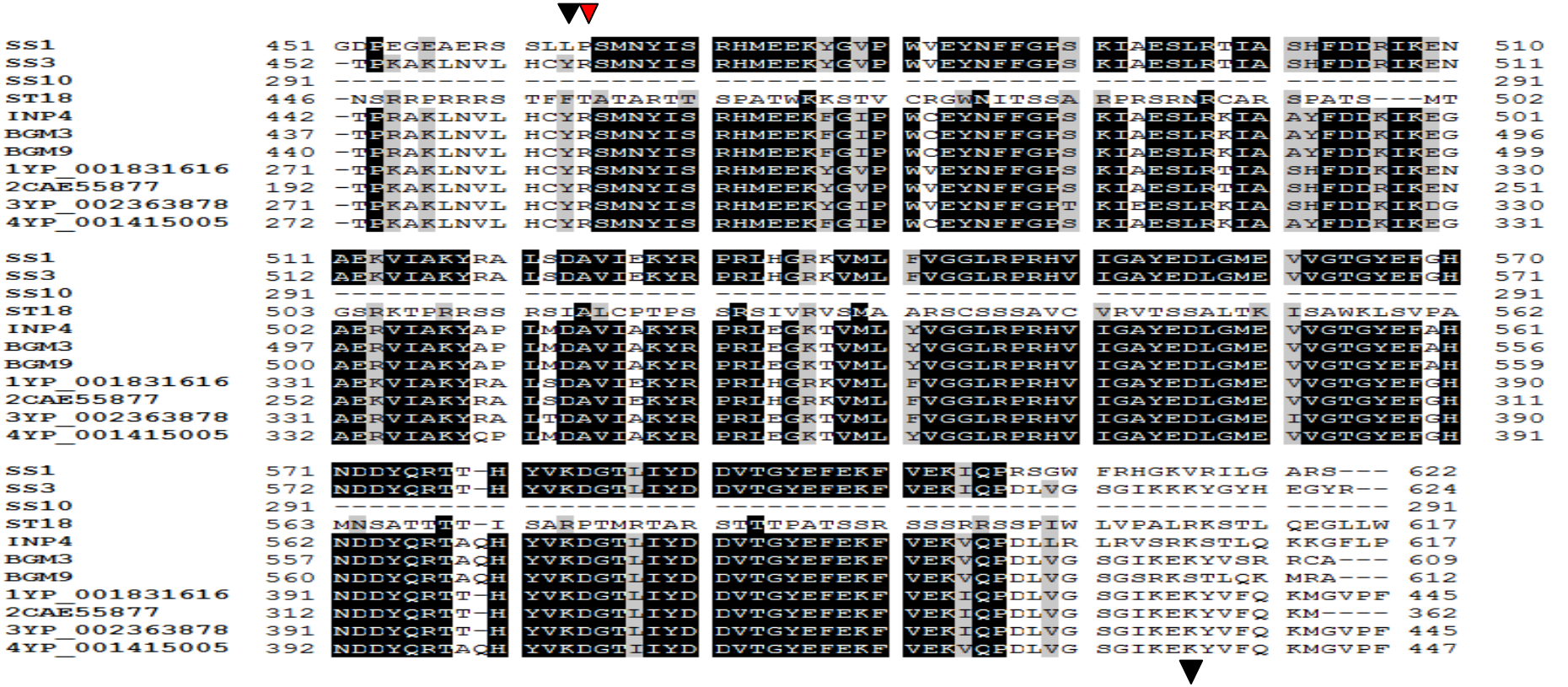


Figure 4. Alignment of deduced amino acid sequences of the approximately 1900 bp of *nif*D partial genes from BGM3, BGM9, SS1, SS3, SS10, ST18, and INP4 reference species that derived from *GenBank*: 1. *Beijerinckia indica* subsp. *indica*, 2. *Beijerinckia mobilis*, 3. *Methylocella silvestris* BL2, 4. *Xanthobacter autotrophicus* Py2 (yellow triangle: αCys62, blue: αCys88, and green: αCys154 were P cluster binding sites; while, red triangle: αCys275 was FeMo-co cluster binding site; black triangles were residues that involved in substrate reduction by FeMo-co cluster). Identical residues are in black boxes, and similar residues are in gray boxes.

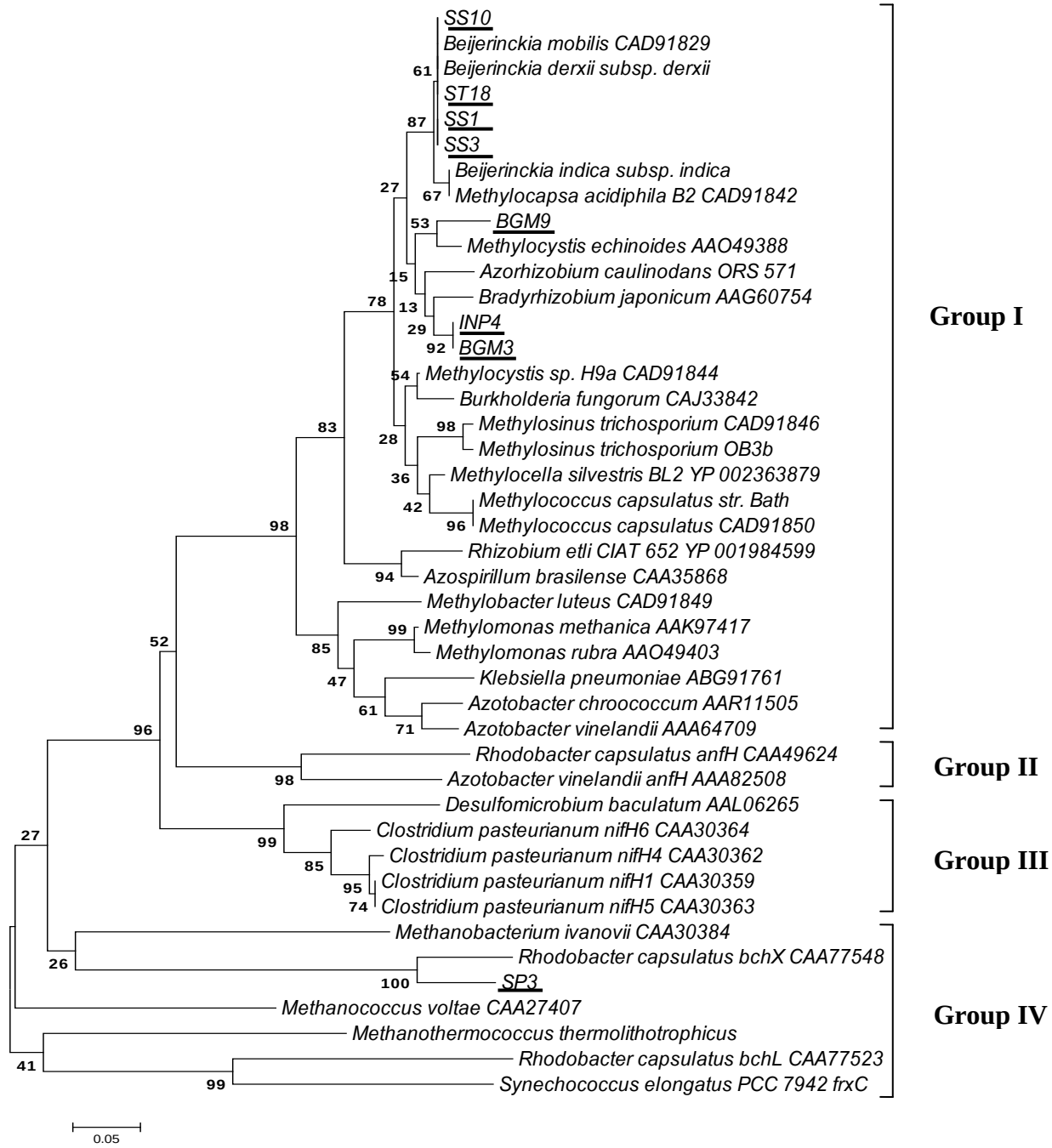


Figure 5. Phylogenetic tree based on amino acid sequences of NifH from eight isolates and other representative nitrogen-fixing bacteria belong to Group I (molybdenum-dependent nitrogenase (NifH) and vanadium-dependent nitrogense (VnfH)), Group II (Molybdenum-independent nitrogenase or iron nitrogenase (AnfH)), Group III (obligat anaerobic nitrogen-fixing bacteria, low GC content, and sulphate-reducing bacteria), and Group IV (uncharacterized nitrogenase, chlorophyllide reductase). Phylogenetic tree was constructed using Neighbour Joining method, numbers indicate the statistical confidence of the branching order determined by bootstrap analysis of 1000 alternative trees. The scale bar represent 0.05 substitution per nucleotide position.

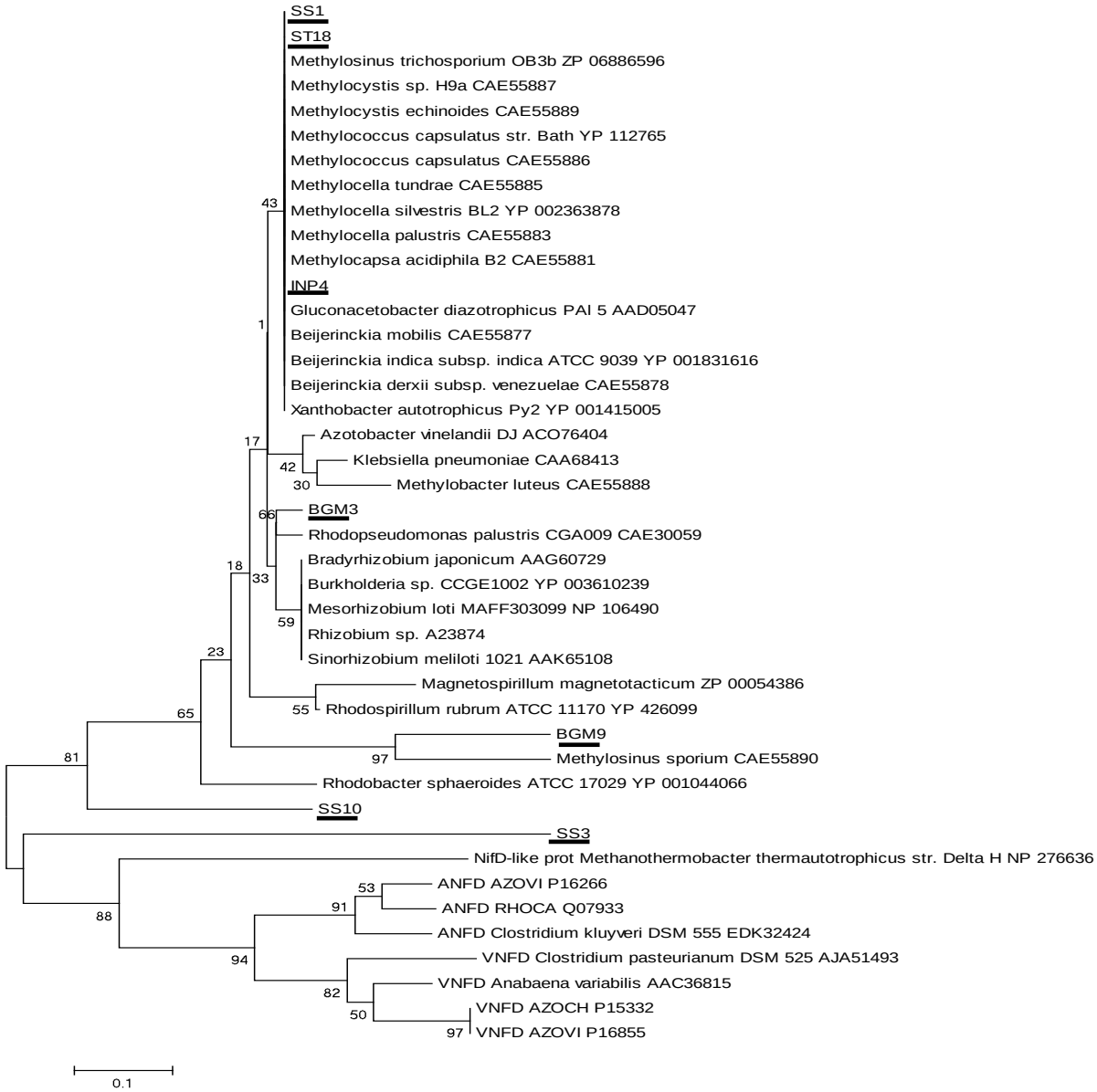


Figure 6. Phylogenetic tree based on amino acid sequences of NifD from seven isolates and other representative nitrogen-fixing bacteria. Alternative nitrogenase are designated VNFD (vanadium type) and ANFD (iron-only type). Species designations are as follows: AZOVI, *Azotobacter vinelandii*; RHOCA, *Rhodobacter capsulatus*; AZOCH, *Azotobacter chroococcum*. Phylogenetic tree was constructed using Neighbour Joining method, numbers indicate the statistical confidence of the branching order determined by bootstrap analysis of 1000 alternative trees. The scale bar represent 0.1 substitution per nucleotide position.

Phylogenic tree based on NifH, NifD, and 16S rRNA gene sequencing

Phylogenetic analysis of NifH sequences indicated that SS1, SS3, SS10, ST18, INP4, BGM3, and BGM9 were classified into Group I that consists primarily of *nif*H sequences from Proteobacteria (α, β, and γ) and Cyanobacteria. This Group I are represent the best-studied nitrogenase. The phylogenetic tree showed that SS1, SS3, SS10, and ST18 clustered together with the genus *Beijerinckia* along with *Methylocapsa acidiphila* B2 with identity values of 87%. Isolates of BGM3 and INP4 closely related each other with identity values of 92% and along with BGM9 clustered with *Methylocystis echinoides*, *Azorhizobium caulinodans*, and *Bradyrhizobium japonicum*. Meanwhile, SP3 belong to Group IV that consists of *nif*-like sequences from Archaea and distantly related chlorophylide reductase genes (Zehr *et al*., 2003). Isolate of SP3

closely related to *Rhodobacter capsulatus* with identity values of 100% (Figure 5).

So far, most of the environmental studies of nitrogen-fixing bacteria has focused on analysis of *nif*H gene sequences (Boulygina *et al*., 2002; Dedysh *et al*., 2004). As a consequence, the number of *nif*D gene sequences in databases are limited. In contrast, *nif*H gene sequences are rapidly expand and dominated by *nif* gene sequences of uncultured microorganism (Dedysh *et al*., 2004). The lack of sufficient number of *nif* gene sequences of culturable microorganism will lead into difficulty of data interpretation and making conclusion of phylogenetic analysis (Dedysh *et al*., 2004). Analysis of 16S rRNA gene sequences indicated that SS1, SS3, SS10, and ST18 had closeness to *Beijerinckia* sp. P310-1, while INP4 closely related to *Xanthobacter* sp. M5C24. Phylogenetic tree analysis showed that SS1, SS10, SS3, and ST18 had very close relationship with genus *Beijerinckia*, Methylocapsa, and *Methylocella* with identity value of 96 % (Figure 7). Meanwhile, BGM3 and BGM9 closely related to *Methylocystis parvus* 57 dan *Methylococcus capsulatus*,Texas respectively (Astuti, 2009). *Beijerinckia* and *Xanthobacter* belong to methylotrophic bacteria that capable to use C-1 compounds as their source of carbon and energy (Radajewski *et al*., 2002). Radajewski *et al*. (2002) also reported that two important substrates of methylotrophic bacteria in the nature are methanol and methan.

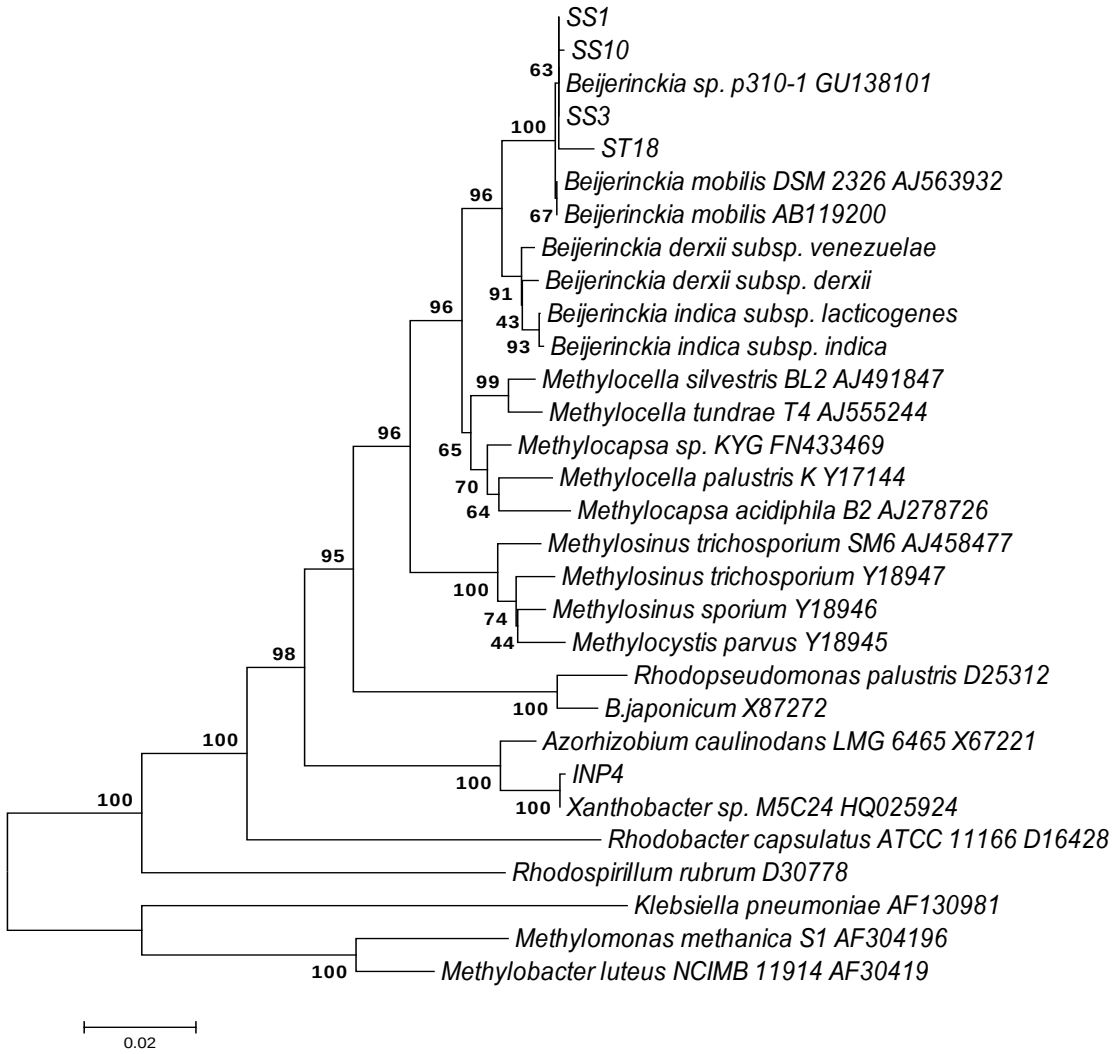


Figure 7. Phylogenetic tree of five isolates based on 16S rRNA gene sequences. Phylogenetic tree was constructed using Neighbour Joining method, numbers indicate the statistical confidence of the branching order determined by bootstrap analysis of 1000 alternative trees. The scale bar represent 0.02 substitution per nucleotide position.

Phylogenetic analysis based on 16S rRNA gene sequences showed similarity with NifH-based phylogenetic analysis which is SS1, SS3, SS10, and ST18 closely related to genus *Beijerinckia*. Those isolates also clustered with methanotrophs type II, genus *Methylocapsa* and *Methylocella*. Several studies also reported that based on 16S rRNA gene sequences, methanotrophs type II, genus *Methylocella* dan *Methylocapsa* closely related to genus *Beijerinckia* and form a monophyletic group of alphaproteobacteria (Dedysh *et al*., 2004; Dunfield *et al*., 2003; Dedysh *et al*., 2005a; Dedysh *et al*., 2005b). Henckel *et al*. (1999) reported that acidophylic-methanotrophs S6 showed similarity of 97% with *B. indica*. Methanotrophs and genus *Beijerinckia* also performed morphological and physiological similarities like acid tolerant and nitrogen fixation (Dedysh *et al*., 2005a).

Conclusions

*nif*H and *nif*D Genes of all isolates were succesfully amplified. Based on primer used to amplify *nif*D, it can be concluded that *nif*H and *nif*D of all isolates located within the same operon, except SP3. *nif*H and *nif*D genes sequences and phylogenetic tree analysis resulted that SS1, SS3, SS10, and ST18 closely related with genus *Beijerinckia*. Strains of BGM3, BGM9, SS1, SS3, SS10, ST18, and INP4 belong to nitrogen-fixing bacteria group I. Identification based on 16S rRNA genes also showed that SS1, SS3, SS10, and ST18 closely related with *Beijerinckia* sp. p310-1 (GU138101), meanwhile strain INP4 closely related to *Xanthobacter* sp. M5 C24 (HQ025924).

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