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Effects of Temperature and Nutritional Changes on the Fatty Acids of *Agmenellum quadruplicatum*[1]

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The fatty acid composition of the blue-green bacterium *Agmenellum quadruplicatum* was examined under a wide variety of growth conditions. The fatty acid composition was found to undergo significant changes with variations in temperature, media composition, and growth phase (log versus stationary). With increasing growth temperature (20 to 43 C) log-phase cells exhibited an increase in saturated fatty acids (38.4% at 20 C to 63.6% at 43 C). Striking changes were seen with some of the individual fatty acids such as 18:3, which made up 16.0% of the total fatty acid at 20 C but was not measurable at 43 C. Fatty acid 12:0 was not measurable at 20 C but made up 16.3% of the total fatty acids at 43 C. Cell lipids were separated into neutral lipid, glycolipid, and very polar lipid fractions. The neutral lipid fraction was composed almost entirely of 12 carbon fatty acids (12:0, 12:1). Glycolipid and very polar lipids were more similar in their fatty acid composition when compared to the total cellular fatty acids, although they did lack 12 carbon fatty acids. The total of 12 carbon fatty acids in the cell can be used as an indicator of the amount of neutral lipid present.

Lipid composition has been proposed as a guide to the classification of bacteria (20). The fatty acid composition of blue-green bacteria has been suggested as an indicator of their phylogenetic position (10). Fatty acid patterns have been examined in several comparative studies of blue-greens. Kenyon et al. (15) found that these organisms can be separated into several classes based on the absence or presence of polyunsaturated fatty acids, especially linolenic acid. However, no comprehensive growth studies have been made with blue-green bacteria with respect to possible changes in fatty acid composition.

The fatty acid composition of a wide variety of organisms has been shown to change as a result of environmental conditions, especially temperature (1, 2, 5, 7). Lowering the growth temperature typically results in a higher proportion of unsaturated fatty acids in the cell membrane.

In this paper we describe the fatty acid composition of the blue-green bacterium *Agmenellum quadruplicatum*, strain BG1, under a wide variety of temperatures and growth conditions. In addition, we have examined the fatty acid compositions of three lipid fractions, neutral lipids, glycolipids, and very polar lipids (phospholipids and sulfolipids), and have found these to be quite different with respect to fatty acid composition. (This was submitted by G. J. O. to the faculty of the Department of Microbiology, University of Florida, in partial fulfillment of the Master of Science degree. Portions of this work were presented at the 1975 Annual Meeting of the American Society for Microbiology, New York.)

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MATERIALS AND METHODS

Organism and culture conditions. *A. quadruplicatum* strain BG1 was originally isolated into axenic culture by Van Baalen (22). The cells were grown in ASP-2, a mineral salts medium, as described previously (11). Unless stated otherwise cells were harvested in log phase at an optical density of 0.2 (750 nm; Beckman DU spectrophotometer). Stationary-phase cultures were grown to an optical density of 1.7. Starvation cultures were produced by diluting cells growing in complete medium into media lacking a single particular component and growing for 48 h at 39 C. Cultures were inactivated by the addition of solid trichloroacetic acid to a final concentration of 5%, and lipids were extracted with chloroform-methanol as described by Kanfer and Kennedy (12). The washed chloroform extract was then used for further analyses (silicic acid column chromatography, thin-layer chromatography, or fatty acid analysis).

Silicic acid column chromatography. The

method of Vorbeck and Marinetti (23) was followed in separating the washed chloroform extract into neutral lipid, glycolipid, and very polar lipid fractions. These fractions were flash evaporated (40 C) and stored in their respective elution solvents under refrigeration until use.

Fatty acid analysis. The washed chloroform extract was evaporated to near dryness under dry N_2 and transesterified using 2% H_2SO_4 in methanol as described by Silbert et al. (21). The methyl esters were extracted twice into an equal volume of *n*-pentane and concentrated using dry N_2 prior to analysis by gas chromatography. One-microliter injections of the methyl esters were examined on a Packard series 7600 gas chromatograph (Packard Instrument Co., Downers Grove, Ill.) equipped with a flame ionization detector and a glass column (2 mm by 183 cm) packed with 10% diethyleneglycolsuccinate on Gas Chrom Q (100 to 120 mesh). The gas chromatograph was run isothermally at 175 C with a carrier gas (N_2) flow of 30 ml/min. Fatty acids were identified using standards obtained from Applied Science Laboratories (State College, Pa.). Relative abundance of the various fatty acids was determined as percentage of total peak area. Individual peak areas were calculated by triangulation. Reported values were the averages of the least two and usually four independent determinations. Mean deviations were always within 10% of the reported percentages.

Thin-layer chromatography. Lipids were identified using thin-layer chromatography with a variety of solvent systems. Thin-layer plates (0.25 mm; Silica Gel F; E. M. Laboratories, Elmsford, N. Y.) were activated for 20 min at 105 C and spotted with the appropriate lipid fraction. The plates were run in either a diethyl ether-benzene-ethanol-acetic acid (40:50:2:0.2, vol/vol/vol/vol) solvent system as described by Freeman and West (4) or chloroform-methanol-water (70:35:7, vol/vol/vol) solvent system as described by Yribarren et al. (24). Lipids were initially detected by iodine vapors and subsequently by using specific reagent sprays for sugars (16), phosphate esters (3), or amines (16). The major phospholipid component, diphosphatidyl glycerol, was identified by two-dimensional, thin-layer chromatography using chloroform-methanol-7 N ammonium hydroxide (65:30:4, vol/vol/vol) in the first dimension, followed by chloroform-methanol-acetic acid-water (170:25:25:4, vol/vol/vol/vol) as described by Hitchcock and Nichols (9). Lipid extracts from plant leaf tissue served as glycolipid and sulfolipid standards. Tripalmitin and phosphatidyl glycerol standards were obtained from the Sigma Chemical Co. (St. Louis, Mo.).

Sugar analyses. The glycolipid and very polar lipid fractions were hydrolyzed in 2.0 N HCl for 2 h at 100 C and then neutralized with 2.0 N NaOH. Glucose and galactose were then determined using glucostat and galactostat test kits (Worthington Chemical Co., Freehold, N. J.).

RESULTS

Lipid composition. Table 1 summarizes the types of lipids detected by thin-layer chromatography of the various lipid fractions that were obtained using silicic acid column chromatography. The neutral lipids migrated near the solvent front at R_f 0.90. Most of the pigments (chlorophyll and carotenoids) were eluted with the glycolipid fraction and also migrated near the solvent front. Two other spots in the glycolipid fraction migrated more slowly and were sugar positive when sprayed with thymol-sulfuric acid reagent (16). These are presumably monoglycosyl and diglycosyl diglycerides, the two main glycolipids found in other blue-green bacteria (19). A plant leaf extract, known to contain these glycolipids, was analyzed as a standard (9). Yribarren et al. described a monogalactosyl diglyceride in *Actinomyces viscosus*

TABLE 1. *Thin-layer chromatography of the lipid fractions of A. quadruplicatum*[a]

Fraction	Spot	R_f	Detection methods			Tentative identification
			I_2	Sugar	Phosphate	
Neutral lipid	1	0.90	+	–	–	Mono-, di-, triglycerides
Glycolipid	2	0.91		Visual detection		Chlorophyll
	3	0.89		Visual detection		Carotenoids
	4	0.85	+	+	–	Monoglycosyl diglyceride
	5	0.74	+	+	–	Diglycosyl diglyceride
	6	0.00	+	–	–	Unknown
Very polar lipids	7	0.91		Visual detection		Chlorophyll
	8	0.74	+	+	–	Diglycosyl diglyceride
	9	0.71	+	+	–	Unknown glycolipid
	10	0.64	+	+	–	Unknown glycolipid
	11	0.62	+	–	+	Phosphatidyl glycerol
	12	0.15	+	+	–	Sulfoquinovosyl diglyceride

[a]Chloroform-methanol-water (70:35:7, vol/vol/vol).

which had a near identical R_f (in the same solvent system) to one of our glycolipid spots (24). Sugar analysis of the glycolipid fraction revealed the presence of glucose and galactose in approximately equal amounts. One additional spot was detected in the glycolipid fraction. It remained at the origin and was negative for sugar and phosphate ester. The immobility of this spot is inconsistent with the column separation, and it may represent oxidation products, possibly from pigments, produced during flash evaporation.

The very polar lipid fraction also contained a trace amount of chlorophyll. In addition, one spot was positive for phosphate esters. Using two-dimensional chromatography, the spot was found to chromatograph in the region of phosphatidyl glycerol. Three faint sugar spots which migrated in the glycolipid region were also detected. These probably represent slight overlap in the column separation of the glycolipid fraction. A major lipid component exhibiting a positive sugar reaction was detected with a low R_f. This is presumably sulfoquinovosyl diglyceride, the plant sulfolipid. Cochromatography of the sample with a peanut leaf lipid extract revealed a major spot with identical R_f. This sulfolipid is known to be a major component of other blue-green bacteria (19). A sulfur spot test confirmed the presence of sulfur. Sugar analysis of the very polar lipid fraction revealed the presence of glucose and galactose in a ratio of 1:4.

Fatty acid composition. The fatty acid composition of each fraction relative to the total is shown in Table 2. There is a striking difference in the fatty acid composition of different fractions at 39 C. Almost all of the lauric acid in this organism is associated with the neutral lipid fraction, which contained little of the other fatty acids. The glycolipid and very polar lipid fractions are more similar and more representative of the total cellular fatty acids. A gravimetric estimation of the relative contribution of the acyl lipids (in each fraction) was impossible due to the large amounts of pigments, especially in the glycolipid fraction. Estimations are based upon total methyl esters.

Effects of nutrient limitation and growth phase on fatty acid composition. The omission of various single components from the growth medium markedly changed the fatty acid pattern (Table 3) as compared with normally grown cells. In general, there was a decrease in the percentages of 12:0 and 18:2 fatty acids, accompanied by an increase in 16:0 and 18:1. Since the neutral lipid is composed almost solely of 12:0 and 12:1 fatty acids at 30 C, a decrease in the total of these fatty acids reflects a decrease in the amount of neutral lipid in the cell under these conditions. Other fatty acids exhibited quite a variation in amount between each starved culture.

A comparison of the fatty acid composition of exponential- and stationary-phase cultures was made at 39 C (Fig. 1C and D). The most significant differences were in the monounsaturated fatty acids 16:1 and 18:1, with stationary-phase cells containing 70% more of these fatty acids. In addition, stationary-phase cells contained 48% less of the polyunsaturated fatty acids (18:2, 18:3). Overall there was a slight increase in the degree of unsaturation of stationary-phase cells. At 30 C, there is a larger increase in unsaturation with stationary-phase cells as compared to exponential-phase cells. Again the monounsaturates show an increase in

TABLE 2. *Distribution of each fatty acid among the lipid fractions (%) at 39 C*[a]

Fatty acid	Neutral lipids	Glycolipids	Very polar lipids
12:0	100.0	Tr	
12:1	Tr	Tr	
14:0	Tr	Tr	Tr
14:1	Tr	Tr	Tr
16:0	Tr	39.7	60.3
16:1	2.0	49.0	49.0
UNK	Tr	Tr	Tr
18:0	Tr	53.3	46.6
18:1	1.3	46.0	52.6
18:2	Tr	54.0	46.1
18:3	Tr	44.1	55.8

[a] Tr, Trace; UNK, unknown.

TABLE 3 *Effects of nutrient limitations on fatty acid composition (%)*[a]

Fatty acid	Control	$-NO_3$	$-PO_4$	-NaCl	-FeCl$_3$	-Mg, -Ca
12:0	15.3	8.0	6.5	6.8	10.2	10.5
12:1	3.9	5.1	5.7	Tr	Tr	Tr
14:0	Tr	Tr	Tr	Tr	Tr	Tr
14:1	Tr	Tr	Tr	Tr	Tr	Tr
16:0	34.6	38.3	41.0	47.3	46.4	40.0
16:1	7.9	5.5	4.2	8.2	5.9	5.0
UNK	Tr	11.1	Tr	Tr	2.0	1.1
18:0	1.8	Tr	4.2	Tr	3.0	2.2
18:1	11.5	12.3	21.0	12.3	21.3	26.2
18:2	23.6	16.9	14.2	21.0	11.2	12.7
18:3	2.7	2.8	3.2	4.4	Tr	1.4
Saturates	51.7	46.3	51.7	54.1	59.6	53.6

[a] Tr, Trace; UNK, unknown.

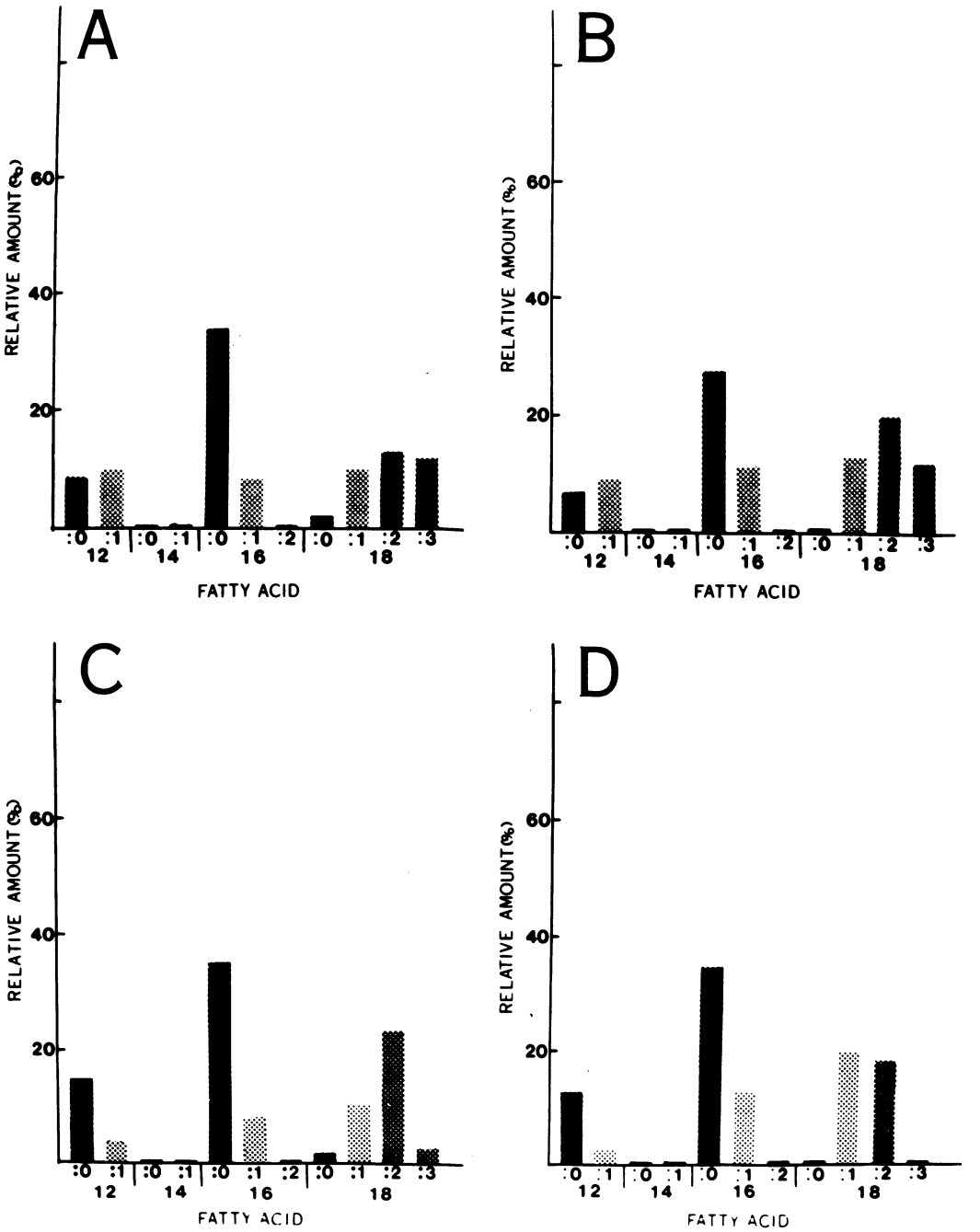

FIG. 1. *Comparison of fatty acid compositions of log- and stationary-phase cells at 30 and 39 C. (A) 30 C, log; (B) 30 C, stationary; (C) 39 C, log; (D) 39 C, stationary.*

relative abundance in stationary phase, but in this case 18:2 also increases in abundance, whereas 18:3 is unchanged.

Effects of temperature on fatty acid composition. Cells harvested in the exponential phase of growth exhibited changes in fatty acid composition at different growth temperatures (Fig. 2). Unsaturates decrease with increasing temperature. Especially dramatic are the trends exhibited by 12:0 and 12:1 as well as by

the polyunsaturated fatty acids (18:2 and 18:3). In going from lower (30 C) to higher (39 C) temperature the stationary-phase cells show an overall decrease in unsaturated fatty acids (Fig. 1). Striking changes are seen in 18:3, which drops from 12% to a trace, and 12:0, which increases from 6% to over 13% of the total fatty acids.

Silicic acid column fractions were also compared at 30 C (Fig. 3) and 39 C (Fig. 4). These fractions further illustrate the dramatic changes which occur. The neutral lipid fraction shows a near-even distribution of both 12:0 and 12:1 fatty acids at 30 C. However, at 39 C the 12:1 disappears almost completely, whereas 12:0 makes up 76.4% of the fatty acids in this fraction. The changes in the other fractions, though not as dramatic, are significant. At higher temperature, the level of 18:3 is reduced considerably in both fractions and is compensated in part by an increase in 18:2. The neutral lipid fraction can be seen to be composed of the total of 12:0 and 12:1 fatty acids. Any net change in the combined total amount of these two fatty acids can thus be interpreted as a change in abundance of the neutral lipids.

DISCUSSION

Fatty acid composition varies considerably from organism to organism. Gram-negative bacteria (with rare exceptions) possess only saturated and monounsaturated fatty acids, whereas higher plants and animals can synthesize these as well as polyunsaturated fatty acids (5). Blue-green bacteria appear to be an intermediate group with respect to fatty acid composition (10). Blue-greens, even though they are prokaryotes, with a gram-negative cell wall and

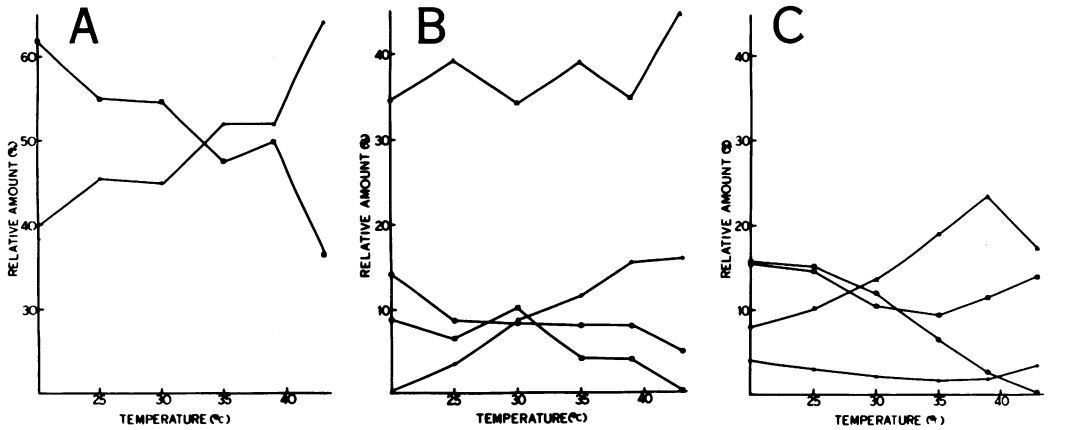

FIG. 2. *Effect of growth temperature on fatty acid composition. (A)* ■, *total unsaturates;* ●, *total saturates. (B)* ▲, *16:0;* ○, *16:1;* ●, *12:0;* ■, *12:1. (C)* ●, *18:0;* ■, *18:1;* ▲, *18:2;* ○, *18:3.*

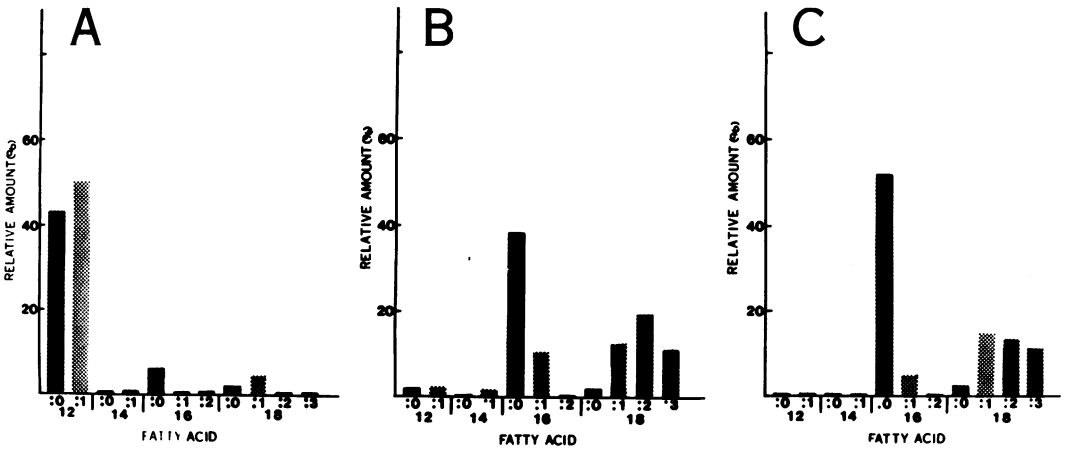

FIG. 3. *Comparison of fatty acid composition of individual lipid fractions at 30 C. (A) Neutral lipids; (B) glycolipids; (C) very polar lipids.*

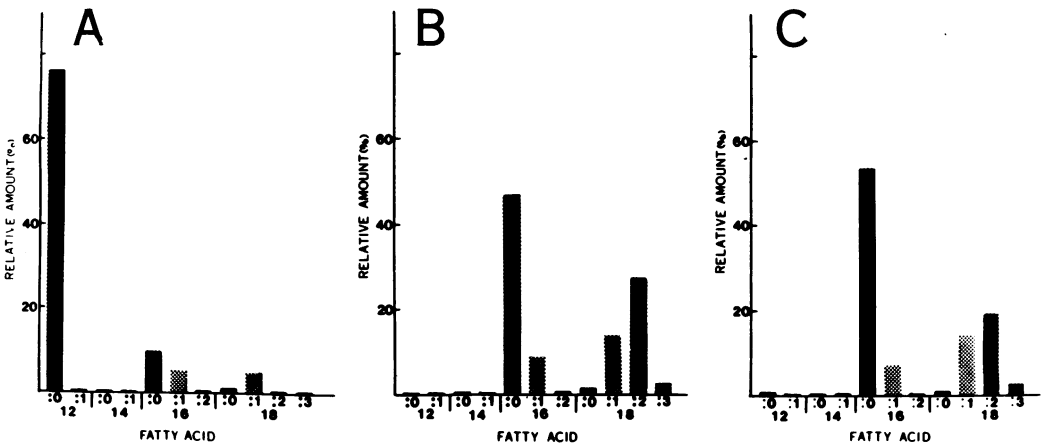

FIG. 4. *Comparison of fatty acid composition of individual lipid fractions at 39 C. (A) Neutral lipids; (B) glycolipids; (C) very polar lipids.*

70*S* ribosomes often have a much more complex fatty acid composition than other bacteria and thus resemble higher organisms in this respect. Some members of this group lack linolenic acid (as do other bacteria); others contain α-linolenic acid characteristic of eukaryotic photosynthesizers; still others synthesize γ-linolenic acid, typically found in animals (10). Thus, the blue-green bacteria as a group seem to represent a transition in fatty acid synthesis between primitive and more complex forms.

The organism examined here, *A. quadruplicatum*, contained appreciable amounts of 18:3 fatty acid. The abundance of this fatty acid, as well as of others, was found to be greatly influenced by a variety of cultural conditions. Some variability with culture conditions was previously reported by Kenyon (14); however, this point was not examined extensively. That study found only minor changes in fatty acid composition, quantitative but not qualitative. In the classification scheme proposed by Kenyon et al. (15) the presence of linolenic acid as a significant fatty acid component was used as a primary characteristic for grouping in conjunction with cultural data. In our system, major changes in this fatty acid were observed under different growth conditions. At 43 C, linolenic acid was hardly detectable, yet it made up 15% of the total cellular fatty acids at 20 C. Lauroleic acid (12:1) was also barely detectable at 43 C, but it made up 9% of the total cellular fatty acids at 20 C (Fig. 2). Lauric acid was absent at 20 C, but it contributed over 15% of the total fatty acids at 39 C. These last two fatty acids also exhibited qualitative changes when grown in deficient culture media (Table 3). Our results indicate that taxonomic studies in blue-greens should not be based on fatty acids alone and may require more extensive investigation than the comparison of patterns observed under a single growth condition.

Several investigators have shown that in a variety of other organisms environmental conditions (especially temperature) profoundly alter fatty acid composition. Bacteria, algae, fungi, and higher plants all exhibit changes in fatty acid composition with changing temperature (5). Marr and Ingraham, in their investigation of *Escherichia coli* (17), found especially striking changes with lowered growth temperature. In *E. coli*, palmitate represented 48% of the fatty acid at 43 C but only 18.2% at only 10 C. At 10 C, the monounsaturates palmitoleic and cis-vaccenic (originally thought to be oleic) acids were found to increase to three times their original abundance at 43 C. In an examination of mesophilic and psychrophilic yeasts, Kates and Baxter (13) found that the saturated fatty acids palmitate and stearate decreased in relative abundance at lower temperatures. This decrease was compensated for by an increase in the polyunsaturated fatty acids linoleic and linolenic acids. In higher plants, Harris and James (7) found that only non-photosynthetic bulb tissue increased in unsaturation of fatty acids at lower growth temperature and that photosynthesizing leaf tissue and the green alga *Chlorella vulgaris* did not undergo a shift to unsaturation with lower growth temperature. Canvin (2) showed that in some seed oils, polyunsaturated fatty acids increase in relative amount with decreasing temperature. Hilditch

and Williams (8) also found that most seed oils become more unsaturated at lower temperatures. Very little work, however, has been done on other parts of the plant, especially the leaves. The changes in *A. quadruplicatum* were similar to those observed in yeast and some higher plant seed oils. In *A. quadruplicatum*, the most dramatic changes occurred with 12:1 and 18:3 fatty acids, which were almost undetectable at 43 C and yet made up more than 9 and 15%, respectively, of the total fatty acid at 20 C. This increase was compensated for by a decrease in 12:0 and 18:2 fatty acids from 17% to a trace amount and from 18 to 8%, respectively. These changes in the levels of 18 carbon fatty acids and 12 carbon fatty acids imply a precursor-product relationship. Other fatty acids showed marked changes: 16:1 decreased from 13 to 5% and 16:0 increased from 34 to 43% as the temperature was raised from 20 to 43 C.

At a given temperature the neutral lipid fatty acids (total of lauric and lauroleic acid) made up a relatively small percentage (9 to 29%) of the total cellular fatty acids, depending on growth temperature (Fig. 3). The lipids of other gram-negative bacteria have been shown to contain 5 to 15% neutral lipid (6). We have also found that fatty acids of glycolipids and very polar lipids are present in similar amounts. In other gram-negative bacteria, glycolipids have been shown to make up anywhere from 1 to 40% of the total lipid, with phospholipids usually making up the majority of the lipids. We are able to detect only a relatively small amount of phospholipid in the form of phosphatidyl glycerol. The striking changes observed in fatty acid composition between neutral lipids versus glycolipids and very polar lipids were surprising, and to our knowledge no one has reported such changes in other organisms. The neutral lipids contained (depending on growth temperature) almost exclusively 12:0 and 12:1 fatty acids. The abundance of these two fatty acids relative to the total fatty acid can be used as an estimate of the total amount of neutral acyl lipids present. This ability to estimate the amount of neutral acyl lipids using the total of these two fatty acids has not yet been reported in other systems.

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LITERATURE CITED

1. Brown, C., and A. H. Rose. 1969. Fatty acid composition of *Candida utilis* as affected by growth temperature and dissolved oxygen tension. J. Bacteriol. **99**:371-378.
2. Canvin, D. T. 1965. The effect of temperature on the oil content and fatty acid composition of the oils from several oil seed crops. Can. J. Bot. **43**:63-69.
3. Dittmer, J. C., and R. L. Lester. 1964. A simple, specific spray for the detection of phospholipids on thin layer chromatograms. J. Lipid Res. **5**:126-127.
4. Freeman, C. P., and D. West. 1966. Complete separation of lipid classes on a single thin layer plate. J. Lipid Res. **7**:324-327.
5. Fulco, A. J. 1974. Metabolic alterations of fatty acids. Annu. Rev. Biochem. **43**:215-241.
6. Goldfine, H. 1972. Comparative aspects of bacterial lipids, p. 1-58. *In* A. H. Rose and D. W. Tempest (ed.), Advances in microbial physiology, vol. 8. Academic Press Inc., New York.
7. Harris, P., and A. T. James. 1969. The effect of low temperatures on fatty acid biosynthesis in plants. Biochem. J. **112**:325-330.
8. Hilditch, T. P., and P. N. Williams. 1964. The chemical constitution of natural fats. John Wiley & Sons, New York.
9. Hitchcock, C., and B. W. Nichols. 1971. Plant lipid biochemistry. Academic Press Inc., New York.
10. Holton, R. W., H. H. Blecker, and T. S. Stevens. 1968. Fatty acids in blue-green algae: possible relation to phylogenetic position. Science **160**:545-547.
11. Ingram, L. O., and C. Van Baalen. 1970. Characteristics of a stable, filamentous mutant of a coccoid blue-green alga. J. Bacteriol. **102**:784-789.
12. Kanfer, J., and E. P. Kennedy. 1963. Metabolism and function of bacterial lipids. J. Biol. Chem. **238**:2919-2922.
13. Kates, M., and R. M. Baxter. 1962. Lipid composition of mesophilic and psychrophilic yeasts (*Candida* species) as influenced by environmental temperature. Can. J. Biochem. Phvsiol. **40**:1213-1227.
14. Kenyon, C. N. 1972. Fatty acid composition of unicellular strains of blue-green algae. J. Bacteriol. **109**:827-834.
15. Kenyon, C. N., R. Rippka, and R. Y. Stanier. 1972. Fatty acid composition and physiological properties of some filamentous blue-green algae. Arch. Mikrobiol. **83**: 216-236.
16. Krebs, K. G., D. Heusser, and W. Wimmer. 1969. Spray reagents. *In* Egon Stahl (ed.), Thin layer chromatography. Springer-Verlag, New York.
17. Marr, A. G., and J. L. Ingraham. 1962. Effect of temperature on the composition of fatty acids in *Escherichia coli*. J. Bacteriol. **84**:1260-1267.
18. Naccarato, W. F., J. R. Gilbertson, and R. A. Gelman. 1974. Effects of different culture media and oxygen upon lipids of *Escherichia coli* K-12. Lipids **9**:322-327.
19. Nichols, B. W. 1973. Lipid composition and metabolism. *In* N. G. Carr and B. A. Whitlon (ed.), Biology of blue green algae. University of California Press, Berkeley.
20. Shaw, N. 1974. Lipid composition as a guide to the classification of bacteria, p. 63-108. *In* D. Perlman (ed.), Advances in applied microbiology, vol. 17. Academic Press Inc., New York.
21. Silbert, D. F., R. C. Ladenson, and J. L. Honegger. 1973. The unsaturated fatty acid requirement in *Escherichia coli*. Biochim. Biophys. Acta **311**:349-361.
22. Van Baalen, C. 1962. Studies on marine blue green algae. Bot. Mar. **4**:129-139.
23. Vorbeck, M. L., and G. V. Marinetti. 1965. Separation of glycosyl diglycerides from phosphatides using silic acid column chromatography. J. Lipid Res. **6**:3-6.
24. Yribarren, M., E. Vilkas, and J. Rozanis. Galactosyl diglyceride from *Actinomyces viscosis*. Chem. Phys. Lipids **12**:172-175.