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Unusual Sulfonolipids Are Characteristic of the *Cytophaga-Flexibacter* Group

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Capnocytophaga spp. contain a group of unusual sulfonolipids, called capnoids (W. Godchaux III and E. R. Leadbetter, J. Bacteriol. 144:592-602, 1980). One of these lipids, capnine, is 2-amino-3-hydroxy-15-methylhexadecane-1-sulfonic acid; the others are, apparently, *N*-acylated versions of capnine. The lipids were found, in amounts ranging from 2.5 to 16 μmol of capnoid sulfur per g of cells (wet weight), in two *Cytophaga* spp. and also in several closely related organisms: several *Capnocytophaga* spp., *Sporocytophaga myxococcoides*, two *Flexibacter* spp., and two *Flavobacterium* spp. With the exception of the flavobacteria, all of these bacteria have been shown to exhibit gliding motility. The two *Flavobacterium* spp. belong to a subset of that genus that shares many other characteristics with the cytophagas. Only the *Capnocytophaga* spp. contained large quantities of capnine as such; in all of the others, most (and possibly all) of the capnoids were present as *N*-acylcapnines. Capnoid-negative bacteria included some gliding organisms that may not be closely related to the cytophagas: two fruiting myxobacters, a gliding cyanobacterium (*Plectonema* sp.), *Beggiatoa alba*, *Vitreoscilla stercoraria*, *Herpetosiphon aurantiacus*, and *Lysobacter enzymogenes*. Nongliding bacteria representing nine genera were also tested, and all of these fell into the capnoid-negative group.

Capnocytophaga spp. contain a group of unusual sulfonolipids (15); one of these, capnine, was shown to be 2-amino-3-hydroxy-15-methylhexadecane-1-sulfonic acid. The other sulfonolipids are, apparently, *N*-acylated versions of capnine, for they contain sulfur, are less polar than capnine, and do not react with ninhydrin, but after acid solvolysis, their sulfur-containing moiety is recovered in a ninhydrin-reactive form that exhibits the chromatographic behavior of capnine. The sulfonolipids, collectively termed the capnoids, were shown to be components of the cell envelope and to be major cell components. They are present (in various strains of *Capnocytophaga* spp.) in amounts ranging from 7 to 16 μmol/g of cells (wet weight) (15) and constitute up to 25% of the cellular lipids.

Recently, we undertook a survey to determine the distribution of these interesting lipids among the bacteria. The organisms were grown in media in which the sulfur sources were labeled with ^{35}S; labeled capnoids were identified by chromatography and chemical tests, and the amounts present were estimated by measuring the radioactivity accumulated in the capnoid fractions after the extensive growth of the organisms in the labeled media. The bacteria tested fall into two well-defined groups: organisms containing large amounts of capnoids (2 to 16 μmol/g of cells [wet weight]) and those containing little or no capnoid. The former group includes only species that are demonstrably related to the cytophagas; except for the flavobacteria, all are recognized gliding bacteria. The group of organisms that contains little or no capnoid includes both gliding bacteria (e.g., the fruiting myxobacters), which are, perhaps, only distantly related to the cytophagas, as well as a large variety of nongliding organisms.

MATERIALS AND METHODS

Cultivation and radiolabeling of bacteria. The organisms used in this study are identified in Table 1. All were grown in the presence of ^{35}S until the cell mass had increased at least 10-fold over that added as inoculum (24 to 96 h, depending on the organism, except for the cyanobacteria, which required 14 days). Unless otherwise indicated, cultures were incubated at 30°C under an air atmosphere, with shaking.

Whenever possible, organisms were grown in defined media in which all of the sulfur compounds were labeled to the same specific activity. *Beggiatoa alba* (shaking omitted) was grown in a medium containing sodium acetate (0.5 g/liter), asparagine (0.5 g/liter), NH_4Cl (1.0 g/liter), mineral base (10 ml; 11), $CaCl_2$ (0.031 g/liter), and K_2HPO_4 (0.1 g/liter); the pH was adjusted to 7.5 with KOH. *Escherichia coli* and the *Mycobacterium-Nocardia* sp. were grown in a standard mixture of salts (34) supplemented with glucose (2.0 g/liter); *Pseudomonas aeruginosa* was grown in

TABLE 1. Identification and sources of bacterial cultures

Organism	Strain	Identifying no.[a]	Source (reference)
Beggiatoa alba	B18LD	ATCC 33555	W. R. Strohl, Ohio State University
Bacillus cereus			University of Connecticut collection
Bacteroides melaninogenicus	2015		S. S. Socransky, Forsyth Institute, Boston, Mass.
Capnocytophaga gingivalis	30N51		E. R. Leadbetter (23)
Cytophaga sp., marine	7B		E. R. Leadbetter
Cytophaga johnsonae		ATCC 17061	J. Pate, University of Wisconsin
Escherichia coli	K-12		R. Vinopal, University of Connecticut
Flavobacterium odoratum		ATCC 4651	O. B. Weeks, New Mexico State University
Flavobacterium breve		NCTC 11099	O. B. Weeks, New Mexico State University
Flexibacter sp.	FS-1		R. P. Burchard, University of Maryland (32)
Flexibacter canadiensis	9D	ATCC 29521	F. D. Cook, University of Alberta, Edmonton
Gleocapsa sp.			M. M. Allen, Wellesley College
Halobacterium sp.	R4	CCM 3361	F. Rodriguez-Valera, Alicante, Spain (31)
Herpetosiphon aurantiacus		ATCC 23779	J. Gibson, Cornell University
Lysobacter enzymogenes	138		F. D. Cook, University of Alberta, Edmonton
Mycobacterium-Nocardia sp.	I-19		E. R. Leadbetter
Myxococcus virescens	M1A2		E. R. Leadbetter
Plectonema sp.			M. M. Allen, Wellesley College
Polyangium cellulosum	M-209		H. McCurdy, University of Windsor, Ontario
Pseudomonas aeruginosa			University of Connecticut collection
Sphaerotilus natans	48		A. Romano, University of Connecticut
Spirochaeta aurantia	J1		E. Canale-Parola, University of Massachusetts (3)
Sporocytophaga myxococcoides			E. R. Leadbetter (18)
Vitreoscilla stercoraria		ATCC 15218	ATCC

[a] Abbreviations used: ATCC, American Type Culture Collection; NCTC, National Culture Type Collection; CCM, Czechoslovak Collection of Microorganisms.

the same medium, but with KNO_3 substituted for the NH_4Cl. *Flexibacter* sp. strain FS-1 was grown in a defined medium (32) that was modified by the substitution of an equimolar quantity of KNO_3 for the $(NH_4)_2SO_4$. The *Gleocapsa* sp. and the *Plectonema* sp. were grown (without shaking, at 35°C under a bright fluorescent light) in the medium of Allen (1), with agar omitted. The *Halobacterium* sp. (incubated at 37°C without shaking) was grown in a medium containing NaCl (156 g/liter), $MgCl_2 \cdot 6H_2O$ (45 g/liter), $MgSO_4$ (1.2 g/liter), $CaCl_2$ (1 g/liter), KCl (4 g/liter), $NaHCO_3$ (0.2 g/liter), sodium bromide (0.5 g/liter), KH_2PO_4 (0.5 g/liter), $FeCl_3$ (0.01 g/liter), KNO_3 (2 g/liter), and glucose (1 g/liter); the pH was adjusted to 7.0 with KOH. *Polyangium cellulosum* was grown in a medium (33) with cellobiose (5 g/liter) as the only organic component; *Sporocytophaga myxococcoides* was grown in the same medium with glucose substituted for the cellobiose. *Sphaerotilus natans* was grown in a medium (26) supplemented with filter-sterilized cyanocobalamin (1×10^{-12} g/liter). The defined media contained inorganic sulfate as the only source of sulfur; they were supplemented with $Na_2{}^{35}SO_4$ to give net sulfate specific activities of 1.0×10^6 to 5.0×10^6 cpm/μmol.

Spirochaeta aurantia, which required both cysteine and inorganic sulfate for growth (and was stimulated by other amino acids), was grown in a defined medium containing the salts of basal medium A (3); mineral base (10 ml); NH_4Cl (1.0 g/liter); glucose (2 g/liter); L-cysteine (0.5 g/liter); a mixture of 18 amino acids, not including those containing sulfur (0.02 g/liter each); thiamine hydrochloride (5×10^{-4} g/liter); and biotin (5×10^{-5} g/liter). The amino acids and vitamins were sterilized by filtration. The medium was supplemented with $Na_2{}^{35}SO_4$ and L-[^{35}S]cysteine to adjust the specific activity of each to 1.5×10^6 cpm/μmol.

For some of the organisms tested, defined media were not available or produced poor growth yields; however, many of these organisms incorporated radioactivity from $Na_2{}^{35}SO_4$ into capnoids when they were grown in undefined media containing large amounts of nonradioactive organosulfur compounds (e.g., peptides; see reference 15 for a discussion of this phenomenon). Hence, it was possible to demonstrate the presence of capnoids in some organisms simply by supplementing their undefined media with the labeled sulfate. *Capnocytophaga* spp. were grown as previously described (15). *Cytophaga johnsonae* and *Flexibacter canadiensis* were grown in enriched *Cytophaga* medium (27) that was modified by the omission of the beef extract and the addition of 2 mM Na_2SO_4. The marine *Cytophaga* sp. was grown in a medium containing 2.5 g of tryptone (Difco Laboratories) per liter, 0.5 g of yeast extract (Accumedia) per liter, and 0.05 g of KH_2PO_4 per liter made up in artificial seawater (that included 20 mM inorganic sulfate). The sulfate concentrations of the growth media for *Capnocytophaga* spp. were determined as previously described (15); in the other cases, it was assumed that the sulfate contributed by the undefined components was negligible compared with that contributed by the defined, inorganic salts. The media were supplemented with $Na_2{}^{35}SO_4$ to adjust the specific activity of inorganic sulfate to 1.5×10^6 to 3.0×10^6 cpm/μmol.

A group of organisms was encountered for which no

defined media could be found and that would not incorporate radioactivity into either capnoids or protein when $Na_2{}^{35}SO_4$ was added to their normal growth media, which contained nonradioactive, organic sulfur sources (peptides). To provide a rigorous test for capnoids in these organisms, we grew most of them in the extract of protease-digested, uniformly labeled *E. coli* cells (described below) at a specific activity of 9.0 × 10^6 cpm/μmol of S. The concentrations (dry weight/volume) of the extract in the medium used for the cultivation of each organism were as follows: *Bacillus cereus* (5.0 g/liter), *Bacteroides melaninogenicus* (17.0 g/liter; grown without shaking in tightly capped tubes at 37°C), *Flavobacterium odoratum* (5.0 g/liter), *Flavobacterium breve* (5.0 g/liter), *Herpetosiphon aurantiacus* (5.0 g/liter), and *Lysobacter enzymogenes* (5.0 g/liter). The medium for *Vitreoscilla stercoraria* contained the extract of *E. coli* (4.0 g/liter) plus the inorganic components of medium L (21); the sulfate ion that is a component of medium L (a defined medium) was labeled to the same specific activity as the sulfur in the extract of *E. coli*. *Myxococcus virescens*, which also fell into this group of organisms that would not utilize inorganic sulfate and could not be tested with defined media, was grown in 10 g of tryptone per liter that was supplemented with L-[^{35}S]cysteine and L-[^{35}S]methionine (1.6 × 10^7 cpm of each per ml, at specific activities exceeding 500 Ci/mol).

Extract of uniformly labeled *E. coli* cells. *E. coli* K-12 was grown aerobically from a small inoculum at 37°C in a medium containing K_2HPO_4 (7.0 g/liter), KH_2PO_4 (3.0 g/liter), trisodium citrate (0.5 g/liter), $MgCl_2 \cdot 6H_2O$ (0.12 g/liter), $FeCl_3$ (0.01 g/liter), NH_4Cl (2.5 g/liter), glucose (2.0 g/liter), and $Na_2{}^{35}SO_4$ (0.10 mM, 1.3 × 10^8 cpm/μmol). The Na_2SO_4 was the sole sulfur source and was growth limiting; the cells grew to a density of approximately 3 g (wet weight) per liter and incorporated approximately 90% of the radioactivity into cellular material. After growth overnight, the cells were harvested by centrifugation (12,000 × *g* for 10 min), suspended in water (10 ml/g of cells [wet weight]), sonicated (Branson Sonifier, 90 s, maximum power), and then heated at 100°C for 15 min. The mixture was adjusted to pH 8.3 with Na_2CO_3; trypsin and chymotrypsin (crystalline) were added at the ratio of 1 mg of each enzyme per 0.25 g of cells (wet weight), and the preparation was incubated at 37°C for 24 h. Every 2 h for the first 10 h, the pH was readjusted to 8.3 with Na_2CO_3. The digest was then centrifuged (12,000 × *g* for 10 min), and the precipitate was discarded. The supernatant was lyophilized, and the residue (0.16 to 0.18 g [dry weight] per g of cells [wet weight]) was stored over a desiccant at −20°C. The labeled material was mixed, on the basis of dry weight, with nonradioactive material that had been prepared in the same way (but using nonradioactive Na_2SO_4) to adjust the sulfur specific activity to the desired level, taking into account the decay of radioactivity during storage. (Growing the cells at the lower specific activity would have generated an excessive volume of radioactive waste.) The material was dissolved in water, adjusted to pH 7.3 with HCl, and autoclaved before use as a growth medium.

Determination of radioactivity. Samples of lipid solutions were dried under N_2 to remove organic solvents, and then mixed with 1 ml of 0.1% (wt/vol) aqueous sodium dodecyl sulfate solution and 10 ml of Scintiverse (Fisher Scientific Co.). Samples (20 to 100 μl) of appropriately diluted culture media were added to 1 ml of water and mixed with 10 ml of Scintiverse. The insoluble residue (mostly protein) remaining after the extraction of cells with solvent A (acetone–methanol–17 N NH_4OH, 5:5:1 [vol/vol]) was mixed with 1% (wt/vol) sodium dodecyl sulfate (approximately 20 ml/g of cells [wet weight]) and heated with stirring at 80 to 90°C until the solids were either dissolved or very finely divided, after which a sample (100 μl) of this preparation was mixed with 1 ml of water and 10 ml of Scintiverse. Radioactivity was measured in an ambient-temperature liquid scintillation counter; the counting of internal standards indicated that the counting efficiency was the same (approximately 80%) for all mixtures used. Autoradiography was carried out as previously described (15), except that the scintillation fluors were omitted.

Radioactivity in capnoids. All determinations were carried out in duplicate. Cells (0.2 to 1.0 g, from 0.1 to 1.0 liter of culture, depending on the organism) were harvested, weighed, washed with trichloroacetic acid solution and then with water, and extracted with solvent A as previously described (15), except that, for cells grown in the extract of *E. coli* cells, the washes with trichloroacetic acid were omitted and two water washes were employed. The washing procedure does not result in the loss of capnoids, and solvent A extracts them quantitatively (15). Samples of the solvent A extracts and of the residual insoluble matter were taken for the determination of radioactivity. Additional samples of the extracts were applied to thin-layer plates for chromatography in system I. Standards consisting of ^{35}S-capnoids, either in the form of crude extracts from *Capnocytophaga* spp. or of purified capnoids, were included on each plate. Zones containing ^{35}S were located by autoradiography; those corresponding to capnoids or otherwise of interest were scraped quantitatively from the plates. The silica gel was washed twice by centrifugation with solvent A (4 ml/ml of packed silica gel), and radioactivity in the eluate was measured. Control experiments using labeled, radiochemically pure capnoids have shown that the sulfonolipids are eluted quantitatively by this procedure. Where no zone could be detected by autoradiography, it was considered that the radioactivity in the corresponding capnoid was less than 100 cpm; calibration using known amounts of radioactive capnoids indicated that this amount could easily be detected when present in a typical capnoid zone.

Calculation of capnoid content. The cellular content of a given sulfonolipid component was calculated as the radioactivity in that component per gram (wet weight) of cells, divided by the specific activity of the sulfur in the growth medium. When no radioactivity could be detected autoradiographically in a zone corresponding to a capnoid, the detection limit of 100 cpm was used in the above calculation. With one exception, experimental conditions for each organism were chosen so that the detection limit thus calculated was no greater than 0.02 μmol of capnoid sulfur per g of cells [wet weight]; the exception was the *Halobacterium* sp., for which the limit was 0.06 μmol/g.

In some cases, bacteria were grown in undefined media containing $Na_2{}^{35}SO_4$ and nonradioactive peptides, and the sulfate specific activity was used to

estimate capnoid content, as described above. This method (used only for organisms that clearly contained capnoids) provided minimum estimates of content, since any capnoid sulfur derived from sources other than sulfate (e.g., from peptides) would not have been detected. In the cases of *Capnocytophaga gingivalis* (strain 30N51) and the marine *Cytophaga* sp., more accurate estimates were made by isolating pure samples of capnine from the cells and determining their specific activity; the specific activity of capnine was taken to be the effective sulfur specific activity and was used to calculate capnoid contents. The procedure for the isolation of capnine from the *Capnocytophaga* sp. and for the determination of its specific radioactivity was previously described (15). The crude lipid extract from the *Cytophaga* sp. was dried under N_2, and the residue was heated in a methanol-water-HCl mixture (as described below), again dried, and redissolved in solvent A before the isolation of capnine.

One organism (*M. virescens*) that would not incorporate ^{35}S from $Na_2{}^{35}SO_4$ into capnoids or protein was grown in a medium consisting of casein peptides (tryptone) supplemented with ^{35}S-amino acids. Protein was prepared by a standard procedure from the residue remaining after the extraction of the lipids from the cells, and its specific activity was determined from counting rates and dry weight. The specific activity of the protein sulfur, calculated on the assumption that the protein was 1% sulfur by dry weight, was taken as the effective sulfur specific activity and was used to estimate capnoid content. The overall specific activity of the sulfur in the growth medium was determined from its counting rate and total sulfur content. The latter value was estimated from the fact that tryptone contains 0.61% sulfur by dry weight; 93% of this is organosulfur (10).

Thin-layer chromatography. Chromatography was carried out in system I (15), which was silica gel G (250-μm thick, for analytical procedures) developed with a mixture of chloroform, methanol, and NH_4OH. For preparative procedures, layers 1-mm thick were employed; samples, representing the material extracted by solvent A from up to 200 mg of cells (wet weight), were streaked across the width of the plate. After development and drying, the *N*-acylcapnine zones were located by autoradiography and scraped from the plates. The silica gel was placed in a chromatographic tube fitted with a fritted glass disk, and the *N*-acylcapnines were eluted with solvent A (4 ml/ml of packed adsorbent).

Deacylation of *N*-acylcapnines. Samples of lipids in solvent A were evaporated to dryness under a stream of N_2. Hydrolysis in 6 N aqueous HCl was carried out as previously described (15). More recently, we have employed an HCl-water-methanol mixture as the deacylating agent. The dried lipids were dissolved in 1 M HCl–10 M H_2O in methanol (14; at least 0.3 ml/mg of lipid [dry weight]) and heated at 70 to 75°C in a tightly capped tube for 16 h. The solvents and HCl were removed under a stream of N_2, and the residue was dissolved in solvent A.

Isolation of capnoids. Capnine was isolated, and radiochemically pure *N*-acyl[^{35}S]capnines were prepared by column chromatography, as previously described (15).

Materials. Radiochemicals were obtained from New England Nuclear Corp., Boston, Mass. The $Na_2{}^{35}SO_4$ was obtained at specific activities greater than 50 Ci/mol, except when it was used to prepare the labeled extract of *E. coli*, in which case carrier-free material was used. The L-[^{35}S]cysteine and L-[^{35}S]methionine, as supplied, had specific activities exceeding 500 Ci/mmol. In all cases (except, possibly, that of *M. virescens*), radiochemicals that were added to growth media were at a sufficiently high specific activity that their contribution to the concentration of the corresponding compounds in the media was less than 5%. Other materials were obtained from sources previously described (15).

RESULTS

Organisms containing large quantities of capnoids. Typical autoradiograms obtained after the chromatography of crude lipid extracts from ^{35}S-labeled cells that contained large amounts of capnoids are shown in Fig. 1, lanes a and b. The preliminary identification of capnoids was made by a comparison of their chromatographic behavior with that of standards (Fig. 1, lane a) obtained from the *Capnocytophaga* spp. which were the source of the capnoids previously characterized (15). *N*-Acylcapnines were usually resolved into two or three zones (Fig. 1, lanes a and b). The identity of all compounds that migrated in these three zones and that are reported in Table 2 to be *N*-acylcapnines was confirmed by degradation. Portions of the extracts were subjected to preparative thin-layer chromatography. The *N*-acylcapnine region, located autoradiographically, was scraped from the plates (without an attempt to separate the three zones), and the labeled lipids were eluted, deacylated by one of the two procedures described above, and rechromatographed with a standard of authentic capnine and a sample of the original *N*-acylcapnines that had not been deacylated. In the case of each organism listed in Table 2, the radioactivity from the mixture of *N*-acylcapnines was converted quantitatively by deacylation to a form that was chromatographically homogeneous and that exhibited the R_f of capnine. An autoradiogram from a typical experiment appears in reference 15. The result implies that the heterogeneity of the *N*-acylcapnines must lie in the *N*-acyl groups and not in the aminosulfonate moiety (which is, presumably, capnine). A possible basis for the heterogeneity is provided by our recent observation (unpublished data) that the *N*-acyl groups include nonhydroxylated and 3-hydroxylated fatty acid residues and, in some organisms, 2-hydroxylated residues as well.

The capnoid content of various organisms is summarized in Table 2. The agreement between replicate determinations was good; nonetheless, the values must be taken merely as indicative of a high content, and comparisons among the

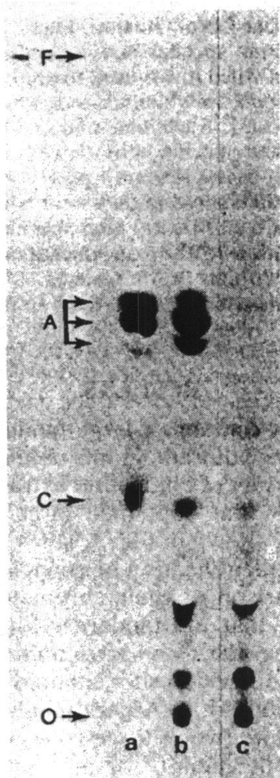

FIG. 1. Chromatography of crude lipid extracts from ^{35}S-labeled bacteria. The figure shows an autoradiogram of a thin-layer plate, prepared as described in the text. Lane a, Capnoid standard: a mixture of crude extracts from cells of *C. gingivalis* 30N51 and *Capnocytophaga* sp. strain P7. The capnoids of these organisms have been previously characterized (15). The former organism contains mostly free capnine, and the latter contains mostly *N*-acylcapnine; the mixture was adjusted so that 66% of the radioactivity was in *N*-acylcapnines. The cells were labeled at a sulfate specific activity of 4×10^6 cpm/μmol; the sample applied (6,000 cpm total) represents the extract from 0.3 mg of cells (wet weight). Lane b, Crude extract from cells of *F. breve*; the sample applied (25,000 cpm) was the material from 0.60 mg of cells (wet weight) labeled at a sulfur specific activity of 9.0×10^6 cpm/μmol of S. Lane c, Crude extract from cells of *H. aurantiacus*; the sample applied (25,000 cpm) was the material from 0.29 mg of cells (wet weight), labeled at a sulfur specific activity of 9×10^6 cpm/μmol of S. Abbreviations: O, origin; C, capnine; A, *N*-acylcapnines; F, solvent front.

organisms listed must be made with caution. Two examples will illustrate this. The capnoid content of *S. myxococcoides* was near the lower end of the range observed. However, the extracellular polysaccharides elaborated by this organism (25) are known to be present in cell pellets, and, therefore, the value based on the wet weight of those pellets undoubtedly represents an underestimate. The values for *F. breve* and *F. odoratum* were, respectively, 2.1 and 3.6 μmol of capnoids per g of cells (wet weight); however, the values for sulfur in nonlipid material (calculated from the amount of radioactivity in the residue remaining after the extraction of the lipids from the cells) were, respectively, 15.9 and 33.3 μmol of sulfur per g of cells (wet weight). Hence, the ratio of capnoid sulfur to nonlipid (protein?) sulfur was nearly the same for the two organisms, and the difference in the values based on wet weight may simply reflect a difference in packing density after centrifugation (although, of course, differences in other sulfur-containing cellular products could play a role). Despite these uncertainties, the data serve to demonstrate a marked dichotomy in capnoid content between the organisms listed in Table 2 (2 to 10 μmol/g) and those that were judged to be capnoid negative, none of which contained more than 0.06 μmol of *N*-acylcapnines or (with two possible exceptions) of capnine per g.

Capnocytophaga spp. contain substantial quantities of free (nonacylated) capnine in addi-

TABLE 2. Capnoid content of bacteria containing large quantities of capnoids

Organism	Capnoid content[a] (μmol/g of cells [wet wt])	*N*-Acylcapnines (mol% of total capnoids)
C. gingivalis	10.0 ± 0.1[b]	15
Cytophaga sp. strain 7B	10.1 ± 0.1	>99[c]
C. johnsonae	≥3.7 ± 0.3[d]	>99
F. breve	2.1 ± 0.2	≥83
F. odoratum	3.6 ± 0.3	≥96
Flexibacter sp. strain FS-1	3.2 ± 0.2	≥98
F. canadiensis	≥7.6 ± 0.3[d]	>99
S. myxococcoides	2.4 ± 0.2	>99

[a] Determined from the incorporation of ^{35}S into capnoids during extensive growth, as described in the text. Values are the mean (± the standard deviation) of at least two independent determinations.

[b] We have previously reported (15) that, among six other *Capnocytophaga* strains, capnoid content ranges from 72 to 156% of the 10 μmol/g found in the type strain listed here.

[c] No radioactive nonacylated capnine zone was detected by thin-layer chromatography under conditions where an amount of radioactivity equal to 1% of the amount that was present as *N*-acylcapnines would have been detected.

[d] These organisms were grown in media containing $Na_2{}^{35}SO_4$ plus nonradioactive organosulfur compounds (peptides). The capnoid content was calculated using the specific activity of the inorganic sulfate; since capnoid sulfur derived from organic sources would not have been detected, the values represent minimum estimates.

tion to *N*-acylcapnines (15); *C. gingivalis* 30N51 had the highest proportion of free capnine yet encountered in any bacterium (Table 2). In this respect, the genus *Capnocytophaga* is unusual. Of the other organisms listed in Table 2, most contained little or no free capnine. With the exception of the flavobacteria and *Flexibacter* sp. strain FS-1, none exhibited, in chromatograms of their extracts, any radioactive zones at R_fs less than that of the slowest *N*-acylcapnine component. The preparation from the *Flexibacter* sp. gave a faint zone having an R_f equal to that of capnine; this represented only 0.06 μmol of sulfur per g of cells (wet weight). Each *Flavobacterium* sp. exhibited a single zone at an R_f close to that of capnine (Fig. 1b). The amounts of sulfur represented were 0.16 and 0.36 μmol/g of cells (wet weight) in *F. odoratum* and *F. breve*, respectively. Because of the low abundance of these components, no further attempt was made to determine whether they were capnine or closely related compounds. The proportions of *N*-acylcapnines given for these organisms (Table 2) were calculated on the assumption that these components were capnine and thus represent minimum values.

Organisms containing little or no capnoid. In contrast to the bacteria listed in Table 2, all of the other organisms listed in Table 1 contained little or no capnoid. In chromatograms of their lipid extracts (Fig. 1, lane c), zones in the *N*-acylcapnine region or within 0.1 R_f unit of its upper or lower extreme, where detected at all, did not contain radioactivity representing more than 0.04 μmol of sulfur per g of cells (wet weight). Only once did the detection limit exceed 0.02 μmol/g: in the *Halobacterium* sp., no zones were found, but the limit was 0.06 μmol/g. In many cases, the limit of the *N*-acylcapnine content could be set at values below 0.01 μmol/g. In one case only, that of *B. alba*, there were intensely radioactive zones of R_fs intermediate between those of capnine and the *N*-acylcapnine standards. On the grounds that these might have been capnine acylated with groups less polar than is common, the material was deacylated and reexamined, but none of the labeled products exhibited the behavior of capnine. Hence, the possible *N*-acylcapnine content of these organisms was, in all cases, more than 20-fold less than the smallest *N*-acylcapnine content found for a capnoid-positive organism (1.5 μmol/g for the *Capnocytophaga* sp.; Table 2).

In the preparations from all but three of the capnoid-negative organisms, radioactivity in compounds that could have been nonacylated capnine also represented no more than 0.04 μmol of sulfur per g of cells, and frequently the limits of content could be set at much lower values. In most cases, simple chromatography sufficed for this determination, but for some organisms additional tests were required. The preparations from the *Halobacterium* sp., the *Gleocapsa* sp., and the *Plectonema* sp. each exhibited an intensely radioactive zone having the R_f of capnine. However, when the lipids from these organisms were carried through the deacylation procedure before chromatography, these zones were absent and were replaced by radioactive zones having R_fs less than 0.1. Therefore, the components originally observed could not have been capnine, which is not affected by the deacylation procedure. For three organisms, the limits established for the content of nonacylated capnine were higher than 0.04 μmol/g. As noted above, the detection limit for the *Halobacterium* sp. was 0.06 μmol/g. The preparations from *H. aurantiacus* (Fig. 1, lane c) and *B. cereus* each showed a faint zone at an R_f very close to that of capnine, representing, respectively, 0.1 and 0.2 μmol of sulfur per g of cells in the two organisms. The larger of these amounts was only 10% of the smallest capnoid content found for a positive organism (Table 2), and no further attempt was made to characterize the compounds.

With the exception of *M. virescens*, all of the capnoid-negative bacteria were tested under the condition that all of the potential sulfur sources in the growth media were uniformly labeled with ^{35}S. As an additional check on the reliability of the tests, we measured the amounts of radioactivity in the nonlipid material (mostly protein) remaining after the extraction of the cellular lipids. The data obtained, when divided by the sulfur specific activity, gave values ranging from 5 to 49 μmol of sulfur per g of cells (wet weight), indicating substantial utilization of the ^{35}S-labeled precursors for the synthesis of cellular products. Those organisms giving values at the low end of the range tended also to be those for which the lowest limits of weight-specific capnoid content were established. In the case of *M. virescens*, the specific activity of the sulfur in cellular protein was 2.9×10^6 cpm/μmol (estimated as described above), whereas the overall specific activity of sulfur in the growth medium was 9.3×10^6 cpm/μmol, indicating that the ^{35}S-amino acids were utilized extensively for the synthesis of protein. Nonetheless, the result for this organism depends upon the assumptions that (i) the nonradioactive sulfur of the peptides in the growth medium would not have been preferred over amino acid sulfur as a precursor of capnoids to a much greater extent than it was preferred as a precursor of protein and (ii) that the tryptone, which was prepared from a purified protein but not under our control, did not contain nonradioactive, heretofore unidentified

sulfur compounds that would be preferred as precursors of the capnoids. The result for *M. virescens* is supported by that for the other fruiting myxobacter, *P. cellulosum*, which was tested under the most rigorous conditions.

DISCUSSION

The results that we present here are of interest and significance for at least two reasons: one relates to the possibility that the capnoids may represent a chemotaxonomic marker for a subset of gliding bacteria, and the other relates to the prospect that these sulfonolipids may play a role in the gliding motility that is so characteristic of the *Cytophaga-Flexibacter* complex.

In the present survey, the capnoids were found to be present in large quantities in gliding bacteria of the genera *Cytophaga*, *Flexibacter*, *Sporocytophaga*, and *Capnocytophaga*—organisms judged on other grounds to have much in common—and absent in representatives of other genera of gliding bacteria, such as *Lysobacter*, *Polyangium*, *Myxococcus*, *Beggiatoa*, *Vitreoscilla*, *Herpetosiphon*, and *Plectonema*, genera with characteristics that distinguish them from those named earlier. Numerous nongliding bacteria were tested as well. With the exception of two *Flavobacterium* spp. (which are discussed below), all were found to lack capnoids.

The properties shared by the bacteria that were found to contain capnoids include DNA with a low guanine plus cytosine (G+C) content (less than 50 mol%; 7, 8, 38), occurrence as single, fusiform (but not filamentous) cells (7, 29), a substantial content of flexirubin-type pigments (30), and a high content of isobranched fatty acids, including 2- and 3-hydroxylated species (13; unpublished data). In contrast, the gliding bacteria in which the capnoids were found to be absent have multiple characteristics which distinguish them from those that contain capnoids. *Lysobacter* spp., although they bear a morphological resemblance to the cytophagas, have DNA with a high G+C content (65 to 68 mol%; 9). The fruiting myxobacters, exemplified by *M. virescens* and *P. cellulosum*, differ from the cytophagas in that they exhibit a complex life cycle and have DNA with a high G+C content (28). The cyanobacteria (represented by the *Plectonema* sp.) differ from the cytophagas in many respects, including the presence of photosynthesis. *Herpetosiphon* spp., although their DNA has a low G+C content, differ from the cytophagas in cellular morphology (they occur as extremely long filaments); it has been proposed that these organisms are more closely related to the cyanobacteria than to the cytophagas (17, 29). Members of the genera *Beggiatoa* and *Vitreoscilla* differ from the cytophagas in that they grow as long, multicellular filaments and exhibit (in some cases, at least) different physiological properties (37). (We should note here that in an earlier report [22], we included *Vitreoscilla stercoraria* among those bacteria which contain capnoids; this report was erroneous, for the organism then studied turned out to have a morphology more akin to that of *Cytophaga* than to that of *Vitreoscilla* and was clearly a mislabeled culture.) There has been a tendency to classify gliding bacteria together on the basis of their means of motility, despite the diversity of their physiological and morphological traits (20); it was recognized, however, that this was largely a matter of convenience and that the relationship among the groups would be clarified by further, detailed studies. We take the lack of capnoids among the *Lysobacter*, *Herpetosiphon*, *Plectonema*, *Beggiatoa*, and *Vitreoscilla* spp. and the fruiting myxobacters to constitute additional evidence that these organisms are distinct from the *Cytophaga-Flexibacter* complex.

The apparent exclusive relationship between the capnoids and what may be termed the simple gliding bacteria may, at first, appear to be negated by our demonstration of these compounds in some of the members of the genus *Flavobacterium*. These organisms are not generally considered to be gliding bacteria (36). In recent years, however, it has become apparent that the members of *Flavobacterium*, which has been regarded as a "catch-all" or "wastebasket" genus (36), fall into two distinct groups, one in which the DNA has a high G+C content and one in which it has a low content. It would appear that, despite the fact that these organisms are not known to glide, those species which have a low G+C content would more appropriately be classified with the cytophagas than with the other flavobacteria. These low G+C flavobacteria share with the cytophagas (but not with the other flavobacteria) a number of properties (5, 12, 24, 30, 35), in addition to the base ratio of their DNA. It is of particular interest, then, that the two members of *Flavobacterium* in which we have detected capnoids—*F. odoratum* and *F. breve*—are in the subset of the genus that has a low G+C content, as well as the other *Cytophaga*-like characteristics (16). It is possible that the low G+C flavobacteria are nongliding derivatives of the cytophagas. Under any circumstances, it clearly becomes important to examine other members of this subset of the genus *Flavobacterium* for capnoids, as well as to survey those members of this genus which lack the *Cytophaga*-like properties.

We have previously suggested (15) that the capnoids might play a role in gliding motility, perhaps by conferring some special surface

property upon the cell. This study provides some circumstantial evidence for that view: with the exception of the flavobacteria, all of the organisms that were found to contain capnoids are gliders, and none of the nongliding organisms was found to contain these sulfonolipids. The finding of capnoids in certain flavobacteria does not disprove the hypothesis. These organisms may be relatives of the cytophagas that have lost the ability to glide (at least, under the laboratory conditions that have been tested). The loss of this ability would not necessarily imply the loss of the capnoids, since there must be many cellular components that are necessary for gliding, a process that is affected by many genes (4). The finding that some gliding bacteria do not contain capnoids does not rule out a role for them in that process in the *Cytophaga*-like bacteria, as different substances could fill their role in different organisms. In fact, Castenholz (6) has reviewed the extant evidence relevant to the mechanisms of gliding and has concluded that, although the mechanisms remain unknown, they cannot be the same in all organisms.

Although the capnoids, per se, appear to be found only in cytophagas, a closely related lipid has been found in a eucaryote, the diatom *Nitzschia alba* (2). This lipid is a 1-deoxyceramide-1-sulfonic acid; it shares the 1-sulfonic acid, 2-*N*-acylamino, and 3-hydroxyl groups of *N*-acylcapnine, but the hydrocarbon chain of the aminosulfonate moiety is that of sphingosine (18 carbons, unbranched and 4,5-unsaturated) rather than that of capnine (17 carbons, isobranched and saturated). The only obvious similarity between the diatoms and the cytophagas is that organisms of both groups move by gliding (19). It will be of interest to examine other eucaryotes for capnoid-like lipids, as well as to investigate the possible role of the capnoids in gliding motility.

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