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Complete genome sequence of natural rubber-degrading, gram-negative bacterium, *Rhizobacter gummiphilus* strain NS21^T

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ABSTRACT

Gram-negative natural rubber-degrader, *Rhizobacter gummiphilus* NS21^T, which was isolated from soil in the botanical garden in Japan, is a newly proposed species of genus of *Rhizobacter*. It has been reported that the *latA1* gene is involved in the natural rubber degradation in this strain. To gain novel insights into natural rubber degradation pathway, the complete genome sequence of this strain was determined. The genome of strain NS21^T consists of 6,398,096 bp of circular chromosome (GenBank accession number CP015118.1) with G + C content of 69.72%. The genome contains 5687 protein-coding and 68 RNA genes. Among the predicted genes, 4810 genes were categorized as functional COGs. Homology search revealed that existence of *latA1* homologous gene (*latA2*) in this genome. Quantitative reverse-transcription-PCR and deletion analyses indicated that natural rubber degradation of this strain requires *latA2* as well as *latA1*.

Natural rubber (NR) is produced by over 2000 plant species from approximately 300 genera [1], and is a biopolymer containing poly(*cis*-1,4-isoprene) as the main component. NR from *Hevea brasiliensis* Müll.Arg. is used industrially for more than 100 years. Waste NR products, such as used tires, have been treated by combustion or stockpiling in landfills; however, these processes are hazardous to the environment and human health. Therefore, for treating rubber-derived wastes, the development of alternative treatment processes such as microbial degradation is required.

It has been reported that NR-degrading bacteria are widely distributed, and to date, NR-degrading Gram-positive and Gram-negative bacteria have been isolated and characterized so far [2–7]. Gram-positive bacteria such as *Streptomyces*, *Gordonia*, and *Nocardia* express the latex clearing protein, Lcp, that is a *b*-type cytochrome that cleaves the carbon-carbon double bond of poly (*cis*-1,4-isoprene) [8,9]. Additionally, an alternative rubber oxygenase (RoxA), encoded by the *roxA* gene, has been reported in the gram-negative bacterium, *Steroidobacter cummioxidans* strain 35Y [10,11]. It has been reported that RoxA is an extracellular *c*-type cytochrome, containing two heme-binding motifs (CXXCH), which constitute the active site of this enzyme [12]. *roxA* orthologs have been found in other Gram-negative bacteria, including *Haliangium, Myxococcus*, *Corallococcus*, and *Chondromyces* species [13]. Recently, another *roxA* ortholog named *roxB* has been identified in strain 35Y [14].

Gram-negative NR-degrading bacterium, *Rhizobacter gummiphilus* NS21^T (= NBRC 109400^T = BCC 58006^T) was isolated from the soil of a botanical garden in Japan [6,15]. Chemotaxonomic and phylogenetic analyses revealed that strain NS21^T is classified as a novel species in the genus of *Rhizobacter*, which also includes *Rhizobacter bergeniae* PLGR-1, *Rhizobacter dauci* H6, and *Rhizobacter fulvus* Gsoil 322 [16–18], and has been presented as the type strain of this genus [15]. The strain NS21^T, in which the *latA* gene encodes a RoxA ortholog, grows on a NR-overlay agar medium forming a clearing zone on it, and depolymerizes poly(*cis*-1,4-isoprene) [19,20]. However, knowledge of the whole genome sequences of gram-negative NR-degrading bacteria is limited except for that of *Haliangium ochraceum* DSM 14365 [21]. To get novel insights into the NR degradation pathway of gram-negative bacteria, the complete genome sequence of *R. gummiphilus* NS21^T was determined and the genes involved in NR degradation were identified.

For DNA extraction, the cells of strain NS21^T were grown on Wx minimal salt medium [22] containing deproteinized NR [23] at a final concentration of 0.4% (v/v) at 30 °C for three days. The cells were harvested by centrifugation at 10,000 ×*g* for 5 min and resuspended into STE buffer (10 mM Tris−HCl pH 8.0, 1 mM EDTA,

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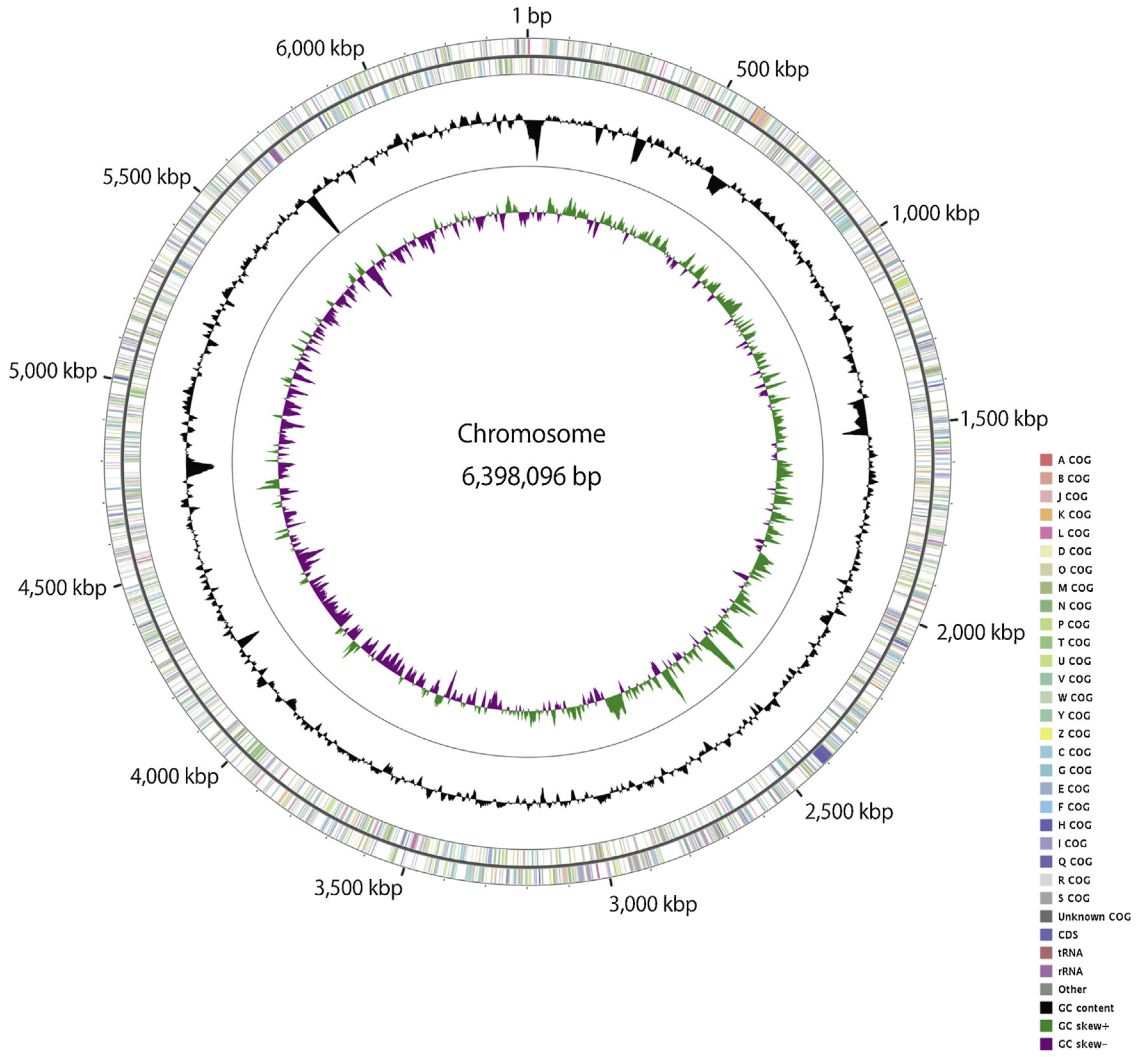

Fig. 1. Chromosome circular map of *R. gummiphilus* strain NS21^{T}. From inner to outer circle: GC skew (green and purple), G + C content (black), and CDS loci. CDS are colored with functional COG categories. CDS on the forward strand and reverse strand are described outside and inside of the black-colored ring, respectively (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article).

and 100 mM NaCl). Then, 1 mg/ml of lysozyme, 0.1 mg/ml of proteinase K, and 5% (w/v) of SDS were added and incubated for three hours at 50 °C to break the cells. After phenol-chloroform extraction, a DNA was extracted by ethanol precipitation. The quality and quantity of genomic DNA obtained were assayed using Qubit 2.0 fluorometer (Life Technologies, MA, USA) and agarose gel electrophoresis, respectively. Genomic DNA was sequenced by single-end sequencing with the 454 GS FLX Titanium system (Roche, Basel, Switzerland) and paired-end sequencing with Illumina HiSeq 1000 system (Illumina, San Diego, CA, USA). A total of 148,252,453 (324,197 reads) and 79,749,549 nucleotides (884,522 reads) were obtained by the GS FLX + and HiSeq 1000 systems, respectively. These sequencing data were assembled by Newbler ver. 2.6 (Roche).

Annotation was performed using NCBI Prokaryotic Genome Annotation Pipeline ver.3.1 [24] and RAST server [25]. The rRNA and tRNA genes were predicted using RNAmmer software [26] and tRNAscan-SE On-line [27], respectively. Signal peptides cleavage site prediction and COG analysis were performed using SignalP 4.1 Server [28] and WebMGA [29], respectively. Pfam domain search was performed using Pfam ver 29.0 [30]. Transmembrane helices were predicted using TMHMM Server ver. 20 [31]. CRISPRfinder program online [32] and CRISPR Recognition Tool (CRT, V1.0) [33]

Table 1
Genome statistics.

Attribute	Value	% of Total[a]
Genome size (bp)	6,398,096	100.00
DNA coding (bp)	5,766,835	90.13
DNA G+C (bp)	4,460,974	69.72
DNA scaffolds	1	-
Total genes	5,775	100.00
Protein coding genes	5,687	98.48
RNA genes	68	1.18
Pseudo genes	88	1.52
Genes in internal clusters	NA	NA
Genes with function prediction	3,425	59.31
Genes assigned to COGs	4,810	83.29
Genes with Pfam domains	4,621	80.02
Genes with signal peptides	739	12.80
Genes with transmembrane helices	1,365	23.64
CRISPR repeats	0	0.00

NA, no analysis.
[a] The total is based on either the size of the genome in base pairs or the protein coding genes in the annotated genome.

Table 2
Number of genes associated with general COG functional categories.

Code	Value	%age[a]	Description
J	188	3.31	Translation, ribosomal structure and biogenesis
A	4	0.07	RNA processing and modification
K	491	8.64	Transcription
L	161	2.83	Replication, recombination and repair
B	4	0.07	Chromatin structure and dynamics
D	33	0.58	Cell cycle control, Cell division, chromosome partitioning
V	66	1.16	Defense mechanisms
T	558	9.82	Signal transduction mechanisms
M	288	5.07	Cell wall/membrane biogenesis
N	211	3.71	Cell motility
U	184	3.24	Intracellular trafficking and secretion
O	200	3.52	Posttranslational modification, protein turnover, chaperones
C	299	5.26	Energy production and conversion
G	318	5.59	Carbohydrate transport and metabolism
E	430	7.56	Amino acid transport and metabolism
F	80	1.41	Nucleotide transport and metabolism
H	185	3.25	Coenzyme transport and metabolism
I	261	4.59	Lipid transport and metabolism
P	311	5.47	Inorganic ion transport and metabolism
Q	167	2.94	Secondary metabolites biosynthesis, transport and catabolism
R	618	10.87	General function prediction only
S	452	7.95	Function unknown
-	176	3.10	Not in COGs

[a] The total is based on the total number of protein coding genes in the genome.

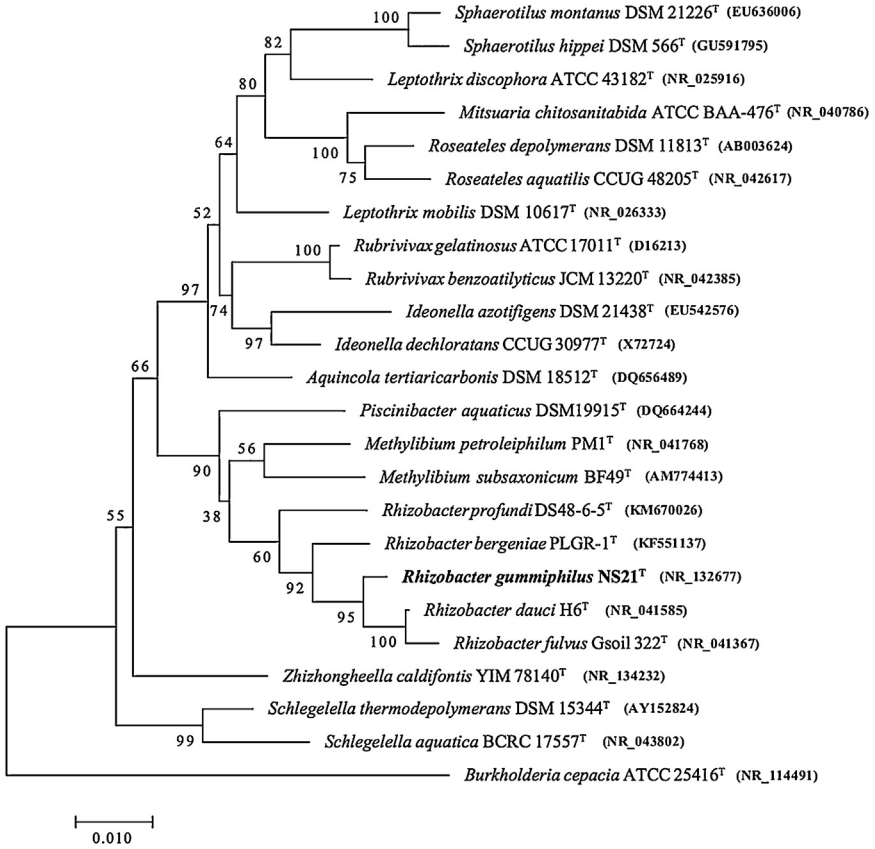

Fig. 2. Phylogenetic tree of 16S rRNA gene of *R. gummiphilus* strain NS21^T with relatively close type strains. A phylogenetic tree was generated by MAFFT program [35] using Neighbor-Joining method. The bootstrap values were calculated with 1000 replicates and values >50 are given above or below the branch nodes. Bar shows 0.01 substitutions per nucleotide position. *Burkholderia cepacia* ATCC 25416^T was used as outgroup. GenBank Accession numbers are shown in parentheses.

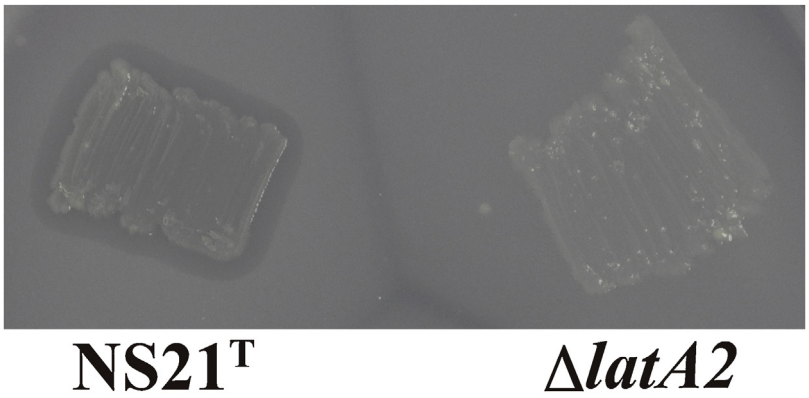

Fig. 3. The Growth of NS21^T and *latA2* mutant strain on deproteinized NR. The cells of NS21^T and *latA2* mutant (Δ*latA2*) were grown for 3 days on a deproteinized NR-overlay agar medium.

were used for the search of the clustered regulatory interspaced short palindromic repeats structures of the NS21^T genome. Circular genome map was generated using CGView [34] based on the predicted open reading frames and RNA genes.

The NS21^T genome contains one circular chromosome that was composed of 6,398,096 bp in length with a G + C content of 69.72% (Fig. 1). The number of genes encoding proteins with a defined function, hypothetical proteins, tRNA, and rRNA were 3,425, 2,262, 59, and 9, respectively. Detailed features of the genome statistics results are shown in Table 1. A total of 4810 CDS were assigned to functional COG categories as shown in Table 2. TMHMM analysis indicated that 1365 amino acids contained a transmembrane helices motif. No CRISPR region was found in the NS21^T genome. The genome sequence analysis revealed that three full-length 16S rRNA gene sequences, which are 100% identical to each other. The 16S rRNA gene sequence of strain NS21^T showed the highest identity with those of *R. dauci* H6 (95.9%) and *R. fulvus* Gsoil 322 (95.2%). The phylogenetic tree of 16S rRNA gene sequence constructed by the MAFFT program [35] using the Neighbor-Joining method revealed that strain NS21^T falls into the *Rhizobacter* species cluster with a high bootstrap value (Fig. 2).

The *latA1* (formerly, *latA*) gene, which encodes rubber oxygenase and is responsible for the initial NR degradation by strain NS21^T has been previously identified [19]. In the present study, a homologous gene (*latA2*) was predicted by the genome analysis, and its amino acid sequence shared a similarity of 37% and 65% with those of *latA1* and *roxA*, respectively. The heme-binding CXXCH motif, which was conserved in the amino acid sequences of LatA1 and RoxA, was also found in that of LatA2. To investigate the role of *latA2* in NR degradation by strain NS21^T, this gene was deleted by gene replacement technique. The resulting *latA2* mutant significantly lost the ability to form a clearing zone on a deproteinized NR-overlay agar medium, suggesting that *latA2* is required for NR utilization by this strain (Fig. 3). The enzymatic activities of *latA1* and *latA2* gene products have a synergistic effect on poly(*cis*-1,4-isoprene) degradation [20]. Furthermore, it has been reported that the *latA1* deletion mutant is unable to utilize NR [19]. These results suggested that a synergistic effect of *latA1* and *latA2* is required for NR utilization by strain NS21^T. To determine the transcriptional induction of *latA2*, the qRT-PCR analysis was carried out. Total RNAs extracted from the NS21^T cells grown with or without NR was used as template. The mRNA level of *latA2* ($14.7 \pm 2.8 \times 10^{-7}$ [mRNA/16S rRNA]) was elevated 7.0-fold in the cells grown on NR, indicating that transcription is induced during the utilization of NR.

To identify the genes which are included in the β-oxidation pathway of strain NS21^T, the functional annotation of the NS21^T genome was performed. A previous study had revealed that the β-oxidation pathway is involved in NR utilization by *Gordonia polyisoprenivorans* VH2 [36]. As shown in Table 3, a total of 94 genes that code for an aldehyde dehydrogenase (26 genes), an acyl-CoA synthetase (1 gene), an acyl-CoA dehydrogenase (24 genes), an NADPH-dependent 2,4-dienoyl-CoA reductase (2 genes), an enoyl-CoA isomerase (4 genes), an enoyl-CoA hydratase (16 genes), an acetyl-CoA acetyltransferase (thiolase) (10 genes), an α-methylacyl-CoA racemase (1 gene), and a 3-hydroxyacyl-CoA dehydrogenase (10 genes) were predicted. This result suggested that the β-oxidation pathway is involved in NR utilization by NS21^T.

In summary, we report for the first time the complete genome sequence of *Rhizobacter* species. The genome sequence of the NS21^T strain contains of a circular chromosome. The functional annotation of the NS21^T genome revealed that the presence of two orthologs, which encode rubber oxygenases. Our data imply that these rubber oxygenase genes are involved in NR utilization by strain NS21^T. Furthermore, β-oxidation pathway genes, which are required for NR utilization were found in the genome.

Author's contributions

MF and DK conceived the project. DVL, NG, and DK wrote the manuscript. DVL, NG, SI, and MT generated all the physiologic data. AH sequenced the genome. AH and AY assembled the genome presented here. MT annotated and analyzed the genome of this strain. All authors read and approved the final manuscript.

Conflict of interest

The authors declare that they have no competing interests.

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Table 3
The genes code for enzymes of NR degradation pathway in NS21[T].

Enzyme	Locus tag	Loci in the chromosome
LatA1	A4W93_01825	437816 to 439855
LatA2	A4W93_07150	1599479 to 1601500 (complement)
aldehyde dehydrogenase	A4W93_00340	88804 to 90324 (complement)
	A4W93_01495	358948 to 360369 (complement)
	A4W93_01595	384819 to 386408
	A4W93_01725	417601 to 419091
	A4W93_06705	1496435 to 1497382
	A4W93_06895	1537119 to 1538585
	A4W93_07140	1597290 to 1598711 (complement)
	A4W93_07700	1719778 to 1722798 (complement)
	A4W93_09690*	2141103 to 2143571
	A4W93_11050	2491018 to 2492457 (complement)
	A4W93_11120	2507176 to 2508666 (complement)
	A4W93_11645	2632623 to 2633756
	A4W93_12100	2728252 to 2729718 (complement)
	A4W93_12750	2870346 to 2871779
	A4W93_14510	3264888 to 3265349
	A4W93_14990	3367084 to 3368604 (complement)
	A4W93_16420	3667766 to 3669268
	A4W93_18540	4129441 to 4130859
	A4W93_21165	4673276 to 4674730 (complement)
	A4W93_22820	5024137 to 5025639 (complement)
	A4W93_24180	5317079 to 5317534
	A4W93_24775	5439914 to 5440303 (complement)
	A4W93_24780	5440300 to 5441820 (complement)
	A4W93_25560*	5612360 to 5614390 (complement)
	A4W93_26880	5887207 to 5888640 (complement)
	A4W93_28705	6278513 to 6279952 (complement)
acyl-CoA synthase	A4W93_10155	2240238 to 2241530
acyl-CoA dehydrogenase	A4W93_04495	1012120 to 1013352
	A4W93_04510	1015195 to 1016379
	A4W93_04515	1016384 to 1017505
	A4W93_04635	1041367 to 1042593 (complement)
	A4W93_04670	1050758 to 1052557
	A4W93_04720	1061887 to 1063032
	A4W93_05565	1241557 to 1242756
	A4W93_05885	1309672 to 1311462
	A4W93_06445	1435444 to 1437270 (complement)
	A4W93_06820	1522324 to 1523595
	A4W93_07065	1576008 to 1577192
	A4W93_07070	1577206 to 1578327
	A4W93_07085	1580581 to 1581741
	A4W93_07170	1605616 to 1606764
	A4W93_10535	2354538 to 2355728
	A4W93_12800	2879730 to 2881523
	A4W93_13675	3069549 to 3071375
	A4W93_20980	4633752 to 4634957 (complement)
	A4W93_22885	5038889 to 5041027
	A4W93_23075	5074893 to 5076056
	A4W93_24270	5333564 to 5334718 (complement)
	A4W93_24350	5353519 to 5354094
	A4W93_24535	5388995 to 5390185 (complement)
	A4W93_24830	5452150 to 5453352
NADPH-dependent 2,4-dienoyl-CoA reductase	A4W93_00465	113515 to 115536
	A4W93_29140	6377365 to 6379413
enoyl-CoA isomerase	A4W93_04655	1045878 to 1047977 (complement)
	A4W93_05895	1311982 to 1314366
	A4W93_07265	1630401 to 1632548
	A4W93_13660	3065594 to 3067687
enoyl-CoA hydratase	A4W93_03405	761173 to 761985 (complement)
	A4W93_03415	763093 to 763911
	A4W93_03430	766220 to 767035 (complement)
	A4W93_04655	1045878 to 1047977 (complement)
	A4W93_05895	1311982 to 1314366
	A4W93_05905	1315719 to 1316486
	A4W93_07080	1579796 to 1580578
	A4W93_07265	1630401 to 1632548
	A4W93_07280	1634563 to 1635372
	A4W93_07295	1637722 to 1638501
	A4W93_11830	2667130 to 2667900 (complement)
	A4W93_13495	3032092 to 3032745 (complement)
	A4W93_13660	3065594 to 3067687
	A4W93_18665	4153545 to 4154360
	A4W93_24265	5332437 to 5333552 (complement)
	A4W93_25385	5581186 to 5581980 (complement)
acetyl-CoA acetyltransferase	A4W93_04650	1044595 to 1045773 (complement)

Table 3 (*Continued*)

Enzyme	Locus tag	Loci in the chromosome
	A4W93_05900	1314393 to 1315589
	A4W93_06005	1334551 to 1335687 (complement)
	A4W93_07250	1627248 to 1628453
	A4W93_07275	1633388 to 1634566
	A4W93_10490	2340573 to 2341751
	A4W93_13665	3067719 to 3068894
	A4W93_17650	3934930 to 3936096 (complement)
	A4W93_24835	5453484 to 5454665
	A4W93_26405	5785296 to 5786498
α-methylacyl-CoA racemase	A4W93_07160	1602924 to 1604048
3-hydroxyacyl-CoA dehydrogenase	A4W93_04500	1013356 to 1014252 (complement)
	A4W93_04655	1045878 to 1047977 (complement)
	A4W93_04710	1059138 to 1060646
	A4W93_05895	1311982 to 1314366
	A4W93_06450	1437263 to 1437508 (complement)
	A4W93_07255	1628458 to 1629357
	A4W93_07265	1630401 to 1632548
	A4W93_13660	3065594 to 3067687
	A4W93_14090	3171173 to 3171931 (complement)
	A4W93_16470	3678392 to 3679309 (complement)

* Categorized as aldehyde dehydrogenase based on the nomenclature (molybdopterin oxidoreductase).

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