Sulfonolipids of Gliding Bacteria

STRUCTURE OF THE N-ACYLAMINOSULFONATES*

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Earlier (Godchaux, W., and Leadbetter, E. R. (1980) J. Bacteriol. 144, 592–602; (1983) J. Bacteriol. 153, 1238–1246) we demonstrated that an unusual class of sulfonolipids are major components of the cell envelope of gliding bacteria of the genus Cytophaga and of closely related genera. One of these lipids, to which we have assigned the trivial name capnine, was purified and was shown to be 2-amino-3-hydroxy-15-methylhexadecane-1-sulfonic acid (which might also be named as 1-deoxy-15-methylhexadecasphinganine-1-sulfonic acid). Though capnine accumulates as such in the cells of some Capnocytophaga spp., most organisms of the Cytophaga-like genera contain, instead, sulfonolipids that are less polar than capnine. These less polar lipids have been purified from a Capnocytophaga sp., a marine Cytophaga sp., Cytophaga johnsonae, and a Flexibacter sp. Acid methanolysis of the lipids yielded both aminosulfonates and a collection of fatty acid methyl esters. The infrared absorption spectra of the lipids indicated that the fatty acids were in amide (and not ester) linkage to the aminosulfonates. In every instance, analysis by mass spectrometry and other methods revealed that most, if not all, of the aminosulfonates obtained by methanolysis were structurally identical to capnine (though small amounts of variants of that compound may be present in some cases). The less polar sulfonolipids are, therefore, predominantly N-fatty acyl capnines, 1-deoxy-1-sulfonic acid analogs of ceramides. The fatty acid methyl esters obtained from the lipids were heterogeneous, but in all cases were rich in hydroxylated fatty acyl groups, which constituted 66 to 95% of the total.

A group of unusual sulfonolipids (Fig. 1) has been found in bacteria of the genera Cytophaga, Capnocytophaga, Sporocytophaga, and Flexibacter (1), organisms that are noted for their gliding motility (they possess the ability to move over solid surfaces but not through liquids and possess no known locomotor organelles, such as flagella). One of the lipids, to which we have assigned the trivial name capnine, has been purified from cells of a Capnocytophaga sp., and evidence has been obtained (2) that it is 2-amino-3-hydroxy-15-methylhexadecane-1-sulfonic acid (which could also be named as 1-deoxy-15-methylhexadecasphinganine-1-sulfonic acid). The other sulfonolipids, which were detected radiochemically after growth of the cells on sulfur sources labeled with 35S, were suspected to be N-acylated versions of capnine (or some closely related aminosulfonate), for they were less polar than capnine and did not react with ninhydrin but, after acid-catalyzed solvolysis, their sulfur was recovered in a form that exhibited the chromatographic properties of capnine and that reacted with ninhydrin. The sulfonolipids are major components of the cell envelope (2). They are present, in various organisms, in amounts ranging from 2 to 10 μmol/g of cells (wet weight) and may constitute as much as 20% of the cellular lipids (1, 2). Though capnine accumulates in non-acylated form in some Capnocytophaga spp., the less polar sulfonolipids are the predominant forms in most of the Cytophaga (1) and Capnocytophaga (2). These lipids have recently been purified from a Capnocytophaga sp., strain P7; Cytophaga johnsonae, ATCC 17061; a marine Cytophaga sp., strain 7B; and a Flexibacter sp., strain FS-1 (3). Preliminary characterization confirmed that they were, in fact, N-acylaminosulfonates. We report, here, on the details of the structures of these lipids. In each organism, the great majority of the aminosulfonate moieties of the lipids are structurally identical to the native capnine previously isolated from a Capnocytophaga sp. The fatty acyl groups of the lipids are exclusively in amide linkage to the aminosulfonate moieties. Hence, the lipids are, predominantly, N-acylcapnines; they are 1-deoxy-1-sulfonic acid analogs of ceramides. Though the fatty acyl groups of the N-acylaminosulfonates are heterogeneous, in all cases examined they are rich in hydroxylated fatty acyl groups.

EXPERIMENTAL PROCEDURES

Purification of Sulfonolipids—Bacterial cells were obtained and cultivated, and their N-acylaminosulfonates were purified, as previously described (3). Briefly, the lipids were extracted into chloroform/methanol/water and partitioned into chloroform; the N-acylaminosulfonates were adsorbed to hydroxylapatite from that solution, the adsorbent was washed with neutral solvents, and the N-acylaminosulfonates were then eluted with chloroform/acetic acid solution. The N-acylaminosulfonates were adsorbed to DEAE-cellulose from the chloroform/acetic acid solution, the adsorbent was washed with neutral and acidic solvents, and the N-acylaminosulfonates were eluted with an alkaline solvent (which deprotonated the O-diethyihyminoethyl groups of the adsorbent), converted to their sodium salts, and dried. Capnine was purified (2) from cells of Capnocytophaga gingivalis, strain 30N51, and was then recrystallized. For this, the capnine was dissolved in methanol/17 N NH4OH (9:1, v/v; 0.5 ml/g) at 45 °C and the solution was stored at room temperature in a desiccator containing, in separate vessels, methanol and citric acid crystals; the citric acid was replaced when it became wet. Capnine crystals, which formed during 1 to 2 weeks, were washed with cold methanol and dried; yields were 80 to 85% of the starting material, by weight.

Decylation of the N-Acylaminosulfonates—N-Acylaminosulfonates (sodium salts, 100 mg) were dissolved in 30 ml of 1 N HCl, 10 ml of H2O in methanol at 70 °C (4) and heated in a sealed tube at that temperature for 20 h; the reaction mixture was then stored at −20 °C overnight. The crystals that formed were collected by centrifugation, washed once with 10 ml of cold methanol, and dried to constant

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weight (under a stream of N2 and then in vacuo) to yield the amino-
sulfonate fraction.

The combined supernatant and wash from this procedure were
extracted twice with 40-ml portions of hexane. The combined hexane
phases were dried over Na2SO4, and the solvent was removed under
a stream of N2. To ensure that all fatty acids were in the form of
methyl esters, the residue was carried through the esterification
procedure (described below) to yield the fatty acid methyl ester
fraction.

**Fatty Acid Methyl Esters from Whole Cells—**Wet packed bacterial
cells (2 g) were suspended in 20 ml of a solution made by diluting 20
ml of concentrated (11.7 N) HCl to 100 ml with methanol. The
mixture was heated in a sealed tube at 75 °C for 5 h; the contents
were mixed occasionally until the solids were uniformly dispersed.
The mixture was then extracted twice with 20-ml portions of hexane.
The combined hexane phases were dried over Na2SO4, the solvent
was removed under a stream of N2, and the residue was subjected to
the esterification procedure for fatty acids (described below).

**Methyl Esterification of Fatty Acids—**Fatty acids (2 to 20 mg) were
dissolved in 2 ml of a 10% (w/v) solution of BC13 in methanol; the
mixture was evaporated under a stream of N2 to approximate-
ly one-half its original volume, acidified with 0.2 ml of 6
HC1 to 100 ml with methanol. The
mixture was allowed to boil for 2 min at 60 °C and was then
vacuum-dried to a volume of approximately 0.5 ml under a stream of
N2. Water (2 ml) was added and the mixture was extracted twice with
3-ml portions of hexane. The combined hexane phases were dried over
Na2SO4 and the solvent was removed under a stream of N2. The fatty
acid methyl esters were dissolved in acetone for gas chromatography.

**Oxidation of Aminosulfonates—**Oxidation with periodate in
solution, was carried out as described previously for capnine (2), except
that the procedure was scaled down to employ 1 mg of aminosulfonate.
For oxidation with periodate and permanganate, the aminosulfonate
(8 mg) was dissolved in a mixture of 5 ml of t-butyl alcohol and 2.5
ml of water at 60 °C. The temperature was reduced to 40 °C, and
then 1.25 ml of 4 mM Na2CO3 and 2.5 ml of 30 mM NaIO₃, 2.5 mM KMnO4
were added. The mixture was stirred vigorously for 4 h at 40 °C, after
which solid sodium metabisulfite was added until the solution was
colourless. The mixture was evaporated under a stream of N2 to
approximately one-half its original volume, acidified with 0.2 ml of 6
N H2SO4, and extracted twice with 5-ml portions of hexane. The
combined hexane phases were dried over Na2SO4 and the solvent
was removed under a stream of N2, after which the residue (fatty acids)
was carried through the esterification procedure.

**N,O(Bis)acetylcapnine—**Capnine (doubly ionic form, 50 mg) was
heated with 2 ml of pyridine/acetic anhydride (1:1, v/v); both freshly
distilled at 70 °C for 1.5 h, after which all solids had dissolved. The
reagents were removed under a stream of N2 and the residue was
exposed to a high vacuum (over a cold trap) for 2 to 3 h. The dry
material was taken up in methanol (approximately 5 ml) and passed
through a column of Dowex 50-X4, sodium form, 200–400 mesh (bed
volume, 1 to 2 ml, equilibrated with methanol). The column was
washed with 5 ml of methanol, and the combined effluent fractions
were evaporated nearly to dryness under a stream of N2. The residue
was dissolved in 1 ml of chloroform/methanol (1:1, v/v) and 9 ml of
acetone were added, with stirring. Crystals formed during storage at
−20 °C for several days; these were washed once with cold acetone
and dried to constant weight in vacuo. The yield was 55 mg (dry
weight) of the sodium salt. In some experiments hexadecenoic
anhydride (99 atom % D) was used.

**Sulfonolipids of Gliding Bacteria—**System I, II, and III have been
described (2). System IV was Silica Gel G developed with petroleum
ether (boiling point, 35 to 60 °C)/diethyl ether (85:15, v/v). Silica gel
layers were 250 μm thick for analytical procedures and 1 mm thick for
preparative procedures.

**Other Methods—**Methods have been described previously for the
application of spray reagents to thin layer chromatograms (2), for
the preparation of methyl iodide and for their detection by autoradi-
ography (1, 2), and for the quantitative ninhydrin reaction (2).

**Thin Layer Chromatography—**—Chromatographic data were recorded as the total ion current (electron impact ionization) integrated over values of m/z from 40 to 400.

**Classification of the fatty acid methyl esters as saturated and
nonhydroxylated, unsaturated and nonhydroxylated, 2-hydroxylated,
or 3-hydroxylated was achieved by use of their electron impact mass spectra (6). The molecular weight of each species (and, therefore, the number of carbons present) was determined from chemical ionization spectra, in which the species (M+H)+ and (M−H)+ were observed. Branched and unbranched species were differentiated by comparison of their retention times with those of a series of standards that included branched and unbranched nonhydroxylated species and unbranched hydroxylated species. Branched nonhydroxylated species (0.2 to 0.4 min in the same solvent system and at the same length) before their unbranched isomers; it was assumed that an
hydroxylated species emerging 0.3 to 0.4 min before the unbranched
standard that was hydroxylated in the same position and had the
same number of carbons was a branched species. The branch structure of nonhydroxylated species was established by use of their electron impact mass spectra (2, 6). The determination of branch position for the hydroxylated species requires extensive derivatization (6) and was not performed.
RESULTS

Purity and Properties of the N-Acylaminosulfonates—These results have been reported in detail elsewhere (3). Yields of the N-acylaminosulfonates (sodium salts) ranged from 0.2 to 0.5 g/100 g (wet weight) of bacterial cells; in one case where recovery was determined with precision, the purified material amounted to 80% of that originally present in the cells. The variation in absolute yield among the different organisms reflected, in general, the variation in their content of the sulfonolipids, which we have previously determined (1). On thin layer chromatography in system I, the purified lipids were resolved into two or three zones (depending on the organism) that were detected with rhodamine and had \( R_F \) values between 0.6 and 0.7. Each of these zones contained sulfur, as indicated by labeling with \(^{35}\text{S}\); after deacylation, the sulfur from each one was recovered in a form that exhibited the chromatographic behavior of capnine \((R_F = 0.33)\). Studies (unpublished, but similar to those described below) of material recovered from preparative thin layer plates have shown that the three zones are, in order of increasing \( R_F \), N-acylamino-sulfonates containing 2-hydroxy, 3-hydroxy, and nonhydroxylated fatty acyl groups, respectively. No zones other than the three just mentioned could be detected on thin layer chromatograms by a variety of detection methods. The purified N-acylaminosulfonate preparations were shown to be virtually free from contamination by compounds containing phosphorus, free amino groups, carboxylic esters, or sulfate esters (3).

The infrared absorption spectra of the N-acylaminosulfonate preparations (cf. Fig. 2a) each showed a complex of intense bands characteristic of sulfonates \((1025 \text{ to } 1260 \text{ cm}^{-1})\); this region of the spectrum is virtually superimposable upon the corresponding regions of the spectra of capnine and taurine (2). The spectra of the N-acylaminosulfonates also showed intense bands characteristic of amides \((1540 \text{ and } 1630 \text{ cm}^{-1})\). The spectra of the four N-acylaminosulfonate prepa-

rations from different organisms were virtually identical, with the exception that the preparation from the marine Cytocytophaga species (not shown) differed from the others in a single feature; it showed a small but well defined band at 960 cm\(^{-1}\) that suggested the presence of trans-double bonds in an amount equal to 10 to 20% of the number of amide or sulfonate groups that were present. The interpretation of this band remains enigmatic (it may well represent an impurity) for, as we report below, after deacylation of the N-acylaminosulfonates no such band was observed in the infrared spectrum of the aminosulfonate fraction nor were any unsaturated fatty acyl groups found among the methanolysis products.

Structure of the Aminosulfonate Moieties—Samples of the various preparations of N-acylaminosulfonates were deacylated by acid solvolysis. Deacylation was virtually complete, as indicated by the fact that chromatography (System I) of an aliquot of the reaction mixture equivalent to 70 \( \mu \)g of starting material yielded no N-acylaminosulfonate zone (at an \( R_F \) of 0.6 to 0.7, as opposed to 0.33 for nonacylated aminosulfonates) that could be detected with rhodamine (detection limit, 3 pg). Yields of the deacylated aminosulfonates, which crystallized upon cooling of the reaction mixtures, ranged from 47 to 50 mg from 100 mg of N-acylaminosulfonates (sodium salts). These yields represent 78 to 83% of the theoretical amounts (assuming that the N-acylaminosulfonates, as sodium salts, had an average molecular weight of 650 and the aminosulfonates obtained after deacylation were capnine in the form of the free acid-amine hydrochloride).

The properties of the aminosulfonates derived from their N-acylated forms were compared to those of authentic capnine (2) that had been isolated as such from Cynocytophaga sp. Each aminosulfonate fraction was chromatographically homogeneous (thin layer systems I and II; detection as an opaque spot after spraying with water or n-butyl alcohol or as a rose-colored spot after spraying with ninhydrin reagent); in no case were any impurities detected using rhodamine,
iodine vapor, or charring after spraying with H2SO4. In all cases, the single component observed had the same Rf as authentic capnine and did not separate from capnine when an equal weight of that compound was added prior to chromatography.

The infrared absorption spectra of the aminosulfonates (in KBr; not shown) were, in each case, virtually superimposable upon that of capnine (2), and showed, among other features, absorption bands characteristic of the primary amino group (1525 and 1590 cm\(^{-1}\)) and of the sulfonate group (a complex of bands in the region from 1025 to 1260 cm\(^{-1}\)). The presence of the amino group was further confirmed by the observation that the various aminosulfonate preparations gave weight-specific color yields in the quantitative ninhydrin reaction that were 97 to 100% as great as that given by authentic capnine. The presence of sulfur was confirmed by the observation that the aminosulfonates (as detected with spray reagents) had the same chromatographic properties as the radioactive product obtained by deacylation of samples of N-acetyl(\(^{35}\)S)aminosulfonates (detected by autoradiography).

Mass spectra (electron impact ionization, sample temperature 300 to 350 °C) were obtained for the underivatized aminosulfonates and were indistinguishable from those obtained for authentic capnine. In particular, they showed prominent ions of m/z = 124, 150, 164, 236, 238, and 252 (the interpretation of these results has been discussed previously (Ref. 2)). At higher values of m/z, however, the spectra were weak and complex, presumably because of degradation resulting from the high temperatures necessary to volatilize the compounds. The molecular ion was not observed reliably, and ions larger than the molecular ion were observed, so that it was difficult to compare the molecular weight of the aminosulfonates that were derived from their N-acetylated forms with that of capnine. Accordingly, more volatile derivatives of the aminosulfonates were prepared. A two-step procedure in which the aminosulfonates were N,O-acetylated and then converted to sulfonate methyl esters was developed, using capnine (Fig. 1) for pilot studies. Fig. 3a shows the negative ion fast atom bombardment spectrum of capnine that had been acetylated and then crystallized as the sodium salt. Three abundant ions were observed: m/z = 434, N,O(bis)acetylcapnine anion, C\(_3\)H\(_4\)NO\(_3\)S; m/z = 392, N-acetylcapnine anion; and m/z = 374, N-acetyldehydrocapnine anion (the origin of this last species is discussed below). Trideuteroacetylation of capnine produced the spectrum shown in Fig. 3b, in which three ions had shifted to m/z values of 440, 395, and 377, respectively, an observation that supports the aforementioned inferences about the extents of acetylation of the three ions. These results confirm the previous conclusion (2) that the molecular weight of capnine is 351 and establish the efficacy of the acetylation procedure.

N,O(bis)acetylcapnine was converted to its methyl ester by treatment of the free acid with diazomethane; that esterification had taken place was confirmed by the observation that the product had an Rf of 0.4 in thin layer system IV (detection with rhodamine), whereas both the free acid and the sodium salt had Rf values less than 0.1. The electron impact mass spectrum of N,O(bis)acetylcapnine methyl ester, which gave strong ion currents at sample temperatures from 100 to 150 °C, is shown in Fig. 4a. N,O(bis)acetylcapnine methyl ester prepared from crystallized N,O(bis)acetylcapnine, sodium salt (method II) gave the same spectrum as did the N,O(bis)acetylcapnine methyl ester prepared from capnine by method I, a conservative method in which the intervening crystallization step was omitted. (Method I was used for preparing the derivatives of the other aminosulfonates.) The spectrum was interpreted with the aid of high resolution measurements, which permitted the assignment of a unique composition to each abundant ion, and with the use of information obtained from the spectrum of N,O(bis)trideuteroacetylcapnine methyl ester, in which the mass shifts permitted determination of the number of synthetically added acetyl groups in each ion (Table I). The results show that the aminosulfonate (prior to derivatization) has 17 carbons. It is a 2-amino, 1-sulfonic acid with no chain branch at carbon 2, as shown by the observation of the ions of m/z = 290 (in which carbon 1 and the sulfonate group, but not the amino group, have been lost) and m/z = 180 and 138 (which include two carbons from the original molecule, plus the sulfonate and amino groups). The results also show that the molecule has an hydroxyl group on a carbon distal to carbon 2 (though the mass spectra alone cannot be used to rule out the presence of some dehydrocapnine) and that, excepting this possibility, the hydrocarbon chain is saturated. The three functional groups mentioned account for all of the hetero atoms in the molecule.

The mass spectra of the acetylated and esterified derivatives of the aminosulfonates that were obtained from the N-acetylaminosulfonates of each of the organisms (cf. Fig. 4b) were nearly identical to those of the derivative of capnine in all
The hydroxyl group is present and is vicinal to the amino group, as indicated by the examination of the infrared absorption spectra of the amino-sulfonates. It has previously been shown (2) that such oxidation of capnine yields a long chain aldehyde. Given the evidence from mass spectrometry that the aminosulfonates contain the sulfonate group on carbon 1, the amino group on carbon 2, no more than one hydroxyl group, and no other substituents that include O or N, this oxidation to an aldehyde can occur only if the hydroxyl group is present and is vicinal to the amino group (periodate does not cleave hydrocarbon chains at double bonds, such as would be found in dehydrocapnine). When the aminosulfonates were chromatographed in system I and the plates were treated with the periodate-Schiff reagents, the aminosulfonate zones (that had been detected as opaque zones after spraying with butanol, and marked) each exhibited a strong color reaction of intensity approximately the same as that given by an equal amount of authentic capnine. Additional portions of each aminosulfonate fraction and of native capnine were oxidized with periodate in solution, and the putative aldehyde products were extracted into diethyl ether and chromatographed in system III. When the plates were sprayed with the Schiff reagent alone, the products obtained from each aminosulfonate preparation (including capnine) exhibited a single intensely staining zone at an RF of 0.7, which is characteristic of long chain aldehydes (7). In each case, the staining intensity was approximately the same as that exhibited by the product obtained from an equal weight of capnine. The results demonstrate that the hydroxyl group is in each case vicinal to the amino group, that is, it is located on carbon 3. The fact that each aminosulfonate preparation gave approximately the same yield of aldehyde as did capnine (which gave 23 mg of aldehyde from 40 mg of capnine, equivalent to 0.9 mol of the expected aldehyde/mol of capnine (Ref. 2)) reinforces the view that dehydrocapnine (which would not have yielded an aldehyde) was not an abundant component of the aminosulfonate fractions.

In order to examine the structure of the distal portions of the hydrocarbon chains of the aminosulfonates, they were oxidized with periodate and permanganate. This mixture cleaves the same linkage of capnine as does periodate when used alone, but yields a fatty acid from the distal portion of the chain. These fatty acids were converted to methyl esters and analyzed by gas chromatography-mass spectrometry. Yields of total fatty acid methyl esters (assessed from peak areas after chromatography in the presence of a weighed internal standard of methyl hexadecanoate) ranged from 2.3
strates that the method was capable of resolving species which, in each organism, most (if not all) aminosulfonate molecules share with capnine the presence of the terminal isopropyl group and the absence of other chain branches distal to carbon 2. The fact that high yields of fatty acids were obtained confirms the conclusion that isomers of capnine bearing the hydroxyl group on carbons distal to carbon 3 were not present in large quantities, since such isomers would not have been oxidized.

**Structure of the Fatty Acid Moieties—Decylation of samples (100 mg) of each N-acylaminosulfonate (sodium salt) preparation gave yields of fatty acid methyl esters ranging from 33 to 38 mg (dry weight). These yields correspond to 72 to 84% of the theoretical yield, if the average fatty acid methyl ester had a molecular weight of 280. The fatty acid methyl esters from the N-acylaminosulfonate preparations, as well as those from methanolyisates of whole cells, were analyzed by gas chromatography-mass spectrometry; the results are shown in Table III. It is apparent that the fatty acid moieties of the N-acylaminosulfonates are heterogeneous within each organism and that they differ in some respects from one organism to the next, as well. However, the most striking
observation is that all of the N-acylaminosulfonates are rich in hydroxylated fatty acyl groups. In all organisms except the Flexibacter sp., the most abundant acyl group is a 3-hydroxy branched 17-carbon species; in the Flexibacter sp., a 2-hydroxy branched 15-carbon species is most abundant. In all cases, the N-acylaminosulfonates are enriched approximately 2-fold in hydroxylated acyl groups, when compared to the fatty acids of cellular lipids as a whole. There are other apparent specificities; for example, in those cases where it is present in the other cellular lipids, the hexadecenoyl group seems to be excluded from the N-acylaminosulfonates.

**Nature of the Linkage Between the Fatty Acyl Group and the Aminosulfonate Moiety**—The zones of putative N-acylaminosulfonates that were originally obtained on thin layer chromatography did not exhibit a ninhydrin reaction, whereas those of their sulfur-containing hydrolysis products did (2). For this reason, it was considered that, in the acylated compounds, the amino groups were in amide linkage. Infrared spectroscopy confirmed this view and also made it possible to rule out the presence of acyl groups in ester linkage (a possibility raised by the finding of hydroxyl groups in the methanalysis products of the N-acylaminosulfonates). The infrared absorption spectra of all N-acylaminosulfonate preparations (cf. Fig. 2a) showed strong amide bands (1630 and 1540 cm\(^{-1}\)); this is in contrast to the spectrum of capnine (2) which shows, instead, bands characteristic of primary amines (1590 and 1525 cm\(^{-1}\)). In no case did the infrared absorption spectrum of an N-acylaminosulfonate preparation (cf. Fig. 2a) show any relief in the region from 1800 to 1690 cm\(^{-1}\) where...

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**Fatty acid products obtained on oxidation of aminosulfonates with NaOH and KMnO\(_4\)**

| Source of aminosulfonate | Oxidation products | Minor products |
|--------------------------|--------------------|---------------|
| Capnocytophaga sp., strain 30N51 | 100 None | None |
| Capnocytophaga sp., strain P7 | 100 None | None |
| Flexibacter sp., strain FS-1 | 97 N-hexadecanolate (3%) | |
| Cytophaga johnsonae | 81 12-Methyltetradecanolate (10%); n-pentadecanolate (9%) | |
| Marine Cytophaga sp., strain 7B | 78 11-Methylpentadecanolate (17%); 12-methyltetradecanolate (5%) | |

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**Fatty acid composition of purified N-acylaminosulfonates and of whole bacterial cells**

| Fatty acid species | Retention time of methyl ester | Capnocytophaga sp., strain P7 | Cytophaga johnsonae | Marine Cytophaga sp., strain 7B | Flexibacter sp., strain FS-1 |
|-------------------|--------------------------------|-----------------------------|---------------------|-----------------------------|-----------------------------|
|                  | Relative amount (peak area %)* | Whole cells N-Acylaminosulfonates | Whole cells N-Acylaminosulfonates | Whole cells N-Acylaminosulfonates | Whole cells N-Acylaminosulfonates |
| C\(_{16}\)         | min                            | 8.3                          | 3.0                  | 2.8                         | 2.8                         |
| 13-Me-C\(_{16}\)   | 9.0                            | 62 5.5                       | 24 1.7               | 14 1.9                      | 11 3.0                      |
| 12-Me-C\(_{16}\)   | 9.1                            | 1.2 5.3                      | 6.4 3.1              | 8.8 1.1                     | 1.1 1.1                     |
| C\(_{18}\)         | 10.0                           | 2.5 14                       | 9.0 1.9              | 2.0 1.1                     | 11 15                       |
| 15-Me-C\(_{16}\)   | 11.0                           | 2.8                          | 2.8                  | 2.8                         | 2.8                         |
| C\(_{18}\)         | 12.1                           | 12                            | 12                   | 21                          | 21                          |
| 2-OH-Me-C\(_{16}\) | 10.4*                          | 1.3 1.3                      | 1.7 1.6              | 3.2                         | 3.2                         |
| 2-OH-C\(_{16}\)    | 11.8                           | 1.3 1.3                      | 1.7 1.6              | 3.2                         | 3.2                         |
| 3-OH-Me-C\(_{16}\) | 12.7                           | 1.3 1.3                      | 1.7 1.6              | 3.2                         | 3.2                         |
| 3-OH-C\(_{16}\)    | 13.7                           | 2.9                          | 2.9                  | 2.9                         | 2.9                         |
| Unidentified       | 10.8                           | 2.9                          | 2.9                  | 2.9                         | 2.9                         |
| Minor components   | 0.8                            | 2.0                          | 1.4                  | 1.5                         | 1.0                         |
| Total hydroxylated | 34                             | 66                           | 38 78                | 55 95                       | 40 68                       |

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*Abbreviations used: Me, methyl; OH, hydroxy; the subscript following C indicates, before the colon, the number of carbons (exclusive of methyl branches) and after it, the number of double bonds. The use of Me that is not preceded by a position number indicates a branch for which the position was not determined. It has previously been shown (6) that in C. johnsonae, in a Flexibacter sp. (though not the one used in this study) and in a marine Cytophaga sp. that is probably similar to the one used in this study, virtually all of the branched chain fatty acids from whole cells have the methyl groups on the penultimate carbon (iso structure).

* Determined from the integrated ion current (40 \( \leq m/z \leq 400 \)) as described under Table II. Chromatography of weighed standards of fatty acid methyl esters including nonhydroxylated and branched and unbranched species, 2-hydroxylated and 3-hydroxylated species covering the entire range of retention times listed in the table indicated that their molar ion yields did not differ from that of methyl n-hexadecanolate by more than 10%.

* A dash indicates less than 1%.

* Where mass spectra indicated that both of these interfering species were present, they were determined after separation of the hydroxylated and nonhydroxylated species by preparative thin layer chromatography (6).

* Includes all, and only, species that were detectable but constituted less than 1% of the total.
carboxylic esters exhibit strong absorption bands. As a control, the spectrum of \(N_{O}(\text{bis})\text{acetylcapnine}\) was examined. This spectrum (Fig. 2b) shows both the amide bands and a strong ester band at 1735 cm\(^{-1}\). If ester linkages had been present in the isolated N-acylaminosulfonates, then, they would have been detected by infrared spectroscopy. Furthermore, it is unlikely that ester linkages were present in native N-acylaminosulfonates but were degraded during the purification procedure (which involves steps using acids and a base, albeit weak ones). A sample of the acetylated capnine was carried through these steps, after which the ester absorbance was undiminished relative to that of the amide and sulfonate bands. We thus conclude that the fatty acyl groups of the N-acylaminosulfonates are virtually all in amide linkage.

**DISCUSSION**

**Structure of the Aminosulfonate Moieties**—The chemical studies of the aminosulfonate fractions derived from the N-acylaminosulfonates, taken together with the high yields of those fractions obtained (approximately 80% of the theoretical values), indicate that the predominant aminosulfonate moiety found in the N-acylaminosulfonates is the same in all organisms studied and that it is structurally identical to the capnine (Fig. 1) that occurs in nonacylated form in Capnocytophaga spp. The remaining uncertainties are the stereochemistry (which was not studied) and the possibility that some structural variants of capnine may be present in low abundance.

Oxidation with periodate and permanganate of the aminosulfonate fractions from some organisms yielded, in addition to the 13-methyltetradecanoyl that was the most abundant product, small amounts of some other fatty acids (Table II). Those acids that were isomers of 13-methyltetradecanoyl might reflect the presence, in the N-acylaminosulfonates, of small amounts of the corresponding isomers of capnine. The observation of 11-methyldecanoylanole as a minor product (17% of the total) in the case of the marine Cytophaga sp. is consistent with any of several variant aminosulfonate structures, including a 15-carbon homolog of capnine. The mass spectra of the derivatized aminosulfonates from this organism provided no evidence for such variant species, but it is not known whether such a compound would have been detected when present as only 17% of the total material. The minor fatty acids obtained on oxidation could, of course, have been derived from impurities that were present but not detected by the methods used (3) to characterize the N-acylaminosulfonate preparations. The convincing demonstration of the presence of any aminosulfonates different from capnine must await the development of methods for their separation prior to analysis.

Capnine is a structural analog of a sphinganine and the N-acylcapnines are structural analogs of ceramides. As we have pointed out (2), the biosynthesis of capnine may be analogous to that of sphingosine. If that is the case, the molecule would be formed by condensation of a fatty acyl group (which would contribute those carbons distal to carbon 2) and an amino acid, perhaps cysteic acid (which would contribute carbons 1 and 2 and their substituents). In this sense, the chain structure of the major aminosulfonate species of each organism, capnine, is not surprising. The fatty acyl group requisite for its synthesis would be the 13-methyltetradecanoyl group, which is the most abundant one in the whole cells of each organism examined (Table III). A parallel to this situation exists in a group of bacteria presently considered to be a subset of the genus Flavobacterium, but for which the new genus Sphingobacterium has been proposed (8). These bacteria contain true sphingolipids, of which the long chain base is 15-methylhexadecasphinganine, structurally identical to capnine save for the presence of the 1-hydroxyl group instead of the sulfonate group. In these organisms, as in those used in the present study, the most abundant fatty acyl group in the cellular lipids is the 13-methyltetradecanoyl group (8). The sphingolipids of Bacteroides melaninogenicus contain 17-methyloctadecasphinganine and 15-methylhexadecasphinganine, and the organisms are rich in branched 15- and 17-carbon fatty acids (9).

To our knowledge, the only other occurrence of a sulfonolipid similar to capnine or N-acylcapnine is in the diatom Nitzschia alba, a eucaryote (10). This lipid is an N-acylated 2-amino-3-hydroxy-4-trans-octadecene-1-sulfonic acid, that is, the aminosulfonate moiety has the structure of sphingosine, typical of eucaryotes, except for the substitution of the sulfonate group for the 1-hydroxyl group of that compound.

**The Fatty Acid Moieties of the N-Acylaminosulfonates**—It is clear (Table III) that, within each organism, the fatty acid moieties of the N-acylaminosulfonates are heterogeneous. However, there is a striking similarity among the N-acylaminosulfonates of the various bacteria in their high content of hydroxylated fatty acyl groups. In all cases, the N-acylaminosulfonates were enriched approximately 2-fold in their relative content of hydroxylated fatty acyl groups when compared to the methanolysate of whole cells. In one case, that of the marine Cytophaga sp., which, among the organisms tested, has the highest cellular content of hydroxylated species, 95% of the fatty acyl groups in the N-acylaminosulfonates were found to be hydroxylated. Mayberry (11, 12) has reported that in the lipids that can be extracted by organic solvents from cells of a Bacteroides sp., 100% of the fatty acyl groups that are in amide linkage are hydroxylated, whereas 100% of those that are in ester linkage are not hydroxylated. Most of the organisms used in the present study do not exhibit such absolute specificity; among four of the five N-acylaminosulfonate preparations, 22 to 34% of fatty acyl groups were not hydroxylated. Those proportions of ester linkages would easily have been detected by infrared spectroscopy, yet ester absorbance was absent. The enrichment in hydroxylated acyl groups was, nonetheless, observed in each case.

The composition of the fatty acyl groups of the bacterial N-acylaminosulfonates stands in contrast to that of the sulfonolipid of the diatom Nitzschia alba (mentioned above) that is an analog of N-acylcapnine; in this lipid, no hydroxylated acyl groups were found (10). The most abundant fatty acyl group found in the N. alba lipid was a 3-hexadecenoyl group; unsaturated acyl groups apparently are rare in the bacterial sulfonolipids (Table III).

**Taxonomic Considerations**—The bacteria examined in this study resemble each other in a number of respects (reviewed in Ref. 1) in addition to their gliding motility and their high content of N-acylaminosulfonates. Though they have been classified together on the basis of these properties, they are quite diverse in other respects. Their habitats range from the human subgingival tooth surface (the Capnocytophaga spp.) to marine sediments (the marine Cytophaga sp.). The organisms of one genus (Capnocytophaga) are strictly fermentative (though aerotolerant), whereas the others are aerobes. The DNA from representative Capnocytophaga sp. exhibits no cross-hybridization with that from Cytophaga sp. (13). It would appear that N-acylaminosulfonate structure, including the structure of the major aminosulfonate component, capnine, as well as the preponderance of hydroxylated fatty acid residues, has been conserved throughout a period when, in
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...other respects, divergence has taken place (though parallel evolution cannot be ruled out as the pathway to the present situation). Such conservation suggests an important function for the capnoids. A role in gliding motility is one possibility that is presently under study. Interest in this potential role for the sulfonolipids is stimulated by the fact that diatoms, the only other group of organisms of which at least one member contains N-acylaminosulfonates, exhibit a form of motility (14) which resembles, at least superficially, that of the gliding bacteria.

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