Chloroflexus islandicus sp. nov., a thermophilic filamentous anoxygenic phototrophic bacterium from a geyser

Vasil A. Gaisin,* Alexander M. Kalashnikov, Denis S. Grouzdev, Marina V. Sukhacheva, Boris B. Kuznetsov and Vladimir M. Gorlenko

Abstract

A novel, thermophilic filamentous anoxygenic phototrophic bacterium, strain isl-2T, was isolated from the Strokkur Geyser, Iceland. Strain isl-2T formed unbranched multicellular filaments with gliding motility. The cells formed no spores and stained Gram-negative. The existence of pili was described in a species of the genus Chloroflexus for the first time, to our knowledge. Optimal growth occurred at a pH range of 7.5–7.7 and at a temperature of 55°C. Strain isl-2T grew photoheterotrophically under anaerobic conditions in the light and chemoheterotrophically under aerobic conditions in the dark. The major cellular fatty acids were C18:1ω9, C16:0, C18:0 and C18:0-ωOH. The major quinone was menaquinone-10. The photosynthetic pigments were bacteriochlorophylls c and a as well as β- and γ-carotenes. The results of phylogenetic analysis of the 16S rRNA gene sequences placed strain isl-2T into the genus Chloroflexus of the phylum Chloroflexi with Chloroflexus aggregans DSM 9485T as the closest relative (97.0% identity). The whole-genome sequence of isl-2T was determined. Average nucleotide identity values obtained for isl-2T in comparison to available genomic sequences of other strains of members of the genus Chloroflexus were 81.4% or less and digital DNA–DNA hybridisation values 22.8% or less. The results of additional phylogenetic analysis of the PufLM and BchG amino acid sequences supported the separate position of the isl-2T phylotype from the phylotypes of other members of the genus Chloroflexus. On the basis of physiological and phylogenetic data as well as genomic data, it was suggested that isl-2T represents a novel species within the genus Chloroflexus, with the proposed name Chloroflexus islandicus sp. nov. The type strain of the species is isl-2T (=VKM B-2978T,=DSM 29225T,=JCM 30533T).

The genus Chloroflexus was proposed by Pierson and Can-tenholz based on the description of the species Chloroflexus aurantiacus [1]. It was the first representative of the filamentous anoxygenic phototrophic (FAP) bacteria group that represents an important ecological guild in the microbial communities of chlorophototrophic mats, including those inhabiting hydrothermal ecosystems [2, 3]. To date, only two species with validly published names and comprehensive physiological description have been included in the genus: Chloroflexus aurantiacus [1] and Chloroflexus aggregans [4]. In addition, the genome sequences of Chloroflexus sp. Y-396–1, Chloroflexus sp. Y-400-fl and Chloroflexus sp. MS-G [5] are available.

Strains of C. aurantiacus have been isolated from different hot springs throughout the world, including those of Iceland [1]. However, information on the Icelandic strains is limited. Data about Chloroflexus-like bacteria from Iceland were mainly derived from the results of environmental studies [6–8]. Previously, these bacteria had been observed in the Icelandic geyser Strokkur as participants in a biominalisation process [9, 10]. In this study, we perform the first full taxonomic characterisation of the Icelandic strain isl-2T representing a member of the genus Chloroflexus.

Strain isl-2T was isolated from a water–sand suspension sampled from the Strokkur Geyser basin (overflow near the channel), Iceland. Water temperature of the sampling site was 50°C. The enrichment medium consisted of the following (per litre): KH2PO4 0.5 g; NH4Cl 0.5 g; MgCl2·6H2O 0.3 g; KCl 0.5 g; NaCl 0.5 g; Na2S·9H2O 0.05 g; CaCl2·2H2O 0.05 g; Na2SO4 0.05 g; yeast extract 0.3 g; sodium acetate 0.5 g; sodium malate 0.5 g; HEPES 3.0 g; trace-element solution 1 ml; and Pfennig’s vitamin solution 1 ml, which was prepared as described by Pfennig and Lippert [11]. The primary enrichment culture was grown at 54°C in a solid (1% agar) medium (pH about 8.0) in the light (500 lux). The pure culture was grown in a liquid medium under the
same conditions, with the exception for the yeast extract, which was increased to 1.0 g l\(^{-1}\). The same conditions were also used for the determination of temperature and pH ranges for growth.

The temperature for growth was tested using a gradient thermostat at 21–65 °C. Growth at various pH values was determined at 55 °C for a pH range of 6.1–9.3. The effects of various substrates on the growth of isl-2\(^{T}\) were tested by adding the following substrates to the base salt solution: glutamate, aspartate, glycylglycine, acetate, pyruvate, lactate, succinate, malate, butyrate, citrate, mannitol, methanol, ethanol, glycerol, yeast extract, casamino acids, soytone, casein hydrolysate, glucose, fructose, sucrose and sodium bicarbonate. The pH of the substrates was aseptically adjusted to 7.5 before usage. The base salt solution consisted of the following (per litre): KH\(_2\)PO\(_4\) 0.5 g; NH\(_4\)Cl 0.5 g; MgCl\(_2\) 6H\(_2\)O 0.3 g; KCl 0.5 g; NaCl 0.5 g; Na\(_2\)S\(_2\)O\(_3\) 0.1 g; CaCl\(_2\) 2H\(_2\)O 0.05 g; HEPES 3.0 g; vitamin solution 1 ml and trace-element solution 1 ml. The final concentration of the substrates was 0.25% (w/v). The final pH of the base salt solution was adjusted to 7.5. The tests were performed at 55 °C and pH 7.5 under three different conditions: anaerobic in the light and anaerobic and aerobic in the dark. To create anaerobic conditions, the glass vials were completely filled with the base salt solution along with resazurin and a carbon source, leaving a small gas bubble under the rubber stoppers. To create aerobic conditions, the vials were filled up to one-fifth of their volume with the base salt solution along with resazurin and some carbon source and sealed with cotton plugs and foil caps. Vitamin requirements were determined by using eight vitamin combinations, each of which lacked one of eight vitamins (nicotinic acid, thiamine hydrochloride, biotin, \(p\)-aminobenzoic acid, calcium pantothenate, pyridoxine hydrochloride, folic acid or riboflavin). The base salt solution (without vitamins) with fructose and sodium bicarbonate (300 mg l\(^{-1}\)) in the light at 55 °C and pH 7.5–7.6 was used in this test, and the final results were determined after three serial transfers. The influence of sulphide on a growth of isl-2\(^{T}\) was tested by using the base salt solution with sodium bicarbonate (300 mg l\(^{-1}\)) and yeast extract (2 g l\(^{-1}\)) in the light at 55 °C and pH 7.5–7.6. Illumination under phototrophic conditions was 500 lux (compact fluorescent lamp, 4200 K). Growth was determined by measuring the OD\(_{560}\) of the stirred cell suspension using a photometer (KFK-3-ZOMZ).

Cell morphology was determined using a phase-contrast microscope (BX 41; Olympus). The transmission electron micrographs of whole cells were prepared by the negative staining method using 2% phosphotungstic acid. The collected biomass for ultrathin cross sections was prepared using the Kellenberger-Ryter fixation method [12] and embedded in a resin (Epon 812) after dehydration. Reynolds’s reagent was used for contrasting [13]. The ultrathin cross sections were prepared using an ultramicrotome (LKB-4800) and placed on a Formvar-coated grid. A transmission electron microscope JEM-100C (JEOL) with an accelerating voltage of 80 kV was used.

The pigment composition of the cells was analysed using a spectrophotometer (SF-56A, OKB Spectr) at a wavelength range of 350–1100 nm. The absorption spectrum was determined both in the sonically disrupted cell suspension in 50% (v/v) glycerol and in acetone/methanol (7 : 2) extract. For the analysis of carotenoids, the pigments were extracted from the cells using acetone/methanol (7 : 2), transferred to petroleum ether, evaporated under nitrogen and redissolved in acetone. The extract was analysed by HPLC as described by Moskalenko [14]. Fatty acids were determined using a gas chromatography–mass spectrometry system (HP-5973; Hewlett-Packard) as described previously [15]. For quinone analysis, wet biomass was homogenized with liquid nitrogen and extracted with cold acetone. The components of the extract were separated using thin-layer chromatography followed by analysis on a tandem mass spectrometer (LCQ Advantage MAX) as described previously [16].

The bacterial DNA was sequenced using a HiSeq1500 with 150 bp read length for both reads. The sequencing methods and main genomic features have been described previously [17]. Phylogenetic trees based on the 16S rRNA gene sequences as well as BchG and PuFLM sequences were reconstructed using the MEGA 5.2 software package [18]. Intraspacial relationships were tested using digital DNA–DNA hybridisation (DDH), and average nucleotide identity (ANI) values were obtained in silico using the Genome-to-Genome Distance Calculator 2.1 (GGDC 2.1) [19] and the web-based ANI calculator with default parameters [20].

Strain isl-2\(^{T}\) grew as green spherical colonies in agar deeps under photoheterotrophic conditions. Cells formed flexible filaments with varied lengths (Fig. 1a). The filaments shortened at late stages of cultivation (Fig. 1b). Usually, empty parts of the filaments were observed in the micrographs. The cell size was about about 0.6 µm in diameter and 4–7 µm in length. The filaments showed slightly gliding motility. The cells stained Gram-negative. Chlorosomes were evident in the electron micrographs of cells contrasted with phosphotungstic acid (Fig. 1c). The ultrathin cross section showed chlorosomes as well as the complex cell envelope, which included the cell membrane, peptidoglycan and the outer layers (Fig. 1d). Intracellular inclusions (probably polyphosphate) were observed (Fig. 1e). Moreover, the ultrathin cross section showed a mesosome-like structure (Fig. 1e). Spore formation was not observed under the tested culture conditions. Pili were observed on the surface of cells that grew in the liquid medium under photoheterotrophic conditions at 55 °C (Fig. 1f). A microphotograph of the cells with pili is given in the supplementary material (Fig. S1, available in the online Supplementary Material). The pili reached up to about 1 µm in length and formed tangled fila
maments (Fig. S1).
Strain isl-2T is a thermophilic bacterium with an optimal growth temperature of 55 °C, which is similar to those of the other members of the genus Chloroflexus. The temperature range for growth was 46–59 °C. Weak growth occurred at low temperatures, starting from 33 °C. The optimal pH range was 7.5–7.7. The strain grew with sodium thiosulphate or sodium sulphate as the sole source of sulphur for constructive metabolism. The nitrogen sources were not determined specifically. Ammonium chloride served as a nitrogen source. The strain required folic acid as a growth factor. Increasing the sodium sulphide concentration led to decreased cell yield (Fig. S2). Under anaerobic conditions (sodium sulphide concentration 150 mg l\(^{-1}\)), the strain possesses photoheterotrophic metabolism in the presence of light. The best growth under chemoheterotrophic conditions was observed with acetate, glucose, fructose, sucrose, glycerol, yeast extract, casamino acids, soytone, and casein hydrolysate and sucrose. Weak growth under photoheterotrophic conditions occurred with glutamate, lactate and malate (Table S1). Photoautotrophic growth of isl-2T with sodium sulphide and sodium bicarbonate was not observed, as in the case of the strains of C. aggregans and most strains of C. aurantiacus [21, 4]. However, genes for the enzymes of the 3-hydroxypropionate cycle are present in the genome of isl-2T [17].

The major fatty acids of cells were 9-octadecen-1-0 (C\(_{18:1}\)ω9 (16.5 %)), hexadecanoic acid (C\(_{16:0}\) (16.4 %)), octadecanoic acid (C\(_{18:0}\) (14.8 %)) and octadecanol (C\(_{18:0}\)·OH (14.3 %)). Other cellular fatty acids were C\(_{14:0}\) (0.2 %), C\(_{15:0}\) (1.1 %), C\(_{16:1}\)·ω9 (2.5 %), C\(_{16:1}\)·ω7 (0.6 %), C\(_{16:0}\)·OH (2.3 %), C\(_{17:1}\) (1.2 %), C\(_{17:0}\) (5.1 %), C\(_{17:0}\)·OH (2.0 %), C\(_{18:1}\)·ω7 (0.6 %), C\(_{18:1}\)·ω7·OH (4.2 %), C\(_{19:1}\)·ω10 (0.6 %), C\(_{19:1}\)·ω8 (0.4 %), C\(_{19:0}\)·cyc (7.4 %), C\(_{19:0}\) (2.4 %), C\(_{19:0}\)·OH (2.0 %), C\(_{20:1}\)·ω11 (2.5 %), and C\(_{20:0}\) (2.0 %). In contrast to C. aurantiacus J-10fl (and OK-70fl), isl-2T had a small amount of heptadecanoic acid (C\(_{17:0}\)) and a relatively small amount of hexadecanoic acid and octadecanoic acid [22]. The major quinone was menaquinone-10 (molecular weight 852) that is probably typical for the strains of species of the genus Chloroflexus [4, 23].

The main phototrophic pigments of isl-2T were bacteriochlorophyll c (absorption maximum at 461 and 741 nm) and bacteriochlorophyll a (absorption maxima at 805 and 868 nm) in the spectrum of the sonically disrupted cell suspension in 50 % glycerol (Fig. S3). The absorption maxima of bacteriochlorophylls c and a in acetone/methanol extract were 667 and 765 nm, respectively (Fig. S3). The carotenoids were β-carotene (28.6 %), derivatives of β-carotene (26.3 %) and γ-carotene (45.1 %). All the above-mentioned pigments formed under photoheterotrophic growth conditions of isl-2T at 55 °C.

According to the results of 16S rRNA phylogenetic analysis, isl-2T together with other species of the genus Chloroflexus belonged to the uniform cluster (Fig. 2). The sequence identity between isl-2T and the most closely related strain C. aggregans DSM 9486T was 97.0 %, which corresponds to the criteria for determining that isl-2T represents a separate species [24]. The analysis of genomic data revealed 81.4 % ANI or less and 22.8 % DDH or less for isl-2T versus other members of the genus Chloroflexus (Table S2). The threshold for separated species based on ANI is 95.0 % or less [25]. The DNA G+C content of isl-2T was 59.65 mol%.

The results of 16S rRNA gene phylogenetic analysis, ANI calculation and in silico DNA–DNA hybridisation clearly confirmed assignment of isl-2T to a separate species. The additional phylogenetic analysis of the PufLM and BchG amino acid sequences supported the separation of the phylotype of isl-2T from other the phylotypes of other members of the genus Chloroflexus (Fig. S4). The main morphological, physiological and chemotaxonomical characters of the members of the genus Chloroflexus are conservative (Table 1). However, in contrast to the closest
phylogenetically related strain, *C. aggregans* DSM 9486\textsuperscript{T}, isl-2\textsuperscript{T} does not form cell aggregates. A notable feature of isl-2\textsuperscript{T} is the formation of pili, and this fact has not, to our knowledge, been described previously for other strains of species of the genus *Chloroflexus*. For instance, Fukushima et al. [26] reported that electron microscopic analysis had not revealed any possible surface structures (for gliding motility) on the cells of *C. aggregans* strain NBF. In the study by Hanada et al. [27] on the aggregation and motility of *C. aggregans*, the formation of pili was not mentioned. To the best of our knowledge, there are no data concerning pili formation by the cells of *C. aurantiacus*.

Table 1. Comparison characteristics of the species of the genus *Chloroflexus*. ND, not determined

<table>
<thead>
<tr>
<th></th>
<th><em>C. aurantiacus</em> DSM 635\textsuperscript{T}</th>
<th><em>C. aggregans</em> DSM 9485\textsuperscript{T}</th>
<th>isl-2\textsuperscript{T}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell diameter (mm)</td>
<td>0.7–1.2</td>
<td>1.0–1.5</td>
<td>about 0.6</td>
</tr>
<tr>
<td>Cell aggregation</td>
<td>–</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>Pili</td>
<td>–</td>
<td>–</td>
<td>+</td>
</tr>
<tr>
<td>Sheath</td>
<td>+/−</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>Gram staining</td>
<td>Negative</td>
<td>Negative</td>
<td>Negative</td>
</tr>
<tr>
<td>Optimal growth temperature (range for growth), °C</td>
<td>55</td>
<td>55</td>
<td>55 (46–59)</td>
</tr>
<tr>
<td>Optimal pH</td>
<td>8.0–8.5</td>
<td>7.5</td>
<td>7.5–7.7</td>
</tr>
<tr>
<td>Photoheterotroph</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Photoautotroph</td>
<td>+/−</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>O₂ respiration</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chlorosomes</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Bacteriochlorophylls</td>
<td>a, c</td>
<td>a, c</td>
<td>a, c</td>
</tr>
<tr>
<td>Remarkable peaks (nm) in in vivo spectra</td>
<td>462, 740, 802, 865</td>
<td>464, 740, 803, 868</td>
<td>461, 741, 805, 868</td>
</tr>
<tr>
<td>β-Carotene, (%)</td>
<td>28.4</td>
<td>Trace</td>
<td>28.6</td>
</tr>
<tr>
<td>Derivatives of β-carotene, (%)</td>
<td>4.1</td>
<td></td>
<td>26.3</td>
</tr>
<tr>
<td>γ-Carotene, (%)</td>
<td>22.6</td>
<td>Main</td>
<td>45.1</td>
</tr>
<tr>
<td>Derivatives of γ-carotene, (%)</td>
<td>43.9</td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>Major cellular fatty acids</td>
<td>C₂₁₀:0, C₁₇₀:0, C₁₈₀:0, C₁₈:1, C₁₈:0-OH</td>
<td>ND</td>
<td>C₁₂₀:0, C₁₅₀:0, C₁₈:9, C₁₈:0-OH</td>
</tr>
<tr>
<td>Vitamin requirements</td>
<td>Thiamine, biotin</td>
<td>Thiamine, folic acid</td>
<td>Folic acid</td>
</tr>
<tr>
<td>Major quinone</td>
<td>MK-10</td>
<td>MK-10 (and MK-4)</td>
<td>MK-10</td>
</tr>
<tr>
<td>DNA G+C content (mol%)</td>
<td>56.9–57.1</td>
<td>56.7–57.0</td>
<td>59.6</td>
</tr>
</tbody>
</table>
DESCRIPTION OF CHLOROFLEXUS ISLANDICUS SP. NOV.

Chloroflexus islandicus [isl.lan’dicus, N.L. masc. adj. islandicus from Iceland, referring to the location of source isolation].

Cells (about 0.6 μm in diameter and 4–7 μm in length) form unbranched multicellular filaments of varying lengths showing gliding motility. The non-spore-forming cells stained Gram-negative. Chlorosomes are present. Active cell aggregation is not observed. Pili are present. Optimum photoheterotrophic growth occurs at 55 °C and pH 7.5–7.7. The colour of cell suspensions is yellowish green under photoheterotrophic conditions. The absorption spectrum of the cell suspension exhibits maxima at 461, 741, 805 and 868 nm, with a shoulder at 407 nm. Photosynthetic pigments are bacteriochlorophylls \(a\) and \(b\) as well as \(\beta\)- and \(\gamma\)-carotenes. The major cellular fatty acids are \(C_{18:1}\) w9, \(C_{16:0}\), \(C_{18:0}\), and \(C_{18:0}\)-OH. The major quinone is menaquinone-10. Carbon and energy sources used under chemoheterotrophic conditions are acetate, glucose, fructose, glycerol, yeast extract, soytone, casamino acids and casamino acids, soytone, casein hydrolysate and sucrose. Carbon sources used under photoheterotrophic conditions are glutamate, aspartate, glycyglycine, acetate, pyruvate, succinate, fructose, glucose, sucrose, mannitol, methanol, ethanol, glycerol, yeast extract, soytone, camasino acids and casamino hydrolysate. Reduced sulphur is not required. Ammonium chloride served as a nitrogen source. Folic acid is required as a growth factor.

The type strain, isl-2T (=VKM B-2978T, =DSM 29225T =JCM 30533T) was isolated from the geyser Strokkur in Iceland. The DNA G+C content of the type strain is 59.65 mol%.

Funding information
This work was supported by Russian Foundation for Basic Research, grant numbers 15-04-07655, 16-34-60071 and 16-04-00830; Russian Federation President Grant NS 9888.2016.4 and Program of the Presidium of the Russian Academy of Sciences The evolution of the organic world and planetary processes.

Acknowledgements
First of all the authors are grateful to Dr N.A. Demidenko from Zubov State Oceanographic Institute (Moscow) who kindly provided the sample from the geyser Strokkur. The authors are grateful to Dr A.A. Ashkhimin for his assistance with determination of carotenoids, Dr G.A. Ospiov for his assistance with determination of cellular fatty acids and Dr B.P. Baskunov for his assistance with determination of quinones. The authors thank Dr I.A. Bryantsseva from the Laboratory of Ecology and Geochemical Activity of Microorganisms, Winogradsky Institute of Microbiology, as well as Drs T.V. Kolganova and R.V. Baslerov from the Molecular Diagnostics Laboratory, Institute of Bioengineering, Research Centre Of Biotechnology, RAS.

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

References


Five reasons to publish your next article with a Microbiology Society journal

1. The Microbiology Society is a not-for-profit organization.
2. We offer fast and rigorous peer review – average time to first decision is 4–6 weeks.
3. Our journals have a global readership with subscriptions held in research institutions around the world.
4. 80% of our authors rate our submission process as ‘excellent’ or ‘very good’.
5. Your article will be published on an interactive journal platform with advanced metrics.

Find out more and submit your article at microbiologyresearch.org.