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DNA base composition of planktonic species of *Anabaena* (Cyanobacteria) and its taxonomic value

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Fifty axenic strains of planktonic *Anabaena*, including 24 strains of the straight form and 26 strains of the coiled form, were examined for their DNA base composition (GC content). The taxonomic value of their GC content at species level was evaluated by comparing their morphological, physiological and biochemical properties. The DNA base composition determined for all fifty strains ranged from 35.9 to 56.4 mol% GC. The straight-form strains were in the range of 35.9–56.4 mol% GC, while coiled forms were in the range of 38.1–50.3 mol% GC. In general, strains assigned to the same species showed similar DNA base composition. However, of three strains of *A. affinis* Lemmermann that were separated into two categories, two had 40.6–40.9 mol% GC, and the third strain 45.6 mol% GC. It is noteworthy that the DNA base composition of the newly established species *A. eucompacta* Li et Watanabe was 45.5 mol% GC, which differed from 39.5 mol% GC of the morphologically close species, *A. compacta* (Nygarrd) Hickel.

Key Words——*Anabaena*; cyanobacteria; DNA base composition; GC content; planktonic; taxonomy

Introduction

DNA base composition has not only become an important taxonomic criterion which has proved useful for both classification and identification of prokaryotes (Tamaoka, 1994), but it is essential to permit the appropriate choice of strains for future DNA/DNA hybridization studies, the latter of which is the only accepted means for the validation of species assignments under the Bacteriological Code of Nomenclature. As concluded in "Guidelines of Characterization of Cyanobacteria" (Castenholz and Waterbury, 1989), DNA base composition is also a very important genetic character in the taxonomy of cyanobacteria. DNA base composition has been determined for nearly 200 strains of cyanobacteria (Herdman et al., 1979). In these strains, GC content ranged between 31–71 mol%, which is a narrower range than that determined for all bacterial groups together (25–80%; Tamaoka, 1994). The taxonomic relevance is obvious; for example, among unicellular cyanobacteria, the *Synechocystis* group was divided into four clusters: marine (30.5–31.7 mol% GC), low-GC (35.8–36.9 mol% GC), high-GC (42.1–48.0 mol% GC) and *Microcystis* (45.4 mol% GC) (Holt et al., 1994).

The DNA base composition of about 6 strains of *Anabaena* has been shown to range from 38.3 to 44% GC, but all these strains are benthic (Herdman et al., 1979). Planktonic species of *Anabaena* are major components of freshwater water-blooms in aquatic environments and may include toxigenic representatives

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(Carmichael, 1981). It remains important to accumulate information about genotypic characterization, and therefore better understand relationships between phenotypic and genotypic characteristics. One genotypic characteristic, DNA base composition, was therefore examined for 50 planktonic strains of *Anabaena* assigned to 22 different morphological species (Li et al., 2000). The results are presented in the present study.

Materials and Methods

Organisms and culture conditions. All 50 planktonic strains of *Anabaena*, including 24 straight-form strains and 26 coiled strains, were axenic. The taxonomy of the strains, based on morphological characteristics, was described by Li et al. (2000). All strains were cultivated in CT medium (Watanabe and Hiroki, 1997). The cultures were grown in 1 L medium in 2-L flasks equipped for aeration with filters of 0.22 μm pore size. All cultures were grown at 20°C under a 12 : 12 L/D cycle with a photon irradiance of 40 $\mu mol \cdot m^{-2} \cdot s^{-1}$ provided by daylight fluorescent lamps. After four weeks, cells were harvested by centrifugation (1,000×*g*, 10 min), washed twice with distilled water and subsequently freeze-dried for 5 days. Purity of all cultures was checked by microscopic examination prior to their being harvested. In the case of the strains having abundant mucilaginous substances, exponentially growing cultures were harvested by filtration, washed three times with distilled water and then used for DNA extraction.

DNA extraction. Ten mg of lyophilized or equivalent wet weight cells were suspended in 0.5 ml of lytic solution [(achromopeptidase (Sigma) 0.5 mg+lysozyme (Sigma) 0.75 mg)/ml of 10 mM Tris-HCl buffer, pH 8.0] and mixed with beads in 2 ml screwed polypropylene vials (1 : 1 with volume), broken by a Beadsbeater (Biospec Products, USA) at 5,000 rpm for 1 min. The samples were incubated at 37°C for 30 min, then 50 μl of 10% Tris-SDS solution (SDS in 1 M Tris, w/v) was added and mixed well. Samples were then incubated at 60°C for 5 min. Lysates were extracted twice with 0.2 ml of water-saturated phenol and 0.2 ml of chloroform. After centrifugation (10,000×*g*, 10 min), 50 μl of RNase solution [(RNaseA (Sigma) 1 mg+RNase T1 (Sigma) 400 units)/ml of 50 mM Tris-HCl, pH 7.5] was added to the upper layer and kept at 37°C for 20 min, and treated with 50 μl of Proteinase K solution (Proteinase K (Sigma) 4 mg/ml of 50 mM Tris-HCl, pH 7.5) at 37°C for 10 min. The samples were then treated again with 0.2 ml of phenol solution and 0.2 ml of chloroform, and centrifuged at 10,000×*g* for 10 min. DNA was precipitated with 0.1 volume of 3 M NaOAC and 2.5 volumes of cold 100% ethanol. Precipitated DNA was pelleted by centrifugation for 15 min at 10,000×*g*, washed with 70% ethanol, then 100% ethanol, dried and stored at −20°C.

GC determination by HPLC method. Dried DNA was mixed with 100 μl of water at 50°C for 1 h. Twenty-five μl of the solution containing 2–25 μg of DNA was heated in boiling water for 5 min and rapidly cooled in an ice water bath. Twenty-five μl of acetate buffer (40 mM, pH 5.3) containing 2 mM $ZnSO_4$, 5 μl P1 nuclease (Sigma) solution (20 U/ml) and 20 μl of water were added. The sample was incubated at 37°C for 2 h. After addition of 2.5 μl of glycine buffer (0.1 M, pH 10.4) and 2 μl of alkaline phosphatase (Sigma) (35 U/ml), the sample was incubated at 37°C for 6 h or overnight. Then, a portion of a 10 μl sample was injected into an HPLC system (Shimadzu, Kyoto, Japan) equipped with an SPD-10A UV-VIS spectrophotometric detector and a column (Cosmosil, 5C18, 4.6 mm i.d., 150 mm, Nacalai, Tokyo, Japan). Nucleosides were eluted by a mixture of 0.4 M $NH_4H_2PO_4$ and acetonitrile (40 : 1, v/v), at a flow rate of 1 ml/min at 40°C controlled by a column oven (Shimadzu CTO-6A). Each nucleoside was detected by its absorbance at 270 nm. Each sample was analyzed at least three times from independent extractions. GC content was calculated from the total composition of DNA or from the ratio of a known base to total nucleosides (Mebash et al., 1989).

Results

The DNA base composition determined for the strains of planktonic species of *Anabaena* ranged from 35.9 to 56.4 mol% GC (Table 1). Strains with straight trichomes were in the range of 35.9–56.4 mol% GC, while coiled forms had GC of 38.1–50.3 mol% GC, slightly narrower than that of straight ones. Majority (70%) of the strains were found to have a mean DNA base composition in the range of 36.9 to 42.4 mol%. Three strains (6%) have a slightly lower GC (35.9–36.2 mol%), and four strains (8%) a slightly higher mean GC content (43.8–45.6 mol%). Eight strains were shown to have an unusually high GC content (between 48.3–56.4 mol%). In general, strains as-

Table 1. DNA base composition of planktonic strains of *Anabaena.*

Taxa	Strain	Source	% GC (mean±SD)
A. affinis	NIES-40	NIES[a]	40.9±0.3
	Inba3	Inbanuma, Chiba, Japan (NIES)	40.6±0.4
	Inba10	Inbanuma, Chiba, Japan (NIES)	45.6±0.2
A. planctonica	TAC422	NSM[b]	40.6±0.2
	Inba2	Inbanuma, Chiba, Japan (NIES)	40.0±0.4
	NIVA 66	NIVA[c]	38.9±0.5
	TAC421	NSM	40.2±0.1
	TAC424	NSM	39.7±0.2
	Inba6	Inbanuma, Chiba, Japan (NIES)	37.3±0.3
	1403/19	CCAP[d]	38.6±0.5
	1403/27	CCAP	37.8±0.5
	TAC434	NSM	36.2±0.3
	TAC435	NSM	38.1±0.7
A. solitaria	NIES-78	NIES	35.9±0.2
	NIES-80	NIES	36.2±0.5
A. smithii	TAC428	NSM	37.1±0.5
	TAC431	NSM	37.1±0.4
	TAC432	NSM	37.7±0.7
	TAC450	NSM	37.9±0.8
	TAC451	NSM	36.9±0.4
A. kisseleviana	NIES-74	NIES	42.0±0.3
	TAC34	NSM	42.4±0.5
A. viguieri	TAC433	NSM	48.7±0.8
A. danica	TAC453	NSM	56.4±0.9
A. spiroides	TAC429	NSM	38.1±0.4
A. ellipsoides	Ana HB	A fish pond, Wuhan, China (NIES)	39.1±0.5
A. crassa	TAC436	NSM	39.7±0.6
	TAC443	NSM	38.9±0.2
	NIES-76	NIES	39.2±0.1
	NIES-263	NIES	38.1±0.4
A. ucrainica	TAC448	NSM	50.1±0.5
	TAC449	NSM	49.0±0.8
	NIES-77	NIES	50.2±0.5
	NIES-79	NIES	48.9±0.3
A. mucosa	TAC425	NSM	40.7±0.9
A. compacta	1403/24	CCAP	39.5±0.3
A. flos-aquae	TAC446	NSM	48.3±0.9
A. mendotae	TAC437	NSM	43.8±0.5
	TAC438	NSM	42.1±0.4
A. lemmermannii	Ana Dalai	Dalai Lake, Inner Mongolia, China (NIES)	40.4±0.3
	Inba4	Inbanuma, Chiba, Japan (NIES)	38.6±0.3
	NIVA82	NIVA	40.2±0.5
A. circinalis	Ana Da	Barato Lake, Hokkaido, Japan (NIES)	44.2±0.2
A. curva	Ana Ao	Aomori, Japan (NIES)	50.3±0.7
A. oumiana	Ana kas1	Kasumigaura, Ibaraki, Japan (NIES)	40.4±0.6
	NIES-73	NIES	42.0±0.2
A. eucompacta	Ana Chiba	Yatsuru Lake, Chiba, Japan (NIES)	45.5±0.6
Anabaena sp 1	Ana Ku-3	Dianchi Lake, Kuming, China (NIES)	38.5±0.6
	Ana Ku-5	Dianchi Lake, Kuming, China (NIES)	39.3±0.5
Anabaena sp 2	CCAP1403/13F	CCAP	38.0±0.5

[a] Microbial Culture Collection, National Institute for Environmental Studies, Tsukuba, Japan.
[b] National Science Museum, Tsukuba, Japan.
[c] Norwegian Institute for Water Research, Oslo, Norway.
[d] Culture Collection of Algae and Protozoa, Windermere, UK.

signed to the same species showed similar DNA base composition (Table 1), such as *Anabaena crassa* (Lemmermann) Komárková et Cronberg (38.1–39.7 mol% GC), *A. ucrainica* (Schkorb.) Watanabe (48.9–50.2 mol% GC), *A. solitaria* Klebahn (35.6–36.2 mol% GC) and *A. kisseleviana* Elenkin (42.0–42.4 mol% GC), *A. smithii* (Komárek) Watanabe (36.9–37.9 mol% GC), *A. mendotae* Trelease (35.9–36.2 mol% GC), *A. lemmermannii* P. Richter (38.6–40.4 mol% GC), and *A. oumiana* Watanabe (40.4–42.0 mol% GC). The strains of *A. planctonica* Brunnthaler showed similar GC contents, 37.2–40.6 mol%. However, three strains of *A. affinis* Lemmermann separated into two categories; NIES-40 and Inba3 had 40.6–40.9 mol% GC, and Inba10 had 45.6 mol% GC. DNA base composition of *A. eucompacta* Li et Watanabe was 45.5 mol% GC, which differed from 39.5 mol% GC of the morphologically related species, *A. compacta* (Nygarrd) Hickel.

Discussion

In bacterial taxonomy, DNA base composition is considered to be one of the required characteristics for a minimum description of genera and species (Lévy-Frébault and Portaels, 1992). It is generally accepted that if two organisms have markedly different base composition values, they must belong to different taxa. However, it is important to recognize that two organisms with similar base composition may not be closely related because the linear arrangement of the nucleotides in the DNA may be different (Tamaoka, 1994).

Previous studies of the DNA base composition of cyanobacteria (Herdman et al., 1979; Paula et al., 1992) have been conducted using indirect methods, such as thermal denaturation of DNA (the T_m method) and buoyant density measurements in caesium chloride (the B_d method). The HPLC method for measuring DNA base composition is popular in microbial taxonomy, because it allows a direct, rapid and precise determination of base composition (Mebash et al., 1989; Tamaoka and Komagata, 1984). Nevertheless, this relatively simple method has rarely been applied for cyanobacterial taxonomy. In the bacteriological view, the DNA base composition of strains of a species shows a narrow range within 1 to 3 mol% GC (Mandel, 1966; Tamaoka, 1994). In the planktonic strains of *Anabaena*, DNA base composition ranged from 35.9 to 56.4 mol%, which is a wider range than that determined for non-planktonic strains of *Anabaena*, 38.3–44 mol% (Herdman et al., 1979). However, about 80% of the planktonic strains of *Anabaena* fell in the range of 37–42 mol% (Table 1). Three strains of *A. affinis* Lemmermann separated into two clusters (NIES-40 and Inba3 had 40.6–40.9 mol% GC, and Inba10 had 45.6 mol% GC) and members of these two clusters also have different growth-temperature ranges and fatty acid patterns (Li, 1998). It is concluded that these two clusters represent different species based on physiological, biochemical and genetic characteristics, although they can not be morphologically distinguished.

Strains of *Anabaena planctonica*, *A. solitaria*, *A. smithii*, and *A. kisseleviana* had narrow range of DNA base composition and there was no significant difference within a given species, although the low GC content (36.2 mol%) of the strain *A. planctonica* TAC434 is closer to the two strains of *A. solitaria* than to the other members of *A. planctonica* (Table 1). These findings seem to support taxonomy based on the morphology of these *Anabaena* representatives. Based on fatty acid composition, strains of *A. planctonica* separated into four clusters (Li, 1998). However, the range of their GC was narrow and members of the four biochemical clusters cannot be distinguished from each other based on GC content.

Anabaena viguieri (Syn.: *A. affinis* Lemm. f. *viguieri*) is morphologically similar to *A. affinis* in trichome and cell size, and akinete shape and size, but *A. affinis* forms bundles and has tapering end cells (Watanabe, 1992). The absence or modification of these diagnostic characteristics in either natural samples or laboratory cultures would make it impossible to identify them based on morphology alone. However, the DNA base composition of *A. viguieri* TAC 433 (48.7 mol% GC) was significantly higher (7.9–8.1 mol% GC difference) than that of two strains of *A. affinis* (NIES 40 and Inba3). *Anabaena danica*, recognized as an independent species by Komárková-Legnerová and Eloranta (1992), is similar to both *A. planctonica* and *A. viguieri* in morphology. The single strain of *A. danica* differed from representatives of the latter two species in various aspects: It grew at a temperature range of 10–25°C, whereas the range was 10–35°C for strains of *A. planctonica* and *A. viguieri*; the strain of *A. danica* belonged to type 2B of fatty acid composition, whereas strains of *A. viguieri* belonged to type 2A (Li, 1998). The strain of *A. danica* had the highest GC content

(56.4 mol%), compared to strains of *A. viguieri* (48.7 mol% GC) and the strains of *A. planctonica* (36.2–40.6 mol% GC). Thus, combining physiological, biochemical and genetic characteristics with morphological features has proved to be a useful method for resolving various problems in cyanobacterial taxonomy.

Strains of the coiled *Anabaena* assigned to the same species, including those of *A. crassa*, *A. ucrainica*, *A. mendotae*, *A. lemmermannii*, and *A. oumiana*, showed very close DNA base composition (Table 1). The strain of *A. curva*, characterized by many filaments embedded in a joint sheath, had a high GC content of 50.4%. *A. mucosa* TAC 425, which is morphologically similar to strains of *A. ucrainica* but differs in salinity tolerance and fatty acid content (Li, 1998), contained 40.7 mol% GC, while those of *A. ucrainica* had 48.9–50.1 mol% GC. The difference in DNA base composition between them supports the treatment of *A. mucosa* as a separate species. The single strain of *A. eucompacta*, comprising the newly established species by Li and Watanabe (1999), differed from the representative of the related species *A. compacta* in DNA base composition (45.5 and 39.5 mol% GC, respectively), strengthening the conclusion that *A. eucompacta* is an independent species.

Two strains (Ku 3 and Ku 5) of *Anabaena* sp 1 were comparable to those of *A. ucrainica* and *A. mucosa* in morphology except for the absence of akinetes. Their DNA base composition ranged between 38.5 and 39.3 mol% GC, which is similar to strains of *A. mucosa* but quite different from the GC determined for the strains of *A. ucrainica* (Table 1). Furthermore, these two strains grew at a wider temperature range (10–35°C) than either strains of *A. mucosa* (10–25°C) or of *A. ucrainica* (10–30°C) and were more similar in fatty acid composition to strains of *A. mucosa* than to strains of *A. ucrainica* (Li, 1998). It is therefore concluded that these two strains can not be assigned to *A. ucrainica*, but they may be closely related to *A. mucosa*, because the only difference so far found is their response to growth temperature.

The DNA base composition of strain CCAP 1403/13F (38.0 mol% GC) was different from that of *A. flos-aquae* (48.3 mol% GC). Although the former strain was also previously assigned to *A. flos-aquae* (Thompson et al., 1988), it was "*Anabaena* sp 2" for the reasons discussed by Li et al.(2000). The two strains of *A. kisseleviana* and of *A. oumiana*, despite their different filament forms, have spherical akinetes adjacent to heterocysts, and both were classified as high-temperature types (Li, 1998). They were shown to have a similar DNA base composition (40.4–42.4 mol%GC), which may imply that they are genetically similar.

The present results indicate that DNA base composition differs greatly among planktonic strains of *Anabaena.* Its taxonomic value has shown to be effective when combined with morphological, physiological and chemical characters. It is suggested that DNA/DNA hybridization studies will be required to confirm the validity of different species, particular for the strains that share a similar mean DNA base composition. The results presented here will be essential for such studies.

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