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Physiological and genomic analyses of cobalamin (vitamin B_{12})-auxotrophy of *Lysobacter auxotrophicus* sp. nov., a methionine-auxotrophic chitinolytic bacterium isolated from chitin-treated soil

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Abstract

A novel bacterium, designated 5-21a^T, isolated from chitin-treated upland soil, exhibits methionine (Met) auxotrophy and chitinolytic activity. A physiological experiment revealed the cobalamin (synonym, vitamin B_{12})(Cbl)-auxotrophic property of strain 5-21a^T. The newly determined complete genomic sequence indicated that strain 5-21a^T possesses only the putative gene for Cbl-dependent Met synthase (MetH) and lacks that for the Cbl-independent one (MetE), which implies the requirement of Cbl for Met-synthesis in strain 5-21a^T. The set of genes for the upstream (corrin ring synthesis) pathway of Cbl synthesis is absent in the genome of strain 5-21a^T, which explains the Cbl-auxotrophy of 5-21a^T. This strain was characterized via a polyphasic approach to determine its taxonomic position. The nucleotide sequences of two copies of the 16S rRNA gene of strain 5-21a^T indicated the highest similarities to *Lysobacter soli* DCY21^T(99.8 and 99.9%) and *Lysobacter panacisoli* CJ29^T(98.7 and 98.8%, respectively), whose Cbl-auxotrophic properties were revealed in this study. The principal respiratory quinone was Q-8. The predominant cellular fatty acids were iso-$C_{15:0}$, iso-$C_{16:0}$ and iso-$C_{17:1}\omega 9c$. The complete genome sequence of strain 5-21a^T revealed that the genome size was 4155451 bp long and the G+C content was 67.87 mol%. The average nucleotide identity and digital DNA–DNA hybridization values between strain 5-21a^T and its most closely phylogenetic relative *L. soli* DCY21^T were 88.8 and 36.5%, respectively. Based on genomic, chemotaxonomic, phenotypic and phylogenetic data, strain 5-21a^T represents a novel species in the genus *Lysobacter*, for which the name *Lyobacter auxotrophicus* sp. nov. is proposed. The type strain is 5-21a^T (=NBRC 115507^T=LMG 32660^T).

INTRODUCTION

The species classified in the genus *Lysobacter* are Gram-negative rods, habiting soil and freshwater, and degrade chitin (polymer of *N*-acetylglucosamine) and often other polysaccharides [1]. This organism is strongly proteolytic, characteristically lysing a variety of micro-organisms (both Gram-negative and Gram-positive bacteria, including actinomycetes, blue-green and green algae, yeasts, and filamentous fungi), as well as nematodes [1]. Members of the genus *Lysobacter* have been reviewed as a source of bioactive natural products including antibiotics [2, 3] as well as a plant disease biocontrol agent [4].

In our past studies on the degradation process of chitin in incubated upland soil, 162 bacterial strains were randomly isolated from chitin-treated incubated upland soil using a non-selective agar medium [5, 6]. Seven strains among them were identified to phylogenetically belong to the genus *Lysobacter* and demonstrated chitinolytic activity [6]. Of them, six strains were found to be

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Keywords: *Lysobacter*; methionine; vitamin B_{12}; cobalamin; auxotroph.
Abbreviations: ANI, average nucleotide identity; Cbl, cobalamin; CDS, protein coding sequence; Cya, cyanocobalamin; dDDH, digital DNA–DNA hybridization; LB, Luria-Bertani; Met, methionine; R2A, Reasoner's 2A; TYGS, Type (Strain) Genome Server; UBCG, up-to-date bacterial core gene.
The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and the complete genome sequence of the type strain 5-21a^T are LC481367 and AP027041, respectively. The raw read sequences have been deposited in the DDBJ Sequence Read Archive under the accession numbers DRR415639 (Illumina MiSeq) and DRR415640 (PacBio RS II).
Three supplementary tables are available with the online version of this article.

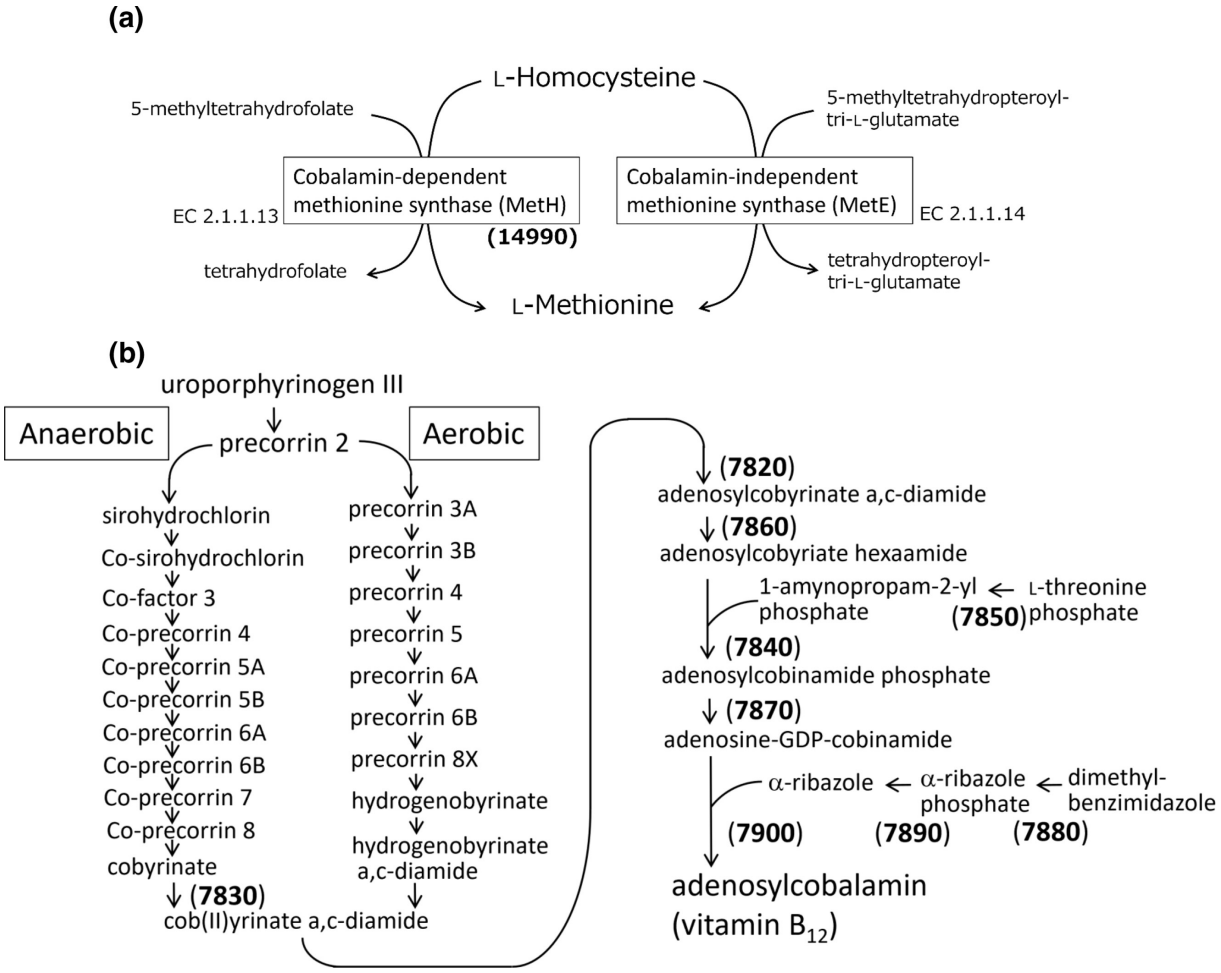

Fig. 1. (a) The reaction of L-homocysteine conversion to L-methionine and the mediating enzymes: Cbl-dependent Met synthase MetH (EC 2.1.1.13) and Cbl-independent one MetE (EC 2.1.1.14). The locus tag number 14990 in parentheses indicates the CDS (LA521A14990) encoding putative MetH on the genome of strain 5-21a^T. No putative CDS for MetE was detected in the genome of strain 5-21a^T. (b) The pathway for synthesizing adenosylcobalamin (vitamin B_{12}) from uroporphyrinogen III. The locus tag numbers for the putative enzymes involved in the biosynthesis of adenosylcobalamin in strain 5-21a^T are indicated in parentheses without the locus tag prefix LA521A for the corresponding enzymatic reactions. If no parenthesized number is indicated, no CDS for the enzyme was predicted on the genome of strain 5-21a^T.

closely related to *Lysobacter panacisoli* [7] and *Lysobacter soli* [8] and exhibited methionine (Met) auxotrophy [6]. In strain 5-21a^T, one of the Met-auxotrophic *Lysobacter* isolates, the Met-auxotrophy was not rescued by homocysteine, which is the precursor of Met in Met biosynthesis. Therefore, we previously assumed that strain 5-21a^T might lack Met synthase that catalyses the reaction synthesizing Met from homocysteine [6].

Nature has evolved two routes for the *de novo* biosynthesis of methionine from homocysteine [9]. One involves the cobalamin (Cbl)-independent methionine synthase (EC 2.1.1.14; MetE) and the other the Cbl-dependent enzyme of the same name (EC 2.1.1.13; MetH) (Fig. 1a) [9]. In most bacteria, the genes *metE* and *metH* coexist; however, several bacteria possess only one of these two genes [10]. In *Myxococcus xanthus* and algae, Cbl-auxotrophy has been reported to be stimulated by the addition of Met because MetH requires Cbl as a cofactor [11–13]. On the genome of *Sphingomonas haloaromaticamans*, which is a Met-auxotrophic bacterial strain, there exists only the putative gene for MetH, but none for MetE [14]. We, therefore, assumed that the Met-auxotrophic property of 5-21a^T may be attributable to the absence of the gene for MetE, implying Cbl-dependence of Met-synthesis in strain 5-21a^T.

In this study, we conducted physiological experiments and determined the complete genome sequence of strain 5-21a^T in order to investigate the physiological and genetic bases of the Met-auxotrophy of this strain. A physiological experiment indicated the Cbl-auxotrophy of strain 5-21a^T. The genome sequence demonstrated that strain 5-21a^T lacks the genes for MetE but has genes for MetH. Moreover, the genes for *de novo* synthesis of Cbl were absent from the genome. Furthermore, the genome sequence

and taxonomic characterization of strain 5-21a^T support our proposal that stain 5-21a^T should be considered to represent a new species of the genus *Lysobacter*.

METHODS

Evaluating auxotrophy in *Lysobacter* strains

Each of the *Lysobacter* strains pregrown on Reasoner's 2A (R2A) agar medium (Nissui) at 30 °C for 3 days was inoculated into 5 ml R2A broth (Nihon Pharmaceutical) in a fraction tube and cultivated for 30 °C overnight with shaking at 150 r.p.m. The cells were then harvested via centrifugation, washed with sterilized MilliQ water, and suspended in 1 ml of sterilized MilliQ water. Ten microlitres of the suspension were inoculated to 5 ml liquid minimal medium (MM) [10 mM K_2HPO_4, 10 mM KH_2PO_4, 1 mM $CaCl_2$, 0.5 mM $MgCl_2$ supplemented with 0.1% (v/v) trace element solution] [15] containing 0.1% (w/w) glucose as the carbon source. L-Met or cyanocobalamin (Fujifilm Wako Pure Chemicals) was supplemented to the liquid MM to evaluate the suspected auxotrophy. Cultivation was performed at 30 °C with shaking at 150 r.p.m., and growth was monitored by assessing turbidity using the McFarland Densitometer DEN-1B (WakenBtech). The reference type strains of the genus *Lysobacter* (*Lysobacter soli* LMG 24126^T, *Lysobacter panacisoli* JCM 19212^T, '*Lysobacter arenosi* JCM 34257^T', *Lysobacter niastensis* NBRC 106399^T and *Lysobacter prati* KCTC 72062^T) [7, 8, 16–18] were supplied by the LMG Bacteria Collection (BCCM/LMG, Belgium), the Japan Collection of Microorganisms (JCM; RIKEN, Japan), the Biological Resource Center, NITE (NBRC, Japan) or the Korean Collection for Type Cultures (KCTC, Republic of Korea).

Determination of the complete genome sequence

The genomic DNA of the bacterial strain 5-21a^T was prepared as per the procedure for DNA preparation for DNA–DNA hybridization [19]. Accordingly, the cells harvested from a 10 ml culture in a Luria-Bertani (LB) liquid medium were lysed by treatment with lysozyme and sodium-dodecyl sulphate. The genomic DNA was purified with cetyl trimethyl ammonium bromide and phenol-chloroform. The complete genome sequence of strain 5-21a^T was determined by using the combination of PacBio long reads and Illumina short reads. A PacBio 20 kb library was prepared using the SMRTbell Template Prep Kit and sequenced on the PacBio RS II instrument (Pacific Biosciences) at Macrogen, Inc. (Seoul, Republic of Korea). An Illumina library was constructed by using the TruSeq DNA PCR-free Library Prep Kit and sequenced on the Illumina MiSeq platform (301 bp paired-end sequencing). Information on the PacBio and Illumina reads used in this study are summarized in Table S1, available in the online version of this article. PacBio subreads were filtered (length, ≥5000 bp; read quality, ≥0.8) using the BamTools version 2.5.1 [20], and the long and high-quality reads were assembled using the Flye version 2.8.3 [21]. The resulting single contig was manually rotated with the Geneious Prime 2022 [22] to place the *dnaA* gene at the first position of the circular chromosome sequence. The PacBio subreads ≥5000 bp were aligned to the circularized sequence using pbalign version 0.3.1, and the polishing was performed with Arrow version 2.2.2 (SMRT Tools Reference Guide, www.pacb.com/wp-content/uploads/SMRT-Tools-Reference-Guide-v8.0.pdf). Illumina reads were cleaned up by trimming the adapter sequences and low-quality ends (quality score ≥15; read length ≥150 bp) with Trimmomatic version 0.38 [23]. The high-quality reads were aligned to the polished chromosome sequence using BWA-MEM version 0.7.17 [24], and the assembly errors were corrected using Pilon version 1.23 [25]. Gene prediction and annotation were performed with DFAST-core version 1.2.18 [26] by running GeneMarkS2 version 1.14_1.25 [27] and MetaGeneAnnotator version 2008/08/19 [28], RNAmmer version 1.2 [29] and tRNAscan-SE version 2.0.5 [30] to predict protein-coding sequences (CDSs), rRNA and tRNA genes, respectively. Average nucleotide identity (ANI) analysis [31] of the genome sequence of strain 5-21a^T was performed using calcANI.pl (https://github.com/Computational-conSequences/SequenceTools) with the ANIb method against 109 genome sequences of the genus *Lysobacter* in the NCBI RefSeq database (last accessed on 27 October 2022). The genome sequence of strain 5-21a^T was also analysed using the Type (Strain) Genome Server (TYGS) [32] for genome-based taxonomic classification of this strain.

Presence of *metE* and *metH* on the chromosomes

To investigate the distribution of the genes for Cbl-independent Met synthase (*metE*) and Cbl-dependent one (*metH*) among *Lysobacter* species, these genes were searched on the chromosomes of the 22 type strains of *Lysobacter*, in RefSeq (NCBI reference sequence database; www.ncbi.nlm.nih.gov/refseq/). The genes were searched by keywords 'metE' and '2.1.1.14' for *metE*, and 'metH' and '2.1.1.13' for *metH*.

Reconstruction of the phylogenetic tree

The nucleotide sequences of 16S rRNA genes were aligned by MUSCLE [33] using the default parameters of the MEGA11 software [34]. Then, phylogenetic trees were reconstructed with the neighbour-joining method by using MEGA11 software. To evaluate tree topologies, bootstrap values were calculated with 1000 replicates. For a further analysis of the phylogenetic relationships of 5–21 a in the genus *Lysobacter*, a phylogenetic tree based on the 92 core genes was reconstructed using an up-to-date bacterial core gene (UBCG) pipeline with a default parameter [35].

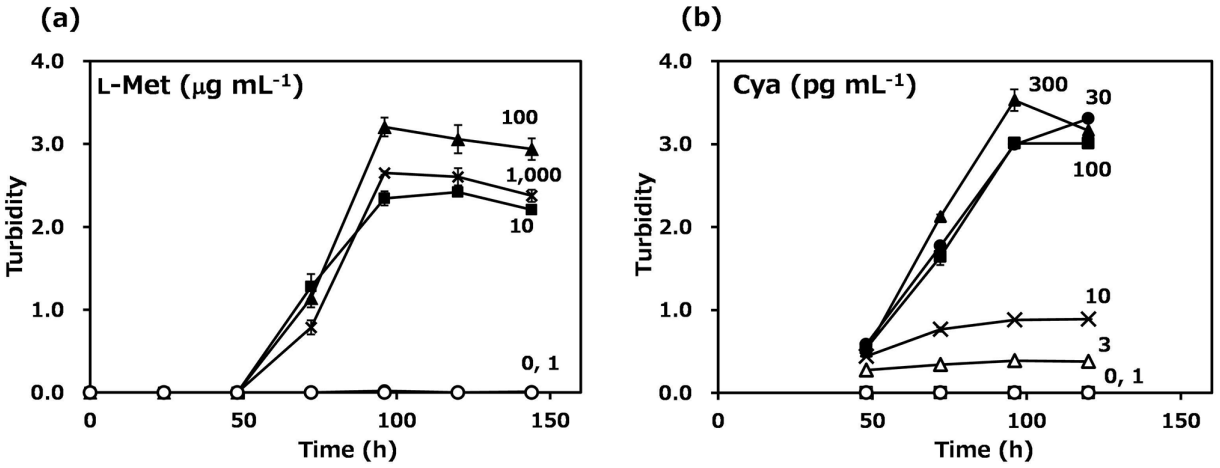

Fig. 2. Growth of strain 5-21a^T in the presence or absence of L-methionine (a) or cyanocobalamin (b) in an MM containing 1 mg ml^{-1} glucose as the carbon source. (a) The cells of strain 5-21a^T were inoculated in the liquid MM containing 0 (open circles), 1.0 (closed circles), 10 (closed squares), 100 (closed triangles), or 1000 (crosses) μg/ml L-methionine (L-Met). (b) The cells of strain 5-21a^T were inoculated into the liquid MM containing 0 (open circles), 1.0 (open squares), 3 (open triangles), 10 (crosses), 30 (closed circles), 100 (closed squares), or 300 (closed triangles) pg ml^{-1} cyanocobalamin (Cya). The bacterium was grown at 30 °C with shaking at 150 r.p.m. Growth was evaluated based on the extent of turbidity.

Physiological tests and chemical analyses for taxonomic characterizations

Gram-staining was performed with Favor G 'Nissui' as per the manufacturer's instruction (Nissui Pharmaceutical). Cell morphology and motility were observed under the Olympus Light Microscope BX50F4, with cells grown on R2A agar for 72 h at 30 °C. Catalase and oxidase activities were determined following the method described by Barrow and Feltham [36]. Growth at pH 4.0–11.0 was assessed after 5 days of incubation at 30 °C on LB medium (Merck) supplemented with agar (Fujifilm Wako Pure Chemicals). Growth at 25, 30, 33, 37 or 42 °C was tested on the LB agar medium (pH 7.0) after 5 days of cultivation. Some enzyme activities and carbon-utilization abilities were tested by using API ZYM, API 50CHB and API 20NE kits according to the instructions of the manufacturer (bioMérieux). The dry cells for isoprenoid quinone and fatty acid analyses were prepared via lyophilization of the cells using a freeze drier FDU-12AS (AS ONE) after harvesting the cells by centrifugation from R2A liquid culture (overnight with shaking at 30 °C). Isoprenoid quinones were extracted from 200 mg dry cells using a chloroform–methanol solution (2:1, v/v). The ubiquinone fraction was separated by TLC using hexane–diethyl ether (8.5:1.5, v/v) as the solvent. The ubiquinone spot was detected under UV light, and the ubiquinone was extracted with acetone, dried using a nitrogen stream, and then analysed using the LCMS-8030 and LC-20AD (Shimadzu) equipped with a Shim-Pack FC-ODS column (150×2.0 mm i.d, Shimadzu). Methanol–isopropanol was used as the mobile phase (25% isopropanol, 60 min) at the flow rate of 0.2 ml min^{-1} with UV detection at 275 nm. The preparation and analysis of cellular fatty acid methyl esters were performed using the protocol of the MIDI Sherlock Microbial Identification System [37] and a gas chromatograph 6890 N (Agilent Technologies) with Sherlock MIDI software (version 6.2) and the TSBA6 database (version 6.2).

RESULTS AND DISCUSSION

Effect of the dose of Met on the growth of strain 5-21a^T

The Met auxotrophy and the dose effect of L-Met on the growth of strain 5-21a^T were investigated in a liquid MM containing 1 mg ml^{-1} glucose as the carbon source. Strain 5-21a^T exhibited significant growth in the presence of 10–1000 μg ml^{-1} L-Met, whereas no growth was observed in the absence or presence of 1 μg ml^{-1} L-Met (Fig. 2a). Thus, the L-Met auxotrophy of strain 5-21a^T was confirmed in the liquid medium as was previously indicated on an agar medium [6]. L-Met (1 μg ml^{-1}) was possibly very low to support adequate growth, as detected based on turbidity, or may be very low to be transported to the cytoplasm.

Cbl-auxotrophy of strain 5-21a^T and its relate strains

The Cbl-auxotrophy of strain 5-21a^T and the dose effect of Cbl on the growth of strain 5-21a^T were investigated in a liquid MM containing 1 mg ml^{-1} glucose as the carbon source. Cyanocobalamin (Cya) was supplemented as Cbl. Strain 5-21a^T indicated significant growth in the presence of 3–300 pg ml^{-1} Cya, and the similar growth rate and density seemed saturated in the presence of 30–300 pg ml^{-1} Cya (Fig. 2b). No growth was recorded in the absence or presence of 1 pg ml^{-1} Cya (Fig. 2b). The concentration of Cya (1 pg ml^{-1}) may be extremely low to support growth, as detected by turbidity, or to be transported to the cells. The results

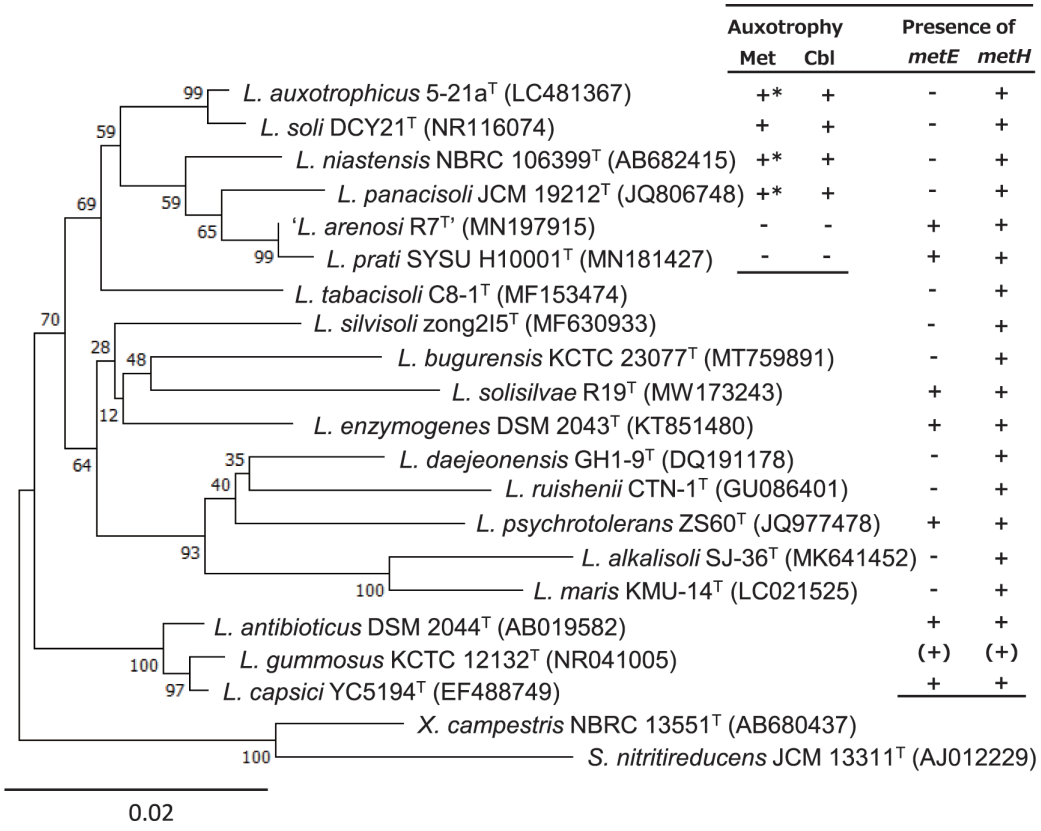

Fig. 3. Phylogenetic position of strain 5-21a^T. The nucleotide sequence of the 16S rRNA gene of the strain was aligned with those of the 11 neighbouring type strains of the known *Lysobacter* species and those of *Xanthomonas campestris* NBRC 13551 and *Stenotrophomonas nitritireducens* JCM 13311 as outgroups. The phylogenetic relationships were calculated by the neighbor-joining method. The bar with the number 0.02 indicates the number of substitutions per site. The number at each node shows the result of 1000 bootstrap analyses in percent (%). Numbers in parentheses are the accession numbers of the nucleotide sequence. '+' and '–', respectively, indicate the Met and Cbl auxotrophy or the presence and absence of *metE* and *metH* on the genome. '(+)' shows the data from the complete genome sequence of *L. gummosus* strain 3-2-11 (accession number CP011131). '*' indicates the data cited from our previous study [6].

indicated that Cya rescued the L-Met auxotrophy of strain 5-21a^T and supports the assumption that strain 5-21a^T contains the gene for Cbl-dependent Met synthase (MetH) but not that for Cbl-independent one (MetE).

We previously reported that among the five type strains of *Lysobacter* tested, *L. panacisoli* JCM 19212^T and *L. niastensis* NBRC 106399^T, which are closely related to strain 5-21a^T (Fig. 3), exhibited Met-auxotrophy, while the three other type strains (*L. rhizospherae* JCM 30321^T, *L. koreensis* NBRC 101156^T and *L. concretionis* NBRC 102010^T) did not exhibit auxotrophic properties [6]. In this study, we tested the Met auxotrophy of additional three type strains to which strain 5-21a^T was phylogenetically closely related: *L. soli* LMG 24126^T, *L. prati* KCTC 72060^T and '*L. arenosi* JCM 34257^T' (Fig. 3). *L. soli* did not grow in a liquid MM supplemented with glucose as the sole carbon source but the growth was rescued by supplementation with L-Met (data not shown), indicating the L-Met auxotrophy (Fig. 3). In contrast, *L. prati* and '*L. arenosi*' grew in the liquid minimal medium with glucose regardless the supplementation with L-Met (data not shown), implying that they are not auxotrophs (Fig. 3).

Like strain 5-21a^T, the Met-auxotrophic type strains *L. soli*, *L. panacisoli* and *L. niastensis* exhibited Cbl-auxotrophy, whereas '*L. arenosi*' and *L. prati* did not (Fig. 3). The Met-auxotrophic *Lysobacter* isolates (strains 45-18, 45-27, 45-28, 45-29 and 45-65), which were isolated from chitin-added upland soil together with strain 5-21a^T [6], belong to the same phylogenetic clade as *L. soli* or *L. panacisoli* and *L. niastensis*, indicating Cbl-auxotrophy (data not shown). Therefore, it can be concluded that not only strain 5-21a^T but also the three *Lysobacter*-type strains and the five *Lysobacter* isolates are Cbl auxotrophs, except for the type strains of '*L. arenosi*' and *L. prati*. The data indicated that the Cbl-auxotrophic property is not unique for strain 5-21a^T, rather it is shared with other members of the genus *Lysobacter*. Although '*L. arenosi*' and *L. prati* grew in the MM in the absence of Met or Cya, the growth was slower than that in the presence of Met or Cya (data not shown). The addition of Met, which is the most expensive amino acid in terms of consumed mol of ATP per molecule produced in *Escherichia coli* [38], could provide an energetic

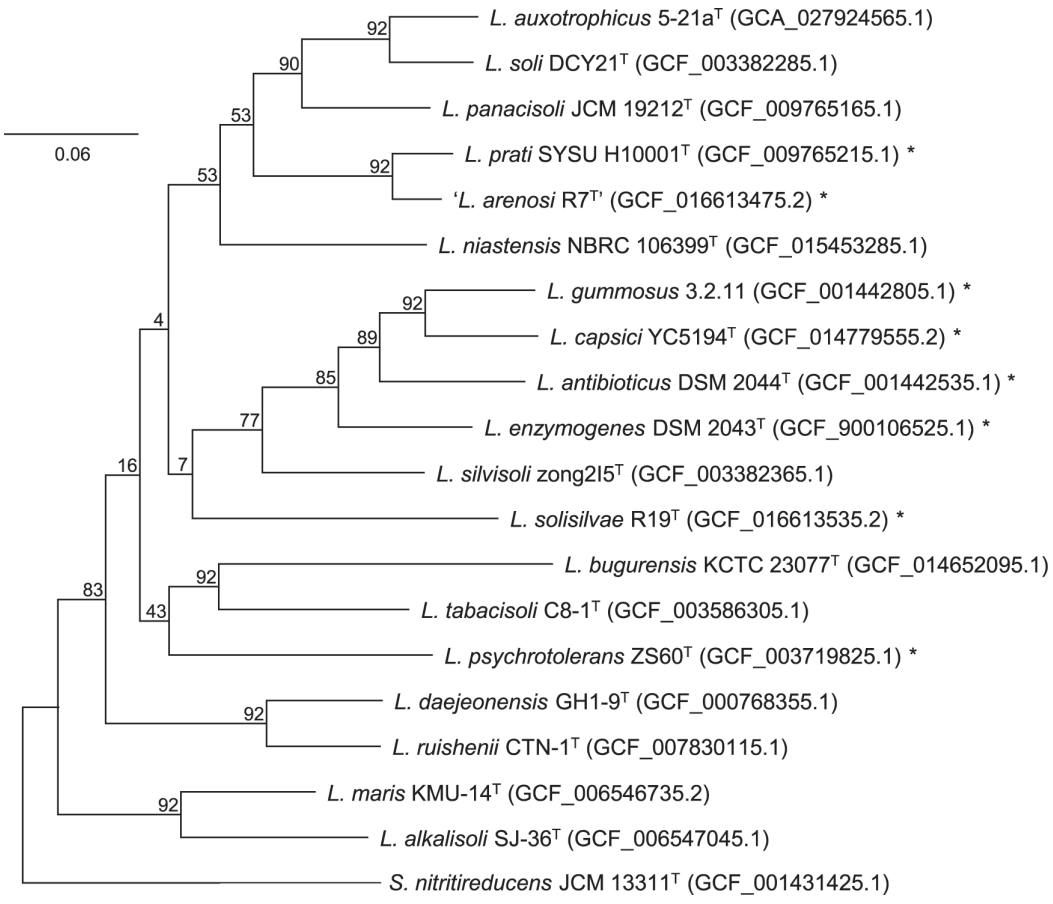

Fig. 4. The UBCG tree of strain 5-21a^T and closely related *Lysobacter* species. The numbers presented on the nodes represent the gene support index, which is the number of single gene trees supporting the branch. *Stenotrophomonas nitritireducens* JCM 13311 was used as an outgroup. Bar, 0.06 substitution per nucleotide. Numbers in parentheses are the accession numbers of the nucleotide sequence. '*' indicates *metE*-possessing strains shown in Fig. 3.

advantage in terms of the growth. Cya acted as the cofactor of Cbl-dependent enzymes other than MetH and can stimulate the growth of those type strains.

Complete genome sequence of strain 5-21a^T

The complete genome sequence of strain 5-21a^T consisted of a circular chromosome of 4 155 451 bp with a G+C content of 67.87 mol%. The genome contains 3728 protein-coding sequences, two sets of rRNA genes and 50 tRNA genes. The nucleotide sequences of the two copies of the 16S rRNA gene demonstrated the highest identity levels of 99.87% (1491/1493) and 99.80% (1490/1493), respectively, with that of *L. soli* DCY21^T (=KCTC 22011^T=LMG 24126^T; accession number NR116074), 98.78% (1295/1311) and 98.70% (1294/1311) with *L. panacisoli* CJ29^T (=JCM 19212^T; JQ806748) and 97.48% (1433/1470) and 97.42% (1432/1470) with *L. niastensis* NBRC106399^T (AB682415). The genome sequence of strain 5-21a^T exhibited the highest ANI value with that of the type strain *L. soli* DCY21^T (88.8%, GCF_003382285.1), followed by *L. soli* XL170 (88.7%, GCF_009755585.1) (Table S2). As of 10 November 2022, taxonomic analysis by TYGS displayed a digital DNA–DNA hybridization (dDDH) value (formula d4, a.k.a. GGDC formula 2) of 36.5% with the type strain *Lysobacter* soli KCTC 22011^T, suggesting a potential new species as a conclusion. These results suggest that stain 5-21a^T represents a new species of the genus *Lysobacter*.

In the genome of strain 5-21a^T, there is one putative CDS for MetH (LA521A14990), while there is no CDS encoding MetE. This genome information can account for the no growth of 5-21a^T in the MM without Met or Cbl (Fig. 2). It can thus be assumed that the strain 5-21a^T cannot synthesize Met without the presence of Cbl because of the lack of genes for MetE.

The nucleotide sequence of the genome of strain 5-21a^T also suggested that the known pathway for *de novo* Cbl synthesis is not present. The biosynthesis of adenosylcobalamin from uroporphyrinogen III requires >30 enzymatic steps via two distinct routes: oxygen-dependent (aerobic) pathway and oxygen-independent (anaerobic) pathway (Fig. 1b) [39, 40]. The 5-21a^T genome

Table 1. Characteristics that differentiate strain 5-21a^T from closely related type strains of the genus *Lysobacter*

Strains: 1, 5-21a^T; 2, *L. soli* DCY21^T; 3, *L. panacisoli* CJ29^T; 4, *L. niastensis* GH41-7^T; 5, '*L. arenosi*' R7^T; 6, *L. prati* SYSU H10001^T. +, Positive; –, negative; w, weakly positive; NA, data not available from the references.

Characteristics	1[†]	2[a]	3[b]	4[c]	5[d]	6[e]
Cell length (μm)	1.0–3.0	0.6–0.9	1.0–2.0	2.0–4.0	1.0–1.2	NA
Cell width (μm)	0.3–0.5	0.2–0.5	0.4–0.45	0.5–0.6	0.35–0.4	NA
Colony colour	Yellow	Yellow	Bright yellow	Light beige	Pale yellow	Yellow
Growth on LB at:						
pH 9.5	+	+*	+*	w*	+*	–*
pH 10	+	+*	w*	–*	+*	–*
pH 11	w	w*	–*	–*	w*	–*
37 °C	+	+*	+*	–*	+*	+*
42 °C	+	+*	–*	–*	–*	–*
Nitrate reduction	+	+	–	+	+	+
Enzyme activity:						
N-Acetyl-β-glucosaminidase	–	+	–	–[b]	–	–
Arginine hydrolase	–	+[b]	–	–[b]	+	NA
Galactosidase	–	–	+	+[b]	–	–
Trypsin	+	–	+	–[b]	+	–
Urease	–	+[b]	–	–[b]	–	–
Valine arylamidase	+	+	–	–[b]	+	+
Acid production from:						
N-Acetylglucosamine	–	+*	–*	–*	–*	–*
L-Arabinose	+	+*	–*	–*	–*	–*
Fructose	–	–*	–*	–*	–*	–*
Fucose	+	+*	–*	–*	–*	–*
Glucose	–	w*	–*	–*	–*	–*
Lyxose	–	+*	–*	–*	–*	–*
Mannose	–	w*	–*	–*	–*	–*
Trehalose	–	+*	–*	–*	–*	–*
Turanose	–	+*	–*	–*	–*	–*
Xylose	–	+*	–*	–*	–*	–*
Assimilation test:						
Glucose	–	+	–	w	+	+
Mannose	–	+	+	–	+	–

*Data confirmed or obtained in this study.
[a, b, c, d] and [e] indicate data cited from references [7, 8, 16, 18], and [17], respectively, unless otherwise stated.

included only one (LA521A07830) of the genes for corrin ring synthesis from uroporphyrinogen III to cob(II)yrinic acid a,c-diamide (Fig. 1b). On the other hand, the genome of strain 5-21a^T contained genes for the final synthesis and repair of Cbl (LA521A07820, 7840, 7850, 7860, 7870, 7880, 7890 and 7900) (Fig. 1b). The data imply that strain 5-21a^T can synthesize Cbl from inactive cobalamides. There are also some CDSs that are presumed to encode subunits of an ABC transporter: 'TonB-dependent

vitamin B_{12} receptor' (LA521A07790 and 33820), which may act as an outer membrane protein; 'cobalamin-binding protein' (LA521A29160) which encodes a putative periplasmic solute-binding protein; 'cobalamin ABC transporter' (LA521A07810), which may encode a transmembrane protein. Some of these CDSs (LA521A07990 and 7810) for the ABC transporter for Cbl form a cluster with those for the final synthesis and repair of Cbl (LA521A07820–7900) on the genome of strain 5-21a^T.

On the genome of strain 5-21a^T, there are some CDSs that may encode Cbl-dependent enzymes other than that for MetH. The genome contains CDSs for 'methylmalonyl-CoA mutase family protein' (LA521A22980) and 'tRNA epoxyqueuosine(34) reductase QueG' (LA521A13090). As 5-21a^T grows in an MM supplemented with Met [6] (Fig. 2a), biochemical reactions catalysed by these possible Cbl-dependent enzymes are assumed to be not essential for the growth of strain 5-21a^T. Alternatively, these reactions might be bypassed or mediated by Cbl-independent enzymes in the absence of Cbl.

Distribution of *metE* and *metH* among *Lysobacter* strains

Shelton *et al.* [10] found that the occurrence of MetH is more common than MetE, but most (>40%) bacteria have both enzymes. About 30% of 44 802 publicly available bacterial genomes have only CDSs for MetH, but not for MetE [10]. To investigate the distribution of the genes for MetE and MetH in *Lysobacter*, we searched the corresponding genes on the complete sequences of the genomes of 18 *Lysobacter*-type strains. Similar to entire bacteria, the *metH* gene is more widely distributed among the *Lysobacter* type strains than *metE*: all of the 18 type strains possess *metH*, whereas eight species among the 18 have *metE* (Fig. 3). Five among the eight *metE*-possessing species are included in the two clades (one formed by '*L. arenosi*' and *L. prati* and the other with *L. antibioticus*, *L. gummosus* and *L. capsici*) (Figs 3 and 4). The other three species possessing *metE* are *L. solisilvae*, *L. enzymogenes* and *L. psychrotolerans* (Fig. 3). Thus, eight species, which is almost half or even less than the numbers of half of the tested 18 *Lysobacter*-type strains, have both *metE* and *metH*. To the best of our knowledge based on the tests for strain 5-21a^T, *L. soli*, *L. panacisoli*, *L. niastensis*, '*L. arenosi*' and *L. prati*, the distribution of *metE* corresponded well with that of the Met- and Cbl-auxotrophic properties. The other seven type strains possessing only *metH* are assumed to be Met and Cbl auxotrophs, which need Cbl to synthesize L-Met from L-homocysteine. Thus, it was inferred that Met- and Cbl-auxotrophic species are not minor among the genus *Lysobacter*. The addition of Met or Cbl to culture media may improve the isolation efficiency of *Lysobacter* strains from the environmental samples and may enhance the growth and activity of *Lysobacter* strains isolated as biocontrol agents against pathogenic micro-organisms.

In our past study [6], we found that the addition of chitin to the soil immediately increased the population of bacteria in the genus *Streptomyces*, which is known as the main decomposers of chitin in soil. Then, as the population of *Streptomyces* decreased over time, the population of *Lysobacter* increased [6]. Because six of the seven *Lysobacter* strains isolated from the chitin-treated upland soil were Met-auxotrophs, and, because the addition of Met to the incubated upland soil increased the population of *Lysobacter*, we assumed that Met is a factor that led to the increase of the population of *Lysobacter* in the chitin-treated upland soil [6]. In this study, these six *Lysobacter* isolates were found to be Cbl-auxotrophs (data not shown). We now speculate that the factor that led to the increase in the counts of *Lysobacter* in the chitin-treated upland soil was Cbl rather than Met. *Streptomyces* is a good producer of Cbl [41, 42]. Thus, it can be hypothesized that Cbl, which was produced and secreted by *Streptomyces*, stimulated the growth of Cbl-auxotrophic *Lysobacter*. We are presently conducting experiments to investigate this hypothesis.

Taxonomic position of strain 5-21a^T

The UBCG-based phylogenetic tree showed that strain 5-21a^T was most closely related to *L. soli* DCY21^T (GCF_003382285.1; Fig. 4), consistent with the phylogenetic analysis based on 16S rRNA genes (Fig. 3). Because of the relatively low ANI level of the genome sequence of strain 5-21a^T with that of *L. soli* DCY21^T (88.8%), the strain was characterized through a polyphasic approach so as to determine the taxonomic position. The cells of strain 5-21a^T were Gram-negative, non-spore-forming, motile, aerobic rods, 0.3–0.5 µm wide and 1.0–3.0 µm long. The strain grew well at 25–37 °C, and growth occurred at 42 °C. Phenotypic and biochemical characteristics that differentiate strain 5-21a^T from other related species of the genus *Lysobacter* are listed in Table 1. The major fatty acids detected (percentages of the total cellular fatty acids) in strain 5-21a^T were iso-$C_{15:0}$ (29.8%), iso-$C_{16:0}$(25.6%) and iso-$C_{17:1}$ ω9c (14.7%), which were similar to those of the relative type strains of the genus *Lysobacter*: *L. soli* LMG 24126^T, *L. panacisoli* JCM 19212^T and *L. niastensis* NBRC 106399^T (Table S3). The major ubiquinone detected was Q-8. This quinone system is a characteristic feature of the members of the genus *Lysobacter* [43]. The genome analyses indicated that the ANI and *in silico* DNA–DNA hybridization relatedness values between strain 5-21a^T and its most closely related genus member *L. soli* DCY21^T were 88.8 and 36.5%, respectively. These phenotypic, physiological, chemotaxonomic and genomic data indicate that strain 5-21a^T is clearly separated from the other members of the genus *Lysobacter*. On the basis of the presented data, strain 5-21a^T represents a novel member of the genus *Lysobacter*, for which the name *Lysobacter auxotrophicus* sp. nov. is proposed.

DESCRIPTION OF *LYSOBACTER AUXOTROPHICUS* SP. NOV.

Lysobacter auxotrophicus (au.xo.tro'phi.cus N.L. masc. adj. *auxotrophicus*, auxotrophic, requiring a specific growth substance).

Cells are Gram-negative, aerobic, motile and rod-shaped, approximately 0.3–0.5 µm wide and 1.0–3.0 µm long. Colonies grown on R2A agar for 72 h are yellow, translucent, butter-like, irregular, with wavy peripheral, raised like lens, and a smooth surface. The pH range for growth is pH 6.0–10.5; weak growth is observed at pH 11 and optimum growth is at pH 6–8. Oxidase and catalase positive. Reduction of nitrate is positive; glucose acidification and indole production are negative. The following carbon sources are utilized in the API 20NE test: *N*-acetylglucosamine, maltose and mannose. Utilization of the following substrates is negative: L-arabinose, caprate, citrate, erythritol, gluconate, glucose, L-malate, mannitol and phenylacetate. In assays with the API ZYM kit, acid phosphatase, alkaline phosphatase, esterase (C4), esterase lipase (C8), α-glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase, trypsin and valine arylamidase activities are present, but *N*-acetyl-β-glucosaminidase, chymotrypsin, cystine arylamidase, α-fucosidase, α-galactosidase, β-glucosidase, β-galactosidase, b-glucuronidase, lipase (C14) and α-mannosidase are absent. Q-8 is the predominant quinone. The major cellular fatty acids are iso-$C_{15:0}$, iso-$C_{16:0}$, iso-$C_{17:1}$ $\omega 9c$, iso-$C_{14:0}$ and iso-$C_{11:0}$ 3-OH.

The type strain, 5-21a^T (=NBRC 115507^T=LMG 32660^T), was isolated from chitin-treated upland soil sampled at a field in Fujieda, Shizuoka, Japan. The DNA G+C content of the type strain calculated from the genome sequence is 67.85mol%.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

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