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Genus

Bacteroidota/Bacteroidia/Bacteroidales/Marinilabiliaceae/

Natronoflexus

Sorokin et al. 2011, VL144

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Na.tro.no.fle'xus; N.L. neut. n. *natron* (arbitrarily derived from the Arabic n. *natrun* or *natron*) soda, sodium carbonate; N.L. pref. *natrono-*, pertaining to soda; L. masc. n. *flexus*, a bending, N.L. masc. n. *Natronoflexus*, bending/flexible cells living in soda.

The genus *Natronoflexus* is a member of the family *Marinilabiliaceae*, order *Bacteroidales*, class *Bacteroidia*, and phylum *Bacteroidota*. It is an obligately anaerobic fermentative saccharolytic bacterium with the ability to utilize polygalacturonates and xylan as carbon and energy sources. The only species of the genus, *N. pectinivorans*, is a moderately salt-tolerant, chloride-independent obligate alkaliphile found in soda lakes in Central Asia. The DNA G + C content is 39.6 mol% (genome).

DNA G + C content (%): 39.6 (genome).

Type species: **Natronoflexus pectinivorans** Sorokin et al. 2011, VL144.

Cells of *Natranoflexus* are **long flexible rods** with gliding motility, forming bundles while growing on polymers. The cell wall consists of a monolayer covered with exopolysaccharide. The identified membrane polar lipids include **phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine**. The dominant **polar lipid fatty acids** include **three isomers of C15**. It is an **obligately anaerobic, fermentative, and saccharolytic heterotroph,** specialized in utilization of **polygalacturonates as growth substrates.** The products of galacturonic acid fermentation are acetate and succinate. It is a **moderately salt-tolerant obligate alkaliphile** optimally growing at pH 9.5–10 and at Na^+ concentrations (as carbonates) of 0.4–0.6 M. Found in anoxic sediments of soda lakes in southwestern Siberia (Altai, Russia). The genus currently **includes a single (type) species, *N. pectinivorans*** (Sorokin et al., 2011). *Natranoflexus* is a **member of the family *Marinilabiliaceae*** and order *Bacteroidales* in the class *Bacteroidia*.

DNA G + C content (%): 39.6 (genome).

Type species: **Natronoflexus pectinivorans** Sorokin et al. 2011, VL144.

Number of species with validly published name: 1.

Family classification: The genus *Natronoflexus* is classified within the family *Marinilabiliaceae*.

Further descriptive information

The cells are flexible rods of highly variable length, often long, bent, and with gliding motility, tending to form aggregates (Figure 1). Colonies on alkaline agar with galacturonic acid are up to 2 mm, slimy, and pink. Cells contain membrane-bound carotenoids with an absorption maximum at 498 nm (Sorokin et al., 2011).

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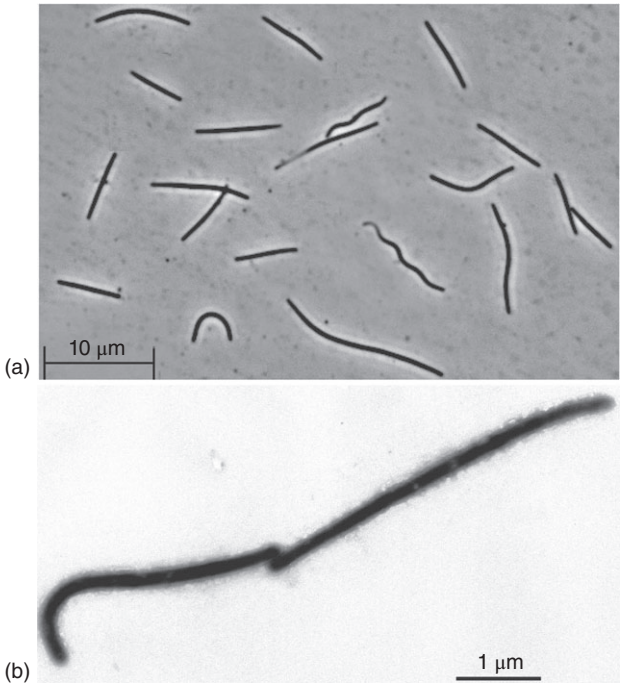
FIGURE 1. Morphology of *Natranoflexus pectinivorans* AP1^T grown at 0.6 M total Na^+ and pH 10 with pectin. (a) Phase-contrast microphotograph. (b) Microphotograph of transmission electron microscopy preparation stained with uranyl acetate.



Natronoflexus is a typical representative of hydrolytic bacteroidetes being able to grow with a wide range of glucans, including polygalacturonates, xylan, laminarin (beta-glucans), starch, glycogen, and pullulan (alpha-glucans). The spectrum of utilized sugars is also relatively broad.

Habitat, enrichment, and isolation

Natronoflexus pectinivorans was enriched from mixed anaerobic sediments taken from several soda lakes in Kulunda Steppe (Altai, Russia). The enrichment medium was based on a sodium carbonate buffer at pH 10 containing 0.6 M total Na^+ supplemented with 1 g/l of apple pectin. After several dilution-to-extinction series the final positive dilutions were plated on alkaline pectin agar, and the dominant pink colony type was transferred back into the liquid medium of similar composition. The procedure was repeated until the colony morphology was uniform and the purity verified by 16S rRNA gene sequencing.

Genome analysis

The draft genome of the type strain *N. pectinivorans* AP1^T was sequenced by Joint Genomic Institute within the GEBA program. The genome is 3.48 Mb and comprises 3,389 genes encoding 3,298 proteins. The genome is not yet analyzed in depth, and here only the main functional content is provided.

Hydrolytic potential

One of the major trends noticeable in the genome is a very high representation of genes encoding polysaccharidolytic enzymes, typical for hydrolytic bacteroidetes. In particular, there is a remarkably large genetic locus (called the *polysaccharide utilization locus*, PUL) encoding various glycoside hydrolases (GH families) with or without carbohydrate-binding modules (CBMs), dominated by different GH43 subfamilies, with the main enzymes as beta-xylosidase and alpha-L-arabinofuranosidase (Mewis et al., 2016), carboxylesterases (CE family 1), and SusCD-TonB modules responsible for oligosaccharide import through the outer membrane. In addition, many other CAZy enzymes are encoded elsewhere in the genome. The major types include:

1. Pectin hydrolysis locus consisting of two multidomain pectate/pectin lyases: PL1, fibronectin III-like CBM and SusD/TonB, which is consistent with the observed growth on pectin.
2. PUL locus, dominated by the GH43 and also including GH families 5, 9, 10, 11, 16, 26, 95, 97, and 146 and CE family 1 + multiple SusCD-TonB modules. Cellulose utilization has not been confirmed; arabinan and mannan utilization was not tested.
3. A separate locus for xylan degradation including two GH10–GH11 endo-β-1,4-xylanases, GH30_1 endoxylanase, GH43_12 beta-xylosidase, and GH115 xylan α-1,2-glucuronidase/α-(4-*O*-methyl)-glucuronidase. This is consistent with the observed growth on xylan.
4. Several homologues of the GH16 family, which include beta-1,3/1,4-endoglucanses, in accordance with the observed growth on laminarin.
5. Several loci encoding alpha-amylases and alpha-glucosidases, apparently responsible for the observed ability to utilize starch and pullulan as growth substrates.

The full list of such enzymes encoded in the genome of *N. pectinivorans* is given in Table 1.

The produced monomeric sugars are fermented via the pentose phosphate pathway.

TABLE 1. Polysaccharide hydrolases encoded in the genome of *Natronoflexus pectinivorans* AP1^T

Locus tag	Predicted polysaccharide degradation	Module	Predicted function
WP_13243 + 2544	Pectin	GH2	Beta-galacturonidase
2727		GH2	
2809		GH28	Exo-polygalacturonase
3503		GH105	Unsaturated rhamnogalacturonyl hydrolase
3362			
3364		CE12	Pectin acetylesterase
4037		CE8	Pectin methylesterase
4038		GH105	Unsaturated rhamnogalacturonyl hydrolase
4061		SusC/RagA	Oligosaccharide export through outer cell-wall membrane
4062		RagB/SusD	
4064		GH28	Exo-polygalacturonase
4075			
4077		GH2	Beta-galacturonidase
4099		PL11	Rhamnogalacturonan endolyase
4153		TonB	Oligosaccharide export through outer cell-wall membrane
4154		RagB/SusD	
4155		GH88/105	Rhamnogalacturonyl hydrolase
4156			
4159		PL1 + CBM13 + CBM66	Pectate lyase/carbohydrate-binding domains
4580		CE8	Pectin methylesterase
5314		GH88/105	Rhamnogalacturonyl hydrolase
5429		CE8/PL1/CBM6	Pectin methylesterase/pectate lyase/carbohydrate-binding domain
5431		CE8/PL1_2/CBM6	
5433			Fibronectin type III-containing protein (carbohydrate binding)
5435		RagB/SusD	Oligosaccharide export through outer cell-wall membrane
5437		TonB	
WP_16592 + 1824		GH88/105	Rhamnogalacturonyl hydrolase
1868		GH106	Rhamnogalacturonan alpha-L-rhamnohydrolase
1869		CE12	Pectin/rhamnogalacturonan acetylesterase
WP_13243 + 0846	Xylan	GH10	Endo-1,4-beta-xylanase
0848		GH43_12	Beta-xylosidase
1054		GH10 + CBM4 + CBM22	Endo-1,4-beta-xylanase + carbohydrate-binding domains
1124		GH10 + CE1 + CBM48	Endo-1,4-beta-xylanase + acetylesterase/carbohydrate-binding domain
1126		GH115	Xylan α-1,2-glucuronidase/α-(4-*O*-methyl)-glucuronidase
1133		GH30_1	Endo-1,4-beta-xylanase
1215		GH43_12	Beta-xylosidase
1227		GH43_29 _CBM6	Endo-1,4-beta-xylanase +carbohydrate-binding domains
1305		CE1 + CBM6	Acetyl xylan esterase + carbohydrate-binding domain
1313, 1315		CE1	Acetyl xylan esterase

TABLE 1. *(continued)*

Locus tag	Predicted polysaccharide degradation	Module	Predicted function
1317		GH10 + CE1+ CBM46	Endo-1,4-beta-xylanase+acetylesterase/carbohydrate-binding domain
1319,1321,1323		GH10 + CBM48	Endo-1,4-beta-xylanase+carbohydrate-binding domain
1325		CE1	Acetyl xylan esterase
1327		GH10 + CE1	Endo-1,4-beta-xylanase + acetylesterase
1343		GH43_10 + CBM22	Endo-1,4-beta-xylanase + carbohydrate-binding domain
1345		GH43_29 + CBM6/22/42	
1351		GH3	Beta-xylosidase
1355		GH3 + CBM6	Beta-xylosidase + carbohydrate-binding domains
1375		GH10 + CBM4+ CBM10	Endo-1,4-beta-xylanase +carbohydrate-binding domains
1381		GH11 +CBM13	
1389		GH10	Endo-1,4-beta-xylanase
2253		GH11	Endo-1,4-beta-xylanase
3419, 3420		GH43_5, GH43_24	Beta-xylosidases/xylanase + carbohydrate-binding domains
3422		GH2	
3423, 3424		GH43_18	
3505		GH43_19 + GH43_34 + CBM42	
4036		GH43_10	
4163			
4210		GH43_26 + CBM42	
4447		GH43_10 + CBM22	
4675		GH2	
4678		GH3 + CBM6	
4682			
4680		GH67	Xylan alpha-1,2-glucuronidase
WP_16592 + 1782		GH115	
WP_13243 + 1110	Starch, pullulan	GH97	Glucoamylase/alpha-glycosidase
1294			
2187			
2189		GH31	Alpha-glycosidase
2191		GH97 + CBM51	Glucoamylase + carbohydrate-binding domain
2193		RagB/SusD	Oligosaccharide export through outer cell-wall membrane
2412	Starch, pullulan	GH13	Alpha-1,4 amylase
2414		GH31	Alpha-glycosidase
2116		GH13	Alpha-1,4 amylase
2118		GH65	Alpha-trehalase
2128		SusE/SusF	Starch-specific carbohydrate-binding/export
2623		GH13	Alpha-1,4 amylase
2729		GH97	Glucoamylase
2838		GH31	Alpha-glycosidase
3869		GH13_5 + CBM20	Alpha-1,4 amylase/pullulanase + carbohydrate-binding domain

TABLE 1. *(continued)*

Locus tag	Predicted polysaccharide degradation	Module	Predicted function
WP_13243 + 1223	Alpha-glucans	GH42	Alpha-L-arabinofuranosidase
1278		GH43_4	Arabinan endo-1,5-alpha-L-arabinosidase
1292			
1290		GH43_5 + GH43_5	Alpha-L-arabinosidase
1341		GH43_5	
1361		GH43_29	Exo-alpha-1,5-L-arabinanase
1369		GH95 + CBM3/32/51	Alpha-L-galactosidase
1377		GH43_12	Exo-alpha-1,5-L-arabinosidase/arabinanase
2523		GH42	Alpha-L-arabinofuranosidase
2725		GH43_5	
3277		GH43_12	Exo-alpha1,5-L-arabinosidase/arabinanase
4198		GH95	Alpha-L-galactosidase
4210		GH43_29 + CBM	Exo-alpha-1,5-L-arabinosidase/arabinanase
5467		GH43_5	Alpha-L-arabinosidase
WP_13243+ 1131	Beta-glucans	GH43_10 + GH5_23 + CBM13	Endo-beta 1,3/1,4-glucanase (multidomain)
1387		GH5_21 + 3 × CBM13	Endo-beta 1,4-glucanase/cellulase + carbohydrate-binding domain
2045		GH5_26	Endo-beta 1,4-glucanase/cellulase
3426		GH5_4	
5320		GH5_55	
1311		GH9	Endo-beta 1,4-glucanase/cellulase
3432		GH9 +CBM 2/4	Endo-beta 1,4-glucanase/cellulase + carbohydrate-binding domains
3478		GH9	Endo-beta 1,4-glucanase/cellulase
4572			
1605		GH16_3 + CBM6/24	Endo-beta 1,3/1,4-glucanase + carbohydrate-binding domains
2731		GH16_3 + CBM6/32/54	
2856		GH16_3	
3170		GH16_3 + CBM6/54	
3171		GH16_3 + PKD type of CBM	
4574		GH16_3	
1597		GH26 + CBM3/35	Beta-mannanase + carbohydrate-binding domains
1707		GH26	
1709		GH 130	Beta-1,2-mannosidase
3717		GH5_10 + CBM32	Endo-beta-1,4-mannanase + carbohydrate-binding domains
3719		GH26	Beta-mannanase/mannobiohydrolase
4228		GH2	Beta-galactosidase
4229		GH53 + CBM61	Endo-beta-1,4-galactanase + carbohydrate-binding domain
1296		GH146	Beta-L-arabinofuranosidase
1347			
1357			
2821		GH3	Beta-glycosidase

TABLE 1. *(continued)*

Locus tag	Predicted polysaccharide degradation	Module	Predicted function
3422		GH2	
3431			
3809		GH3	
3810			
3917		GH146	Beta-L-arabinofuranosidase
4077		GH2	Beta-glycosidase
4675			
4678		GH3	
4682		GH3 + CBM6	Beta-glycosidase + carbohydrate-binding domains
5316		GH2 + CBM35/67	

Haloalkaliphilic adaptations

1. Single subunit Na^+/H^+ and K^+/H^+ antiporters NhaC and NhaP2, respectively.
2. Multisubunit Na^+/H^+ antiporter MrpABCDEF.
3. Two copies of Na^+/Ca^{2+} antiporter CaCA.
4. Potassium import transporters TrkAH and two separate copies of TrkA.
5. Search for the osmolyte biosynthesis pathway indicated two potential candidate pathways. A two-step pathway for the production of *N*ε-acetyl-beta-lysine via the lysine 2,3-aminomutase and β-lysine acetyltransferase (AblB, one copy in an operon with AlbA and a singleton AlbB). This beta-amino acid osmolyte is commonly found in halophilic and halotolerant methanogens (Roberts, 2005). The second pathway consists of two *S*-adenosyl methionine(SAM)-dependent *N*-methyltransferases with moderate homology to glycine/sarcosine and sarcosine/dimethylglycine methyltransferases, performing a three-step sequential *N*-methylation of glycine to betaine (Nyyssölä et al., 2001).

Similar to many examples of the soda lake anaerobes, the bioenergetics of *Natronoflexus* seems to be dominated by primary sodium pumps (except for the ATPases), which include the following:

1. Sodium-translocating (exporting) NADH-dehydrogenase NqrABCDEF.
2. Sodium-translocating (exporting) pyrophosphatase.
3. Sodium-translocating (importing) bifurcating complex RnfABCDEG.
4. Sodium-translocating (exporting) oxalacetate decarboxylase OadABG.

Other bioenergetic complexes include a proton-pumping F1F0 ATP synthase and V-type ATPase NtpABDEFI; a bifurcating heterodisulfide reductase HdrABC; and a NAD(P)-dependent H_2-forming Fe-only hydrogenase HndABCD/HydB.

Nitrogen metabolism (not yet proven phenotypically)

1. Potential for dissimilatory reduction of nitrite to ammonia, represented by NrfAH ammonifying nitrite reductase.
2. Potential for diazotrophy, represented by the nitrogenase operon coding for NifHDK/NifNBE with two copies of P-II family nitrogen regulator.

Among the structural features, the genome contains multiple genes coding for gliding motility proteins, some of them with an additional cell-absorption module PKD (polycystic kidney disease) and three megaproteins in a single operon with a homology to porins, which might be involved in oligosaccharide import.

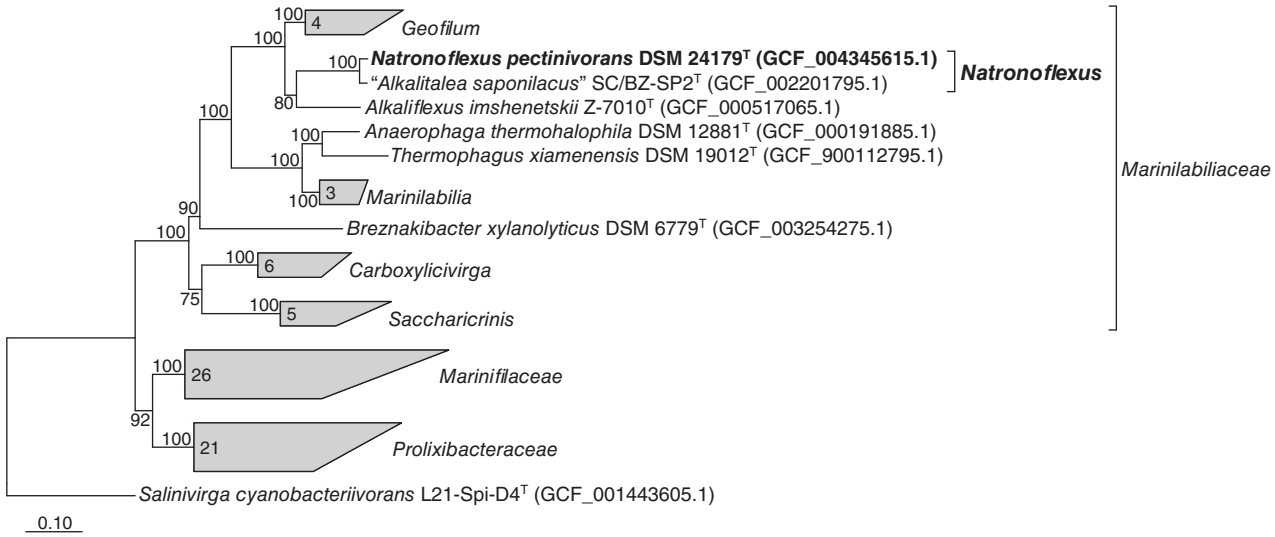
Oxidative stress response

The O_2 defense mechanism includes three SodB homologues of the [Mn,Fe] superoxide dismutase, a glutathione peroxidase, a catalase/peroxidase of the HPI type, and cytochrome *bd* quinol oxidase.

Taking into account the genomic content, the following phenotypic properties were reexamined:

1. diazotrophy: the acetylene reduction test gave positive results for nitrogenase with xylose as substrate.

FIGURE 2. Phylogenetic position of *Natronoflexus* species (in bold) within the order *Bacteroidales* based on the sequence analyses of concatenated alignment of 120 single-copy conserved bacterial protein markers (Parks et al., 2020). According to the phylogenomic analysis, *Alkalitalea saponilacus* could be reclassified as a species of the genus *Natronoflexus*. The trees were built using the IQ-TREE 2 program (Minh et al., 2020) with fast model selection via ModelFinder (Kalyaanamoorthy et al., 2017) and ultrafast bootstrap approximation (Minh et al., 2013) as well as approximate likelihood-ratio test for branches (Anisimova and Gascuel, 2006). Bootstrap consensus tree is shown, with values above 85% placed at the nodes. Bar, 0.10 changes per position.



2. dissimilatory nitrite reduction to ammonia: ammonia production not observed with xylose or maltose as substrates and 5 mM KNO_2.
3. growth tests with amorphous cellulose, beta-1,4-mannan, gluco- and galactomannans, alpha-arabinan, arabinoxylan, and arabinogalactan were negative.

Maintenance and preservation

Active liquid cultures of *Natronoflexus* can be kept viable at 4°C for up to 2 months. Long-term preservation by deep freezing at −80°C is possible with 15% glycerol as a cryoprotectant.

Taxonomy

Phylogenomic reconstructions based on 120 single-copy bacterial conserved protein markers (according to the GTDB taxonomy) confirmed the original placement of the genus *Natronoflexus* into the family *Marinilabiliaceae*, order *Bacteroidales*, class *Bacteroidia*, and phylum *Bacteroidota*. Moreover, another soda lake anaerobic xylanolytic bacterium, currently known as *Alkalitalea saponilacus* (Zhao and Chen, 2012), apparently belongs to the genus *Natronoflexus* and could be reclassified as its second species (Figure 2).

List of species of the genus *Natronoflexus*

Natronoflexus pectinivorans Sorokin et al. 2011, VL144

pec.ti.ni.vo'rans N.L. neut. n. *pectinum*, pectin; L. pres, part. *vorans*, devouring; N.L. part. adj. *pectinivorans*, pectin-devouring.

Cells are long, flexible rods, 0.3 × 3–10 µm, single or in bundles, and capable of gliding movement on solid surface. The cells contain pink carotenoids with absorption peaks in methanol–acetone extract at 468 (shoulder), 492 (main), and 523 nm. The identified polar lipids include phosphatidylcholine, phosphatidylethanolamine, and phosphatidylserine, and a range of unidentified phospho-, amino-, and aminophospholipids are also present. The predominant fatty acids in the membrane polar lipids are $C_{15:0}$, iso-$C_{15:0}$, and anteiso-$C_{15:0}$. It has a genetic potential to produce two organic osmolytes: *N*ε-acetyl-β-lysine and glycine betaine. It is a strictly anaerobic, fermentative, and saccharolytic bacterium, utilizing the following carbohydrates: D-galacturonic acid, D-glucuronic acid, dextrose, fructose, glucose, α,α-trehalose, α-methyl-glycoside, 2-deoxyglucose,

D-mannose, sucrose, D-maltose, D-cellobiose, D-glucosamine, *N*-acetyl glucosamine, galactose, and xylose. The utilized polymers include pectin, polygalacturonate, xylan, laminarin, arabinan, glucomannan, galactomannan, soluble starch, pullulan, and glycogen. Substrates not utilized: alginate, amorphous cellulose, arabinogalactan, arabinoxylan, agarose, arabinose, arabinitol, CMC, casein, dextrin, *meso*-erythritol, glycerol, *meso*-inositol, lactose, lichenan, β-mannan, melibiose, melezitose, D-raffinose, D-rhamnose, D-ribose, and L-sorbose. The products of galacturonic acid fermentation are acetate and succinate. Diazotrophic. Obligately alkaliphilic with a pH range for growth between 8.0 and 10.5, and an optimum at pH 9.5. Moderately salt tolerant with a range from 0.1 to 2.0 M Na^+ (optimum at 0.4–0.6 M). Mesophilic, with an optimum and maximum temperatures for growth at 30 and 41°C, respectively. Species description is based on a single isolate from anoxic soda lake sediments in Kulunda Steppe (Altai, Russia).

DNA G + C content (%): 39.6 (genome).

Type strain: AP1 (=DSM 24179=UNIQEM U807).

EMBL/GenBank accession number (16S rRNA gene): GQ 922844.

Genome assembly in GenBank: GCF_004345615.1.

Acknowledgment

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