

## ***Gordonia desulfuricans* sp. nov., a benzothiophene-desulphurizing actinomycete**

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**The taxonomic position of two actinomycetes isolated from soil was established using a polyphasic approach. The organisms, designated 213E<sup>T</sup> and 213F, were found to have chemical and morphological properties consistent with their assignment to the genus *Gordonia*. Nearly complete sequences of the 16S rDNA genes of the two strains were determined following the isolation and direct sequencing of the amplified genes. The tested strains were found to have identical 16S rDNA sequences and formed a phylogenetic line within the evolutionary radiation occupied by the genus *Gordonia* that was most closely related to *Gordonia rubropertincta* DSM 43197<sup>T</sup>. However, DNA–DNA relatedness data showed that strain 213E<sup>T</sup> and *Gordonia rubropertincta* DSM 43197<sup>T</sup> belonged to distinct genomic species. Strains 213E<sup>T</sup> and 213F also shared an identical phenotypic profile which distinguished them from representatives of validly described *Gordonia* species. The combined genotypic and phenotypic data show that strains 213E<sup>T</sup> and 213F merit recognition as a new species of *Gordonia*. The name proposed for the new species is *Gordonia desulfuricans*, for which the type strain is 213E<sup>T</sup> (= NCIMB 40816<sup>T</sup>).**

**Keywords:** *Gordonia*, polyphasic taxonomy, mycolic acids, benzothiophene, desulphurization

### **INTRODUCTION**

The application of the polyphasic taxonomic concept has led to far reaching changes in the taxonomy of the mycolata, that is, the mycolic acid containing actinomycetes (Chun *et al.*, 1996; Goodfellow *et al.*, 1998; Kämpfer *et al.*, 1999). These organisms are currently assigned to nine taxa, namely, the genera *Corynebacterium*, *Dietzia*, *Gordonia*, *Mycobacterium*, *Nocardia*, *Rhodococcus*, *Skermania*, *Tsukamurella* and *Williamsia*, members of which can be separated from one another using a combination of biochemical, chemical and morphological properties. Representatives of the genera *Gordonia*, *Nocardia*, *Rhodococcus* and *Tsukamurella* have also been distinguished from one another by two-dimensional gel electrophoresis of ribosomal protein AT-L30 (Ochi, 1992), by DNA

amplification and restriction endonuclease analysis (Steingrube *et al.*, 1997) and by taxon-specific 16S rDNA–RNA signature nucleotides (Stackebrandt *et al.*, 1997).

The genus *Gordonia* (formerly *Gordona*) was proposed by Tsukamura (1971), fell into abeyance but was re-established by Stackebrandt *et al.* (1988) for actinomycetes classified as *Rhodococcus bronchialis*, *Rhodococcus ruber* and *Rhodococcus terrae*. *Gordoniae* are aerobic, Gram-positive to Gram-variable, non-motile, catalase-positive actinomycetes which generally form short rods and cocci. They are usually partially acid–alcohol-fast. Rough brownish, pink or orange to red colonies are formed on glucose yeast extract and Sauton's agars. The organism has an oxidative carbohydrate metabolism, is arylsulphatase-negative, sensitive to lysozyme and forms mycobactins. The peptidoglycan is of the A1 $\gamma$  type, contains *meso*-diaminopimelic acid (*meso*-A<sub>2</sub>pm) as the diamino acid and muramic acid with *N*-glycolyl residues. The polysaccharide fraction of the wall is rich in arabinose

**Abbreviation:** *meso*-A<sub>2</sub>pm, *meso*-diaminopimelic acid.

The GenBank accession numbers for the 16S rDNA sequences of strains 213E<sup>T</sup> and 213F are AF101416 and AF101417, respectively.

and galactose. The wall envelope contains mycolic acids with 44–66 carbon atoms with up to four double bonds; fatty acid esters released on Py-GC of mycolic acids have 16–18 carbon atoms. Major proportions of straight-chain, saturated, monounsaturated and 10-methyl (tuberculostearic) cellular fatty acids are also formed. Cells contain diphosphatidylglycerol-, phosphatidylethanolamine- and phosphatidylinositol mannosides as major phospholipids and have dihydrogenated menaquinones with nine isoprene units as the predominant isoprenologue. The range of G + C content of the DNA is 63–69 mol%. *Gordoniae* have been isolated from a variety of habitats including activated sludge foam, biofilms, mangrove rhizosphere and sputa of patients with bronchiectasis and cavitary pulmonary tuberculosis. The type species is *Gordonia bronchialis*.

Nine validly described species are currently assigned to the genus *Gordonia*, viz. *Gordonia aichiensis* (Tsukamura 1982) Klatte *et al.* 1994, *Gordonia amarae* (Lechevalier and Lechevalier 1974) Klatte *et al.* 1994, *Gordonia bronchialis* (Tsukamura 1971) Stackebrandt *et al.* 1988, *Gordonia hirsuta* Klatte *et al.* 1996, *Gordonia hydrophobica* Bendinger *et al.* 1995, *Gordonia rubropertincta* (Hefferan 1904) Stackebrandt *et al.* 1988, *Gordonia rhizosphaera* Takeuchi and Hatano 1998, *Gordonia sputi* (Tsukamura 1978) Riegel *et al.* 1994 and *Gordonia terrae* (Tsukamura 1971) Stackebrandt *et al.* 1988, which form a distinct monophyletic clade within the evolutionary radiation occupied by the mycolata (Goodfellow *et al.*, 1998). Available DNA–DNA relatedness data support the taxonomic integrity of these species (Mordarski *et al.*, 1977, 1980; Zakrzewska-Czerwinska *et al.*, 1988; Klatte *et al.*, 1994; Riegel *et al.*, 1994; Takeuchi & Hatano, 1998). The improved classification provides a sound framework for the recognition of additional species of *Gordonia*.

The tested organisms, strains 213E<sup>T</sup> and 213F, were isolated in a screening program designed to yield micro-organisms capable of desulphurizing benzothiophene (Gilbert *et al.*, 1998). The aim of the present investigation was to clarify the relationships of these strains using the polyphasic taxonomic approach. It was evident from the resulting genotypic and phenotypic data that the organisms should be recognized as a new species of *Gordonia* for which the name *Gordonia desulfuricans* is proposed.

## METHODS

**Isolation of strains 213E<sup>T</sup> and 213F.** The tested strains were isolated by enrichment culture in glycerol *Rhodococcus* broth supplemented with benzothiophene, using as inoculum a soil sample collected in the vicinity of an oil shale spoil heap near a disused mine located at West Calder, West Lothian, Scotland, UK (Gilbert *et al.*, 1998).

**Maintenance and cultivation of organisms.** Strains 213E<sup>T</sup> (NCIMB 40816<sup>T</sup>) and 213F (NCIMB 40817) were maintained on modified Bennett's agar (Jones, 1949) or on yeast extract/malt extract/agar (ISP medium 2; Difco) plates at

30 °C and as cell suspensions in 20% (w/v) of glycerol at –20 °C. The tested strains were also examined together with the type strains of all of the validly described species of *Gordonia* for the phenotypic properties shown in Table 1. The cells for the chemical studies were washed in distilled water and freeze-dried; those needed for the molecular systematic investigations were washed in NaCl/EDTA buffer (0.1 M EDTA, pH 8.0, 0.1 M NaCl) and stored at –20 °C until needed.

**Phenotypic characterization.** The ability of the tested strains to use eight carbon compounds (Table 1) as sole sources of carbon for energy and growth was examined using Stevenson's basal medium (Stevenson, 1967). The organisms were also examined for their capacity to grow in the presence of 0.1% oleic acid and 0.001% zinc chloride using peptone/glucose/yeast extract agar (PGYEA; Bacto peptone, 5 g; glucose, 5 g; yeast extract, 5 g; agar, 15 g; distilled water, 1 l; pH 7.0) as the basal medium. The biochemical tests and the degradation of starch, tributyrin and Tween 80 were carried out following the procedures of Williams *et al.* (1983), and the degradation of hypoxanthine, tyrosine, xanthine and uric acid were examined on ISP 2 plates. All of the tests were read after incubation for 2 weeks at 30 °C. The colonial properties of strains 213E<sup>T</sup> and 213F were examined on PGYEA plates incubated at 30 °C. Smears from these plates were Gram-stained using Hucker's modification (Society for American Bacteriologists, 1957) and examined for micromorphological features. Smears were also stained using a modification of the Ziehl–Neelson method (Gordon, 1967).

**Chemotaxonomy.** The isomeric form of the diaminopimelic acid of the tested strains were determined by TLC of whole-organism hydrolysates (Staneck & Roberts, 1974). Standard procedures were also used for the extraction and analysis of menaquinones (Minnikin *et al.*, 1984) and mycolic acids (Minnikin *et al.*, 1980). Mass spectra of the purified mycolic esters were taken on an Autospec M instrument (Micromass, Warrington, UK) operating in electron impact mode with an ionizing voltage of 55 eV and a probe temperature of 150–300 °C.

**Small subunit rDNA sequencing.** Isolation of chromosomal DNA, PCR amplification and direct sequencing of purified PCR products were carried out as described previously (Kim *et al.*, 1998). The resultant 16S rDNA sequences of strains 213E<sup>T</sup> and 213F were aligned manually with the corresponding sequences of the type strains of the validly described *Gordonia* species, and representatives of other mycolata genera, retrieved from the Ribosomal Database Project (Maidak *et al.*, 1997) and EMBL/GenBank/DBJ databases by using the AL16S program of Chun (1995). The resultant dataset contained information on 1448 nucleotide positions for 19 *Gordoniae* and related mycolata genera. Evolutionary trees were inferred by using the least-squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1981), maximum-parsimony (Kluge & Farris, 1969) and neighbour-joining (Saitou & Nei, 1987) treeing algorithms. Evolutionary distance matrices for the least-squares and neighbour-joining methods were generated according to Jukes & Cantor (1969). The PHYLIP package (Felsenstein, 1993) was used for all of the analyses. The resultant unrooted tree topologies were evaluated by carrying out bootstrap analyses (Felsenstein, 1985) of the neighbour-joining method data based on 1000 resamplings using the SEQBOOT and CONSENSE programs in the PHYLIP package (Felsenstein, 1993). The root position of the unrooted tree based on the

neighbour-joining method was estimated by using four outgroup organisms (*Arthrobacter globiformis*, accession no. M23411; *Bacillus subtilis*, K00637; *Escherichia coli*, J01695; *Nocardia asteroides*, Z36934), as described by Swofford & Olsen (1990).

**DNA relatedness.** DNA–DNA hybridization was carried out by the identification service of DSMZ (Braunschweig, Germany). DNA was isolated by chromatography on hydroxyapatite (Cashion *et al.*, 1977) and hybridization was performed as described by De Ley *et al.* (1970) with modifications (Escara & Hutton, 1980; Huß *et al.*, 1983) using a Gilford System model 2600 spectrophotometer equipped with a Gilford model 2527-R thermoprogrammer and plotter. Renaturation rates were calculated using the TRANSFER.BAS program (Jahnke, 1992).

**DNA base composition.** Chromosomal DNA from the tested strains was digested, dephosphorylated and analysed by the HPLC method (Tamaoka, 1984), as described by Kim *et al.* (1998).

## RESULTS AND DISCUSSION

Strains 213E<sup>T</sup> and 213F have chemical and morphological properties which are consistent with their classification in the genus *Gordonia* (Stackebrandt *et al.*, 1988; Goodfellow *et al.*, 1998). The organisms are aerobic, Gram-positive, amycelial, non-motile, and form pink colonies on modified Bennett's agar. They contain meso-A<sub>2</sub> pm, arabinose and galactose in whole-organism hydrolysates (wall chemotype IV *sensu* Lechevalier & Lechevalier, 1970), predominant amounts of dihydrogenated menaquinones with nine isoprene units [MK-9(H<sub>2</sub>)], mycolic acids with 56–62 carbon atoms and have DNA with a G+C ratio of 64.1 ± 0.4 mol%. The strains have identical 16S rDNA nucleotide sequences which place them in the genus *Gordonia*.

Comparison of nearly complete 16S rDNA sequences (1448 nucleotides) of the tested strains with the corresponding sequences of representative mycolata strains shows that they form a monophyletic clade with *Gordonia rubropertincta* DSM 43197<sup>T</sup> in the least-squares, maximum-likelihood and neighbour-joining trees (Fig. 1). These relationships are also supported by the high nucleotide similarity values between the tested strains and *Gordonia rubropertincta* (98.7% similarity which corresponds to 19 nucleotide differences) and by the high bootstrap value (76%) obtained in the neighbour-joining analysis. However, strain 213E<sup>T</sup> and *Gordonia rubropertincta* DSM 43197<sup>T</sup> share a DNA–DNA relatedness value of 36.8% and hence belong to distinct genomic species (Wayne *et al.*, 1987). Strains 213E<sup>T</sup> and 213F also showed close relationships with *Gordonia bronchialis* DSM 43247<sup>T</sup> (98.3% similarity which corresponds to 25 nucleotide differences) and *Gordonia alkanivorans* (98.2%, which corresponds to 27 nucleotide differences). The mean 16S rDNA similarity value between the tested strains and members of the *Gordonia* clade was 97.6%.

16S rDNA nucleotide similarity values such as those

cited above have been reported for several validly described *Gordonia* species. *Gordonia bronchialis* DSM 43247<sup>T</sup> and *Gordonia terrae* DSM 43249<sup>T</sup>, for example, share a nucleotide similarity value of 98.3% which corresponds to 25 nucleotide differences; members of these species show DNA–DNA relatedness values within the range 16–21% (Zakrzewska-Czerwinska *et al.*, 1988). Similarly, *Gordonia aichiensis* DSM 43978<sup>T</sup> and *Gordonia sputi* DSM 43896<sup>T</sup> share a 16S rDNA nucleotide similarity value of 99.7% (which corresponds to four nucleotide differences) and DNA–DNA relatedness values within the range 38–40% (Goodfellow *et al.*, 1978; Klatte *et al.*, 1994).

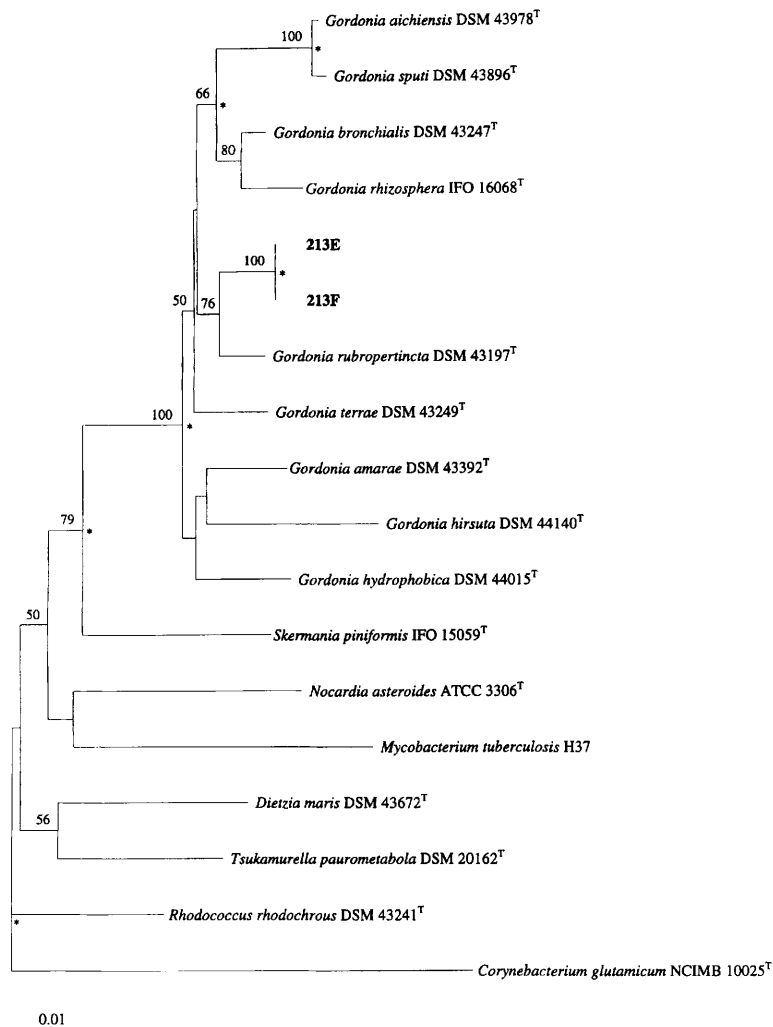
The tested organisms and the type strains of all of the validly described species of *Gordonia* were examined for a number of phenotypic properties found to be of differential value in an extensive numerical taxonomic study of mycolata strains (Toboli, 1995). Strains 213E<sup>T</sup> and 213F gave identical responses in the carbon utilization, degradation and tolerance tests (Table 1). It is also evident from this table that these strains can be distinguished from representatives of the validly described *Gordonia* species using a combination of phenotypic properties.

It is apparent from the genotypic and phenotypic data that strains 213E<sup>T</sup> and 213F form a distinct centre of taxonomic variation within the genus *Gordonia*. It is, therefore, proposed that these organisms be recognized as a new species. *Gordonia desulfuricans* is proposed for this taxon.

### Description of *Gordonia desulfuricans* sp. nov.

*Gordonia desulfuricans* (de.sul.fu'ri.cans. L. pref. de from; L. n. sulfur sulphur. M.L. part. adj. *desulfuricans* reducing sulphur compounds).

Aerobic, Gram-positive, slightly acid–alcohol-fast actinomycete which forms short rods and coccoid elements. Rough pinkish colonies are formed on modified Bennett's and peptone glucose yeast extract agars. Neither aerial hyphae nor diffusible pigments are produced. Adipic acid, D-arabinose, arbutin, glycerol, 1-propanol, sodium propionate and sodium salicylate are used as sole sources of carbon for energy and growth but not N-acetyl-D-glucosamine, betaine, D-cellobiose, sodium fumarate or sodium oxalate. Growth occurs in the presence of adenine (0.25%), oleic acid (0.8%) and zinc chloride (0.001%) but not in the presence of picric acid (0.3%). Cells contain major amounts of meso-A<sub>2</sub> pm, arabinose and galactose. The predominant menaquinone is MK-9(H<sub>2</sub>) though minor amounts of MK-8(H<sub>2</sub>) are present. The mycolic acids have 56–62 carbon atoms with up to three double bonds. The G+C content of the DNA is 64.1 mol%, as determined by the HPLC procedure. The organism was isolated from a soil sample collected in the vicinity of an oil shale spoil heap near a disused mine located at West Calder, West Lothian, Scotland, UK. The type strain is strain 213E<sup>T</sup> (NCIMB 40816<sup>T</sup>). Strain 213F



**Fig. 1.** Neighbour-joining tree (Saitou & Nei, 1987) based on nearly complete 16S rDNA sequences of gordoniae and related mycolic acid containing taxa. Asterisks indicate branches of the tree that were also formed using the least squares (Fitch & Margoliash, 1967), maximum-likelihood (Felsenstein, 1993), and maximum-parsimony (Kluge & Farris, 1969) treeing algorithms. The numbers at the nodes indicate the level of bootstrap support based on a neighbour-joining analysis of 1000 resampled datasets; only values more than 50% are given. The scale bar indicates 0.01 substitutions per nucleotide position. T, Type strain.

(NCIMB 40817) gave identical results to strain 213E<sup>T</sup> in both the genotypic and phenotypic tests.

### Significance of desulphurization in gordoniae

*Gordonia desulfuricans* strains 213E<sup>T</sup> and 213F, which were isolated for their ability to grow on benzo-thiophene as a sole source of sulphur, have a unique kind of sulphur-scavenging pathway whereby benzo-thiophene is desulphurized to 2-(2'-hydroxyphenyl)-ethan-1-al and an inorganic sulphur compound, probably sulphite (Gilbert *et al.*, 1998). This reaction corresponds to the dibenzothiophene desulphurization pathway first identified in *Rhodococcus* sp. strain IGTS8 (now classified as a strain of *Rhodococcus erythropolis*; M. Goodfellow and others, unpublished). Strain IGTS8 desulphurizes dibenzothiophene (DBT) to 2-hydroxybiphenyl and sulphite (Oldfield *et al.*, 1997), as do a large number of subsequent isolates, nearly all of which have been classified in the genus *Rhodococcus*. In both cases the organic product, which is basically the carbon skeleton of the original organo-

sulphur molecule, is not further metabolized (Kayser *et al.*, 1993; Oldfield *et al.*, 1997; Gilbert *et al.*, 1998).

Desulphurization pathways are of prime importance in the development of microbial fuel desulphurization technologies, as a cheap and environmentally friendly alternative to chemical processes. Strains such as 213E<sup>T</sup> and 213F may find particular application in the desulphurization of diesel fuel, which contains a high proportion of benzothiophenes (McFarland *et al.*, 1998).

The discovery of desulphurization pathways in gordoniae, together with a report of a *Gordonia* sp. with the DBT desulphurization phenotype (Rhee *et al.*, 1998), highlights the potential importance of gordoniae as a source of metabolic diversity. Much attention has been given to rhodococci in this context (see e.g. Warhurst & Fewson, 1994), but not to the closely related gordoniae or tsukamurellae. This is not surprising since, except in taxonomic terms, these genera remain little studied; nevertheless it now seems that members of these taxa will, like rhodococci, be found to have novel enzymic capabilities for the

**Table 1.** Phenotypic characteristics separating the test strains from other validly described *Gordonia* species

Strains: 1, 213E<sup>T</sup> and 213F; 2, *G. aichiensis* DSM 43978<sup>T</sup>; 3, *G. amarae* DSM 43392<sup>T</sup>; 4, *G. bronchialis* DSM 43247<sup>T</sup>; 5, *G. hirsuta* DSM 44140<sup>T</sup>; 6, *G. hydrophobica* DSM 44015<sup>T</sup>; 7, *G. rhizosphaera* IFO 16068<sup>T</sup>; 8, *G. rubropertincta* DSM 43197<sup>T</sup>; 9, *G. sputi* DSM 43896<sup>T</sup>; 10, *G. terrae* DSM 43249<sup>T</sup>

Character	Strain:									
	1	2	3	4	5	6	7	8	9	10
Colony colour	Pink	Pink/orange	Tan/white	Brown	White/light yellow	Tan/white	Pink/orange	Orange/red	Pink	Pink/orange
Biochemical tests:										
Aesculin hydrolysis	-	-	+	-	-	+	-	-	+	+
Allantoin hydrolysis	-	-	+	-	-	+	-	-	-	-
Arbutin hydrolysis	-	+	+	-	-	+	-	+	+	+
Nitrate reduction	+	+	-	+	+	+	-	-	+	+
Urea hydrolysis	+	+	+	+	-	-	-	+	+	+
Decomposition of (% w/v):										
Hypoxanthine (0.4)	+	-	+	-	-	+	-	+	+	+
Starch (1)	+	-	+	+	+	-	+	+	+	+
Tributyrin (0.1)	-	-	-	-	-	-	-	+	-	-
Tween 80 (1)	+	+	-	-	+	+	-	-	-	-
Tyrosine (0.5)	-	-	+	-	-	+	-	-	-	-
Uric acid (0.5)	+	+	+	+	-	+	+	+	+	+
Xanthine (0.4)	-	-	-	-	-	-	-	-	+	+
Growth on sole carbon sources (% w/v):										
Arbutin (1)	+	-	+	-	+	+	+	-	-	-
D-Cellobiose (1)	-	-	-	-	+	-	+	-	-	-
Glycerol (1)	+	+	+	+	+	+	-	+	+	+
N-Acetyl-D-glucosamine (0.1)	-	-	+	-	+	+	+	+	-	-
Adipic acid (0.1)	+	+	-	-	-	+	+	-	+	-
Betaine (0.1)	-	-	-	-	-	-	+	-	+	+
Oxalic acid (0.1)	-	-	-	-	-	-	-	-	+	+
Propan-1-ol (0.1)	+	-	+	-	-	+	+	-	-	+
Sodium fumarate (0.1)	-	-	-	+	-	-	+	+	-	+
Growth in the presence of (% v/v):										
Oleic acid (0.1)	+	+	+	+	+	+	-	+	+	+
Zinc chloride (0.001)	+	+	+	+	+	+	-	+	+	+
Mycolic acids* (no. carbons)	56-62	60-64	48-54	60-64	60-64	56-61	60-62	54-62	60-64	54-62
G + C content (mol%)*	64	65	60-66	63-65	69	69	66-8	67-69	65	64-69

\* Data taken from previous studies (Bendinger *et al.*, 1995; Klatte *et al.*, 1994, 1996; Riegel *et al.*, 1994; Stackebrandt *et al.*, 1988; Takeuchi & Hatano, 1998).

transformation and degradation of diverse classes of substrate.

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