REGULAR PAPER

Composition Analysis of Polar Lipids in Halobacteria with Mass Spectrometry

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(Received August 2, 2004; Accepted October 9, 2004)

The structures of the polar lipids in halobacteria including their mutants were determined with ESI-MS and FAB-MS. The composition-analyses with ESI-MS gave the results fairly close to these with TLC analyses. The percentages of the composite polar lipids by the mass spectrometric analyses of *Halobacterium salinarum S-9* were 20, 77, and 5% for glycolipid sulfate (S-TGD), methyl ester of phosphatidyl glycerophosphate (PGP-OMe), and phosphatidylglycerol (PG), respectively. The MS/MS analysis in the FAB-MS provided an analyzing tool for the structural elucidation of the functional groups in the lipids. Newly proposed methylation in the phosphate was investigated with stable isotope experiments. We found that the Australian halobacteria, *Halorubrum* sp. *aus-1*, mainly consist of an isolated glycolipid sulfate with two sugar parts of glucopyranoses. In relationship with the lipid-composition analysis, the chemotaxonomy of halobacteria was discussed. The methylation of the lipids was proved to be essentially independent of that in the photosensory protein of halobacteria on the basis of the wide distribution of the methylated lipids.

1. Introduction

Even in an extremely high concentration of salt, some kinds of bacteria can be still alive resisting the highly osmotic pressure outside. Halobacteriaceae is a kind of the archaebacteria having lived in a salt lake under the extremely severe circumstances since the Paleozoic era.[1] For living, the bacteria are armored with the robust membranes and have partly gained the energies through solar light, like plants absorbing light for photosynthesis. The light-absorbing substance in the halophilic bacteria mainly consists of the purple-colored biomembrane, which is called Purple Membrane (PM), containing bacteriorhodopsin (75%) and lipids (25%).[2] The latter components have the chemical structures strikingly different from these in the common bacteria. The characteristics of the lipids are exhibited in the structures of the *ether*-linkages of fatty alcohols in the glycerophospholipids (PGP) and sulfated glycerotriglycoside (S-TGD).[3] On the other hand, the colored protein, bacteriorhodopsin (BR), plays an important role in the photoenergy conversion originated from proton gradient. Both chemical species of the components have seemed to have no relationship like an independent partner so far. Seemingly, the lipids behave themselves to the proteins as a solvent. However, Oesterhelt *et al.* recently showed the structural stabilization of BR with lipid-mediation, where the S-TGD binds into the central compartment of BR trimers, which has been elucidated with the X-ray structure analysis.[4] In contrast to the S-TGD, the structures of the phospholipids were ambiguously visualized in the X-ray analysis because of the mobility of the phospholipids. As a whole, the assemblies of the lipids in the purple membrane form lipid-bilayers with space filling among the BR-trimers.[5] Thus, the analysis of the lipid-composition in microenvironment gives fundamental data in the structural discussion.[6] For the basic approaches, mass spectrometry can supply a powerful tool for the structural elucidation and the semi-quantification of the components. Here, we report the mass spectrometric analyses of the lipid-composition in the several kinds of halobacteria.

Moreover, the analysis of lipid-composition enables us to develop the taxonomical discussion of the bacteria. Although the direct interaction between the genes and the lipids has not been established, the chemitaxonomy of the halobacteria based on the 16S RNA has been reported in relationship to the lipid-composition.[7] As a separation method, however, the chromatographic analysis of the lipids had hardly provided the detailed information on the chemical structures. This paper describes that the *qualitative* classification of the strains is exhibited on the basis of the lipid-composition with the rapid analysis by the mass spectrometry. Among all those mass spectrometric methods, the recently developed soft-ionization is effective in affording us the structural information about protonated or deprotonated molecules and the fragment ions of the lipids. In particular, the negative ion mass spectrometry with ESI-, MALDI-, or FAB-MS is effective for the polar lipids. We also discuss the application and limitation of the mass spectrometric methods for the analysis of the polar lipids in halobacteria.

2. Experimental

2.1 Mass spectrometers and measuring conditions

All the negative mass spectra were measured with

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the following spectrometers; the Finnigan TSQ-700 spectrometer for the FAB or ES ionization, the VG AutoSpec for LSIMS and MIKES, the Hitachi M-80B spectrometer for liquid-type secondary ion mass spectrometry (LSIMS), and the Fison TofSpec Spectrometer for matrix-assisted laser desorption ionization (MALDI).

For the measurement of the ESI-MS, the solution of the lipids in MeOH–$CHCl_3$ (1 : 1) was sprayed at 1.0 μL/min flow rate under 4 kV voltage. Triethanolamine (TEA) or 3-nitrobenzyl alcohol (3-NBA) was used as a matrix for FAB-MS measurement with cesium or xenon ions generated at 6 kV. The LSIMS was operated at 3 kV of the accelerating voltage equipped with 8 kV gun (xenon ion). The MALDI mass spectra were recorded on the spectrometer equipped with 337 nm-laser light and α-cyano-4-hydroxycinnamic acid (CHCA) was used as a matrix.

2.2 Materials and bacterial membranes

The reagents, MeOH, $CHCl_3$, and TEA, were purchased from Kanto Chemicals as GR grade. The matrix, CHCA, was purchased from Aldrich Chemical Co., Inc. The TLC plates (Merck & Co., Inc.) were used as precoated aluminum sheets with silica gel (1.05554). Methionine-d_3 was purchased from Merck and the deuterated amino acids were obtained from Wako Chemicals. All reagents were used without purification.

The membranes including the proteins and the lipids were isolated from the culture (1 dm^3) for the strains, *Hb. salinarum R1*, *S-9*, and *Hr.* sp. *aus-1* according to the described method.[8] The artificial medium including amino acids was prepared according to Onishi's method.[9] The mixtures of the polar lipids were extracted by MeOH–$CHCl_3$ and precipitated with acetone in total amount of 10 mg.[10]

3. Results and Discussion

3.1 LSIMS of polar lipids

Since 1950s, energetic works by Kates and his coworkers have revealed that most of the polar lipids in halobacteria consist of phosphatidyl glycerocompounds and glycolipid sulfates.[11] The isolation and structural determination of the lipids had required persistent manipulation of the chemical separation before the mass spectrometric approach became feasible and effective in the soft ionization and MS/MS analysis. This approach gave us the prospective development of the separation and determination for more detailed molecular information on the lipids *in situ*. Thus the *negative*-ion LSIMS of the extracted polar fraction with TEA as a matrix showed *m/z* 1217 (8% for the most intense peak) for the highest mass in the spectrum as shown in Fig. 1. The major peaks were appeared in *m/z* 921 (100), 907 (29), 899 (41), 885 (10), and 805 (21) for the peaks above *m/z* 750, whereas mass numbers are used for masses hereinafter. The similar spectrum was acquired for 3-NBA as the matrix while indicating the different peak intensities in the low mass region. On the other hand, the peaks in the *positive* mode of LSIMS appeared as multi-metalated ions at *m/z* 923 (mono-metalated) and 945 (di-metalated) as well as 901 (unmetalated) as shown in Fig. 2. For the structural elucidation, however, the negative spectra are simpler and more informative on the molecular structures of the mixture than those of the positive ones. On the basis of Kates' report, the structure of S-TGD was deduced for *m/z* 1217 in the negative spectra, whereas the peak at *m/z* 907 was assigned to that of PGP-Na in correspondence with the peak at *m/z* 885 to that of the unmetalated PGP, and *m/z* 805 to that of PG.[12), 2)]

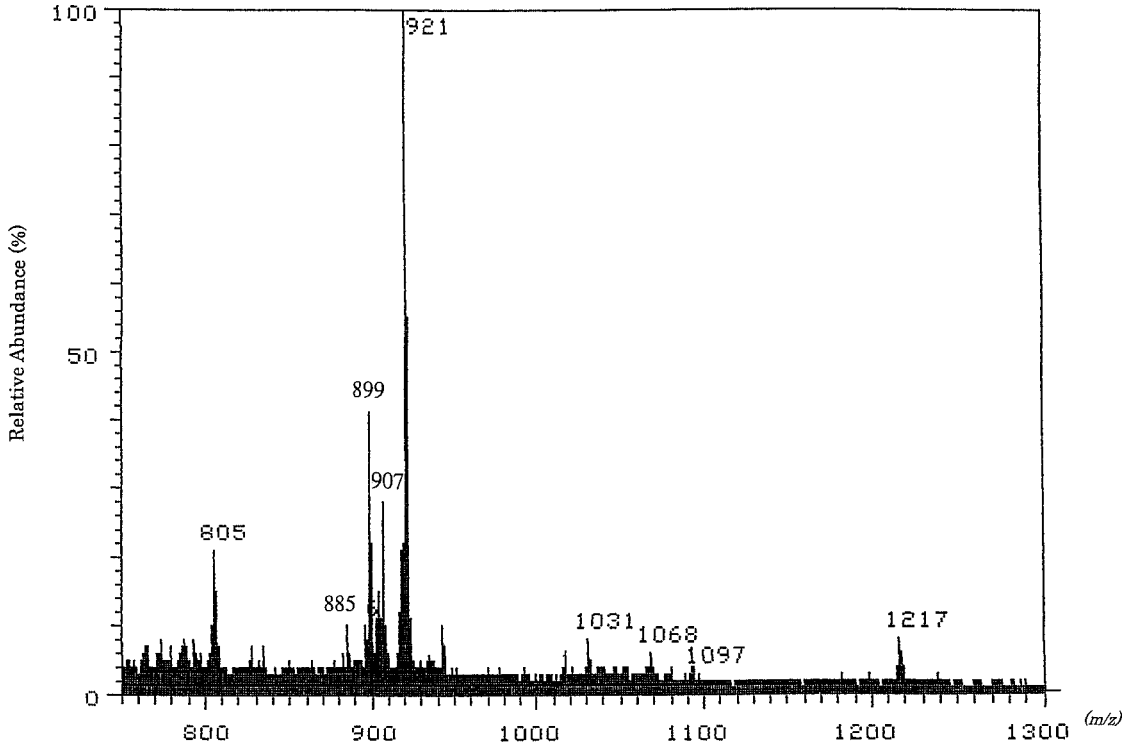


Fig. 1. Negative LSIMS of the polar lipid mixture in halobacteria.

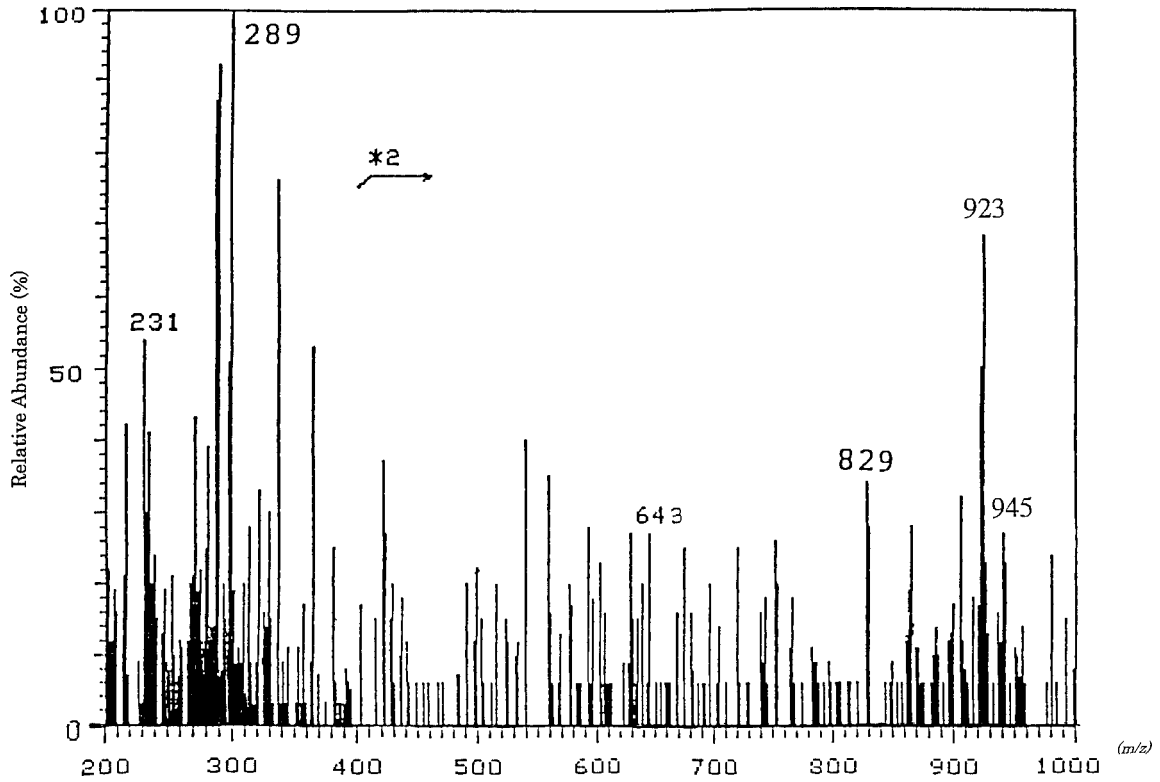


Fig. 2. Positive LSIMS of the polar lipid mixture in halobacteria.

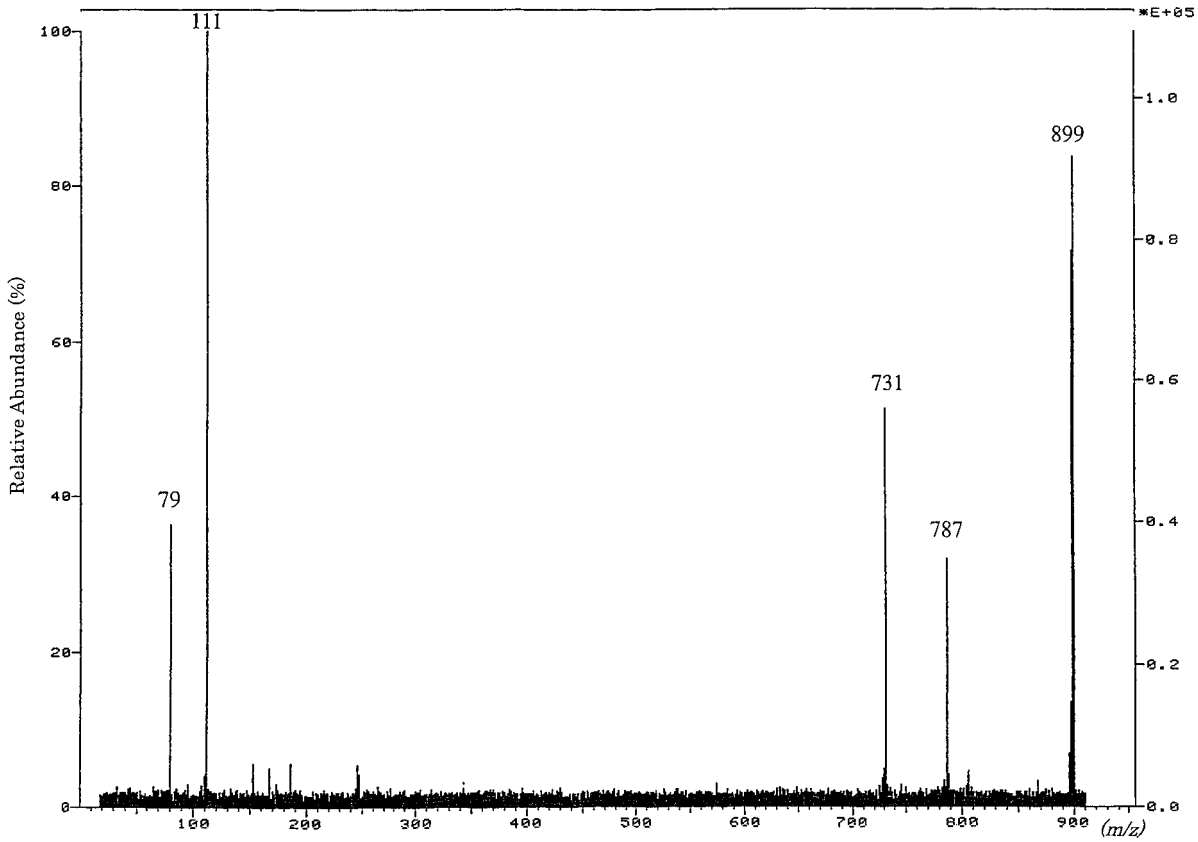
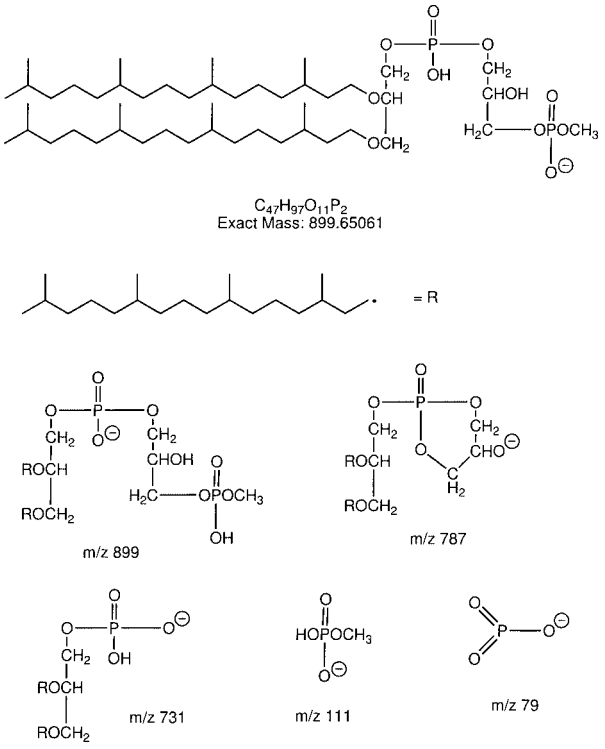


Fig. 3. Product ion spectrum for the ions at *m/z* 899 in the negative FAB-MS.

Since the other intense peaks at *m/z* 899 and 921 in the high mass region of the spectra indicate the increase of 14 u in *m/z* 885 and 907, the partial structures were postulated as methylated PGP and PGP+Na ions, respectively. For the evidence, the MS/MS analysis of the ion at *m/z* 899 gave the fragment ions at *m/z* 805, 787, 731, 111, and 79 as shown in Fig. 3. The fragmentation scheme was drawn in Scheme 1. The ion at *m/z* 111 strongly suggests the structure of *methyl* phosphate ion concomitant with the phosphate ion at *m/z*

79. The same spectrum was obtained in the case of the precursor ion, (PGP+Me+Na) at *m/z* 921. These facts suggest that the lipid consists of a methyl phosphate group. The methylation of phosphoric acid should be accounted for the biochemical synthesis with stable isotopes. In the analysis, the mass spectral elucidation is effective for deduction of the methylated location.



Scheme 1. The structures of the fragment ions produced in fragmentation of PGP-OMe.

3.2 Mass shifts in methyl phosphate of lipid

The finding of methyl phosphtidylglycerophosphate in halobacterial lipid had not been established before 1989.[13] Historically, the structure of *un*methylated phospholipids had been proposed and objected to the incomplete explanation on the results of acid-titration so far.[14] For determination of the PGP–OMe structure, the deuterated methyl ester was biologically synthesized. For biological synthesis of lipids, halobacteria were incubated in an artificial culture of the amino acids with S-trideuterio-methylated methionine instead of undeuterated methionine.[9] After extraction of the polar lipid fraction with ethanol instead of methanol, the sample solution of the extract was prepared for measurement of the (−)-FABMS. The obtained spectrum indicated that the mass peaks shift from *m/z* 899 to 902 for the M−H ion and from *m/z* 921 to 924 for the M−2H+Na ion in comparison with that of the non-deuterium treated lipids, respectively. However, the peak at *m/z* 1217 for S-TGD still remains at the same mass of the native ion as shown in Fig. 4. The shifts similar to the above-mentioned were observed in the (+)-FABMS (Fig. 5).

For the further evidence of the methylated structure, the methylation of the lipid was also confirmed with ^{1}H-NMR in comparison of the spectrum for the non-deuterated phospholipid with that for the deuterated one. The difference between them was appeared in the signals at 3.52 and 3.56 ppm (J_{P-H}=11.4 Hz) for the undeuterated methyl ester; the signals were disappeared for the *methyl*-$^{2}H_3$ ester. These facts suggest that the terminal phosphate is biochemically methylated with the methyl group of methionine.[13]

3.3 Relationship of methylation between lipid and protein

The above-mentioned results implied that the major

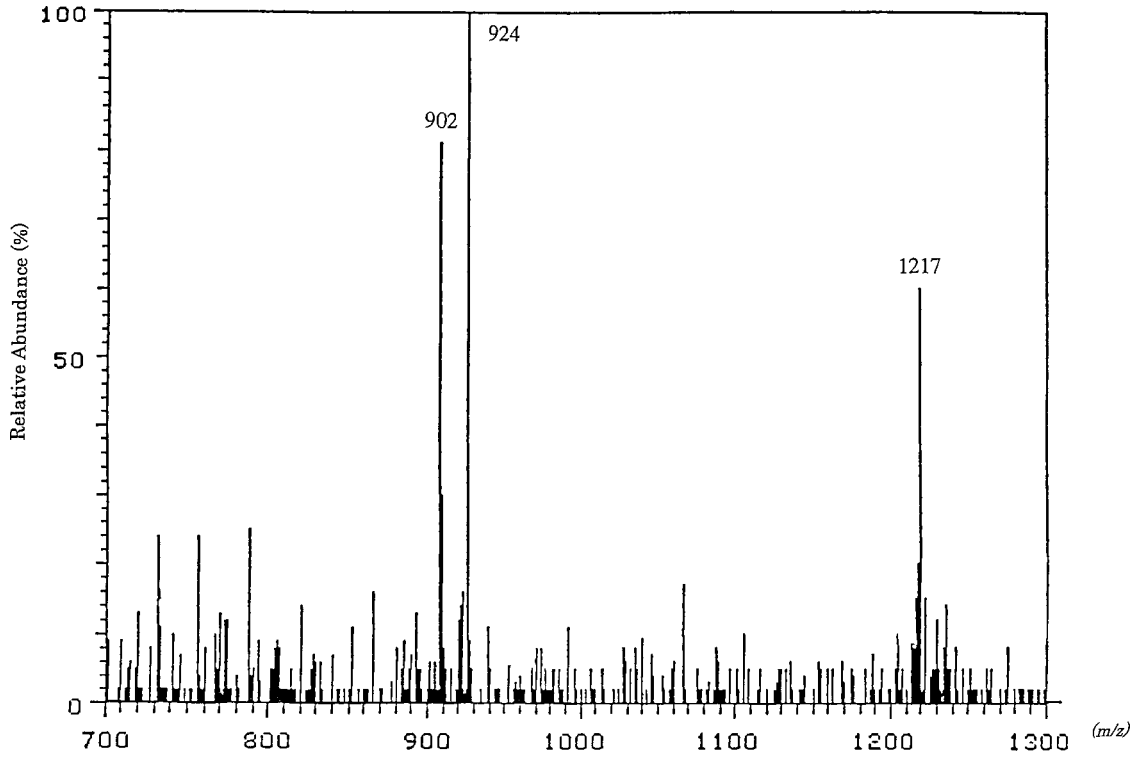


Fig. 4. Negative LSIMS of the deuterated polar lipids.

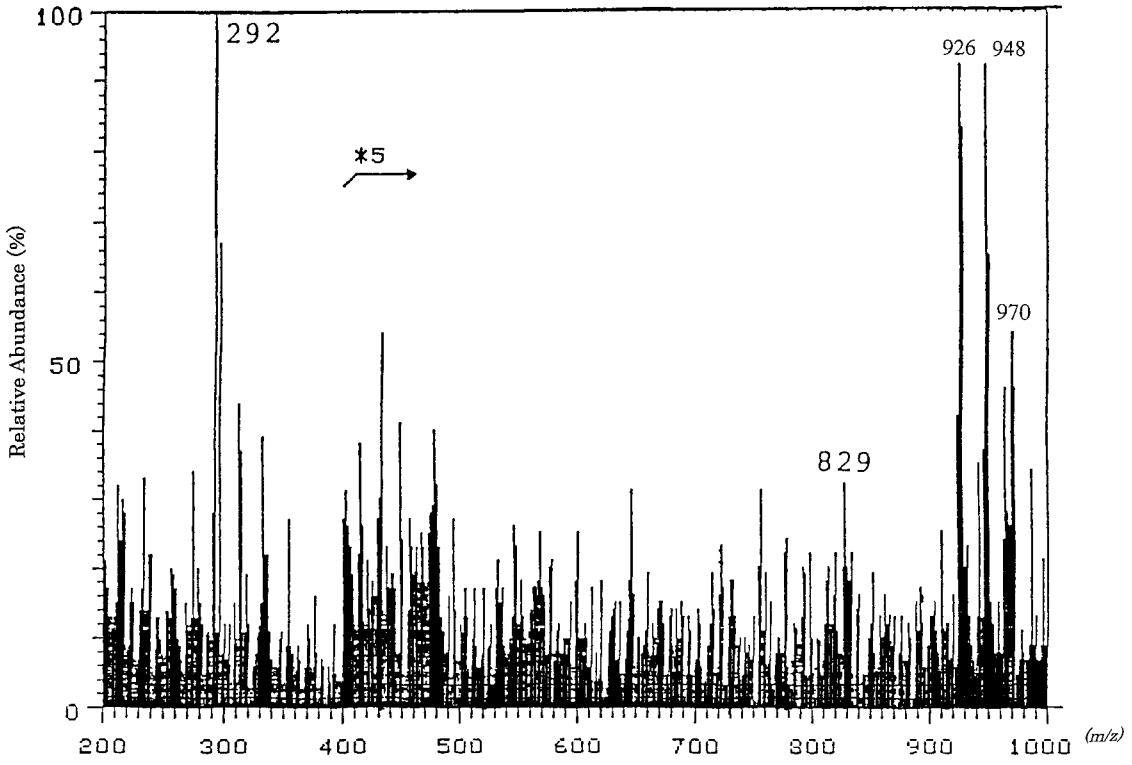


Fig. 5. Positive LSIMS of the deuterated polar lipids.

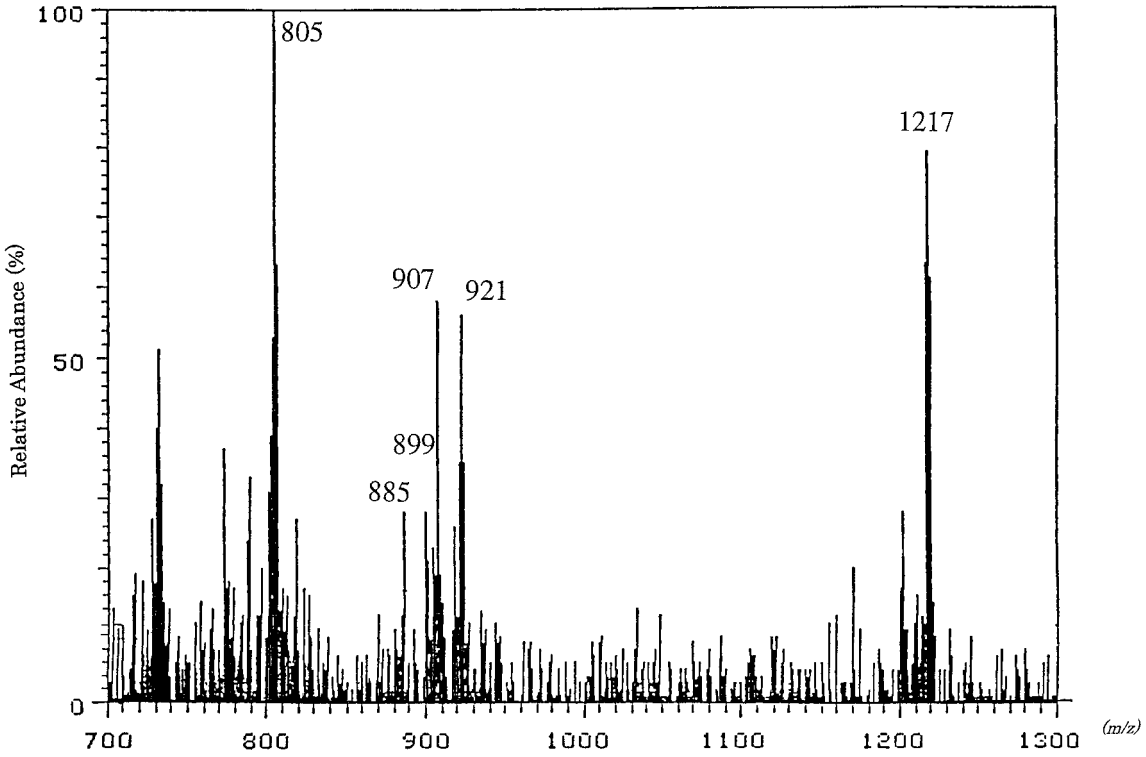


Fig. 6. Negative LSIMS of the polar lipids isolated from halobacteria in artificial culture without methionine.

fraction of the phospholipid in common *Hb. salinarum* is methylated phospholipid. Hereby is raised an important problem whether the methylation of lipids relates to the that of protein or not, although it is known that the methylation of the protein is related to the chemotaxis.[15] For the elucidation of the relationship, halobacteria were grown for several weeks in a methionine-deficient culture.[13] After extraction, a small amount of the polar lipid was isolated using ethanol as an alternative to methanol. The (−)-LSIMS of the lipid mixture showed the intense peak at *m/z* 805 (PG) together with the weak peaks at *m/z* 899 and 921 as shown in Fig. 6. This fact suggests that methionine is a requisite for the formation of the methylated phospholipid and that the methionine-deficiency results in the predominant formation of phosphatidylglycerol (PG). Basically, methyl-

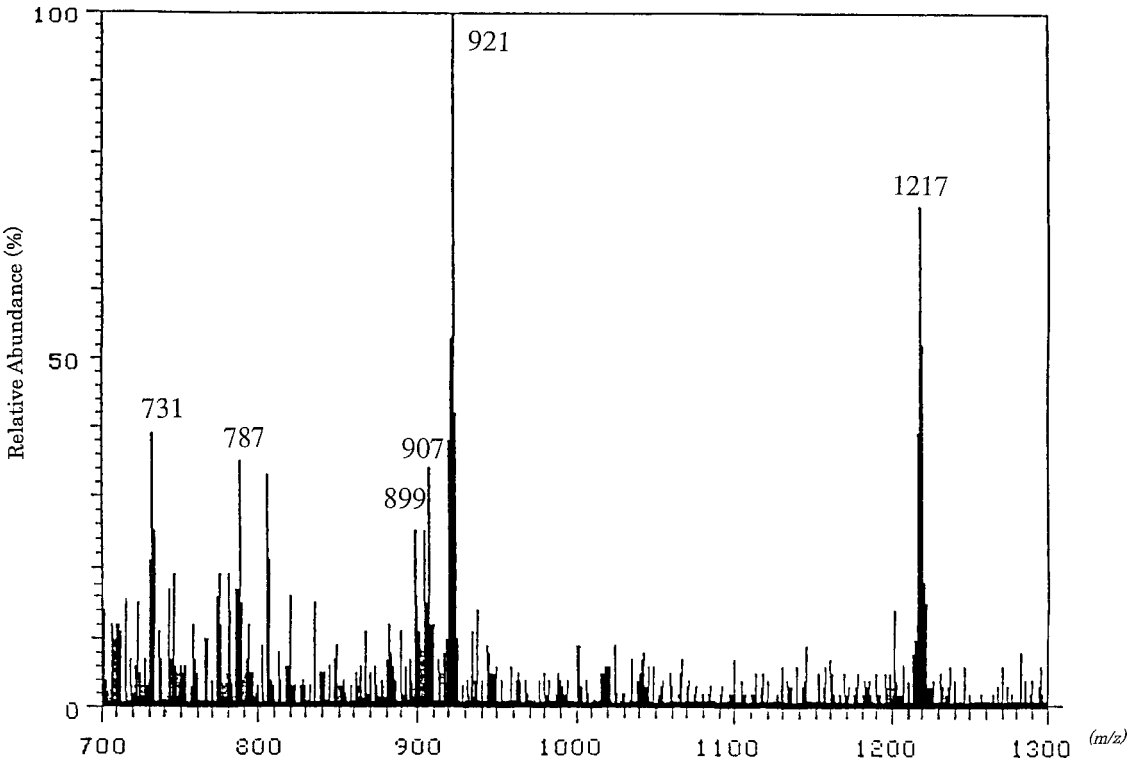


Fig. 7. Negative LSIMS of the polar lipids of the strain *ON-1*.

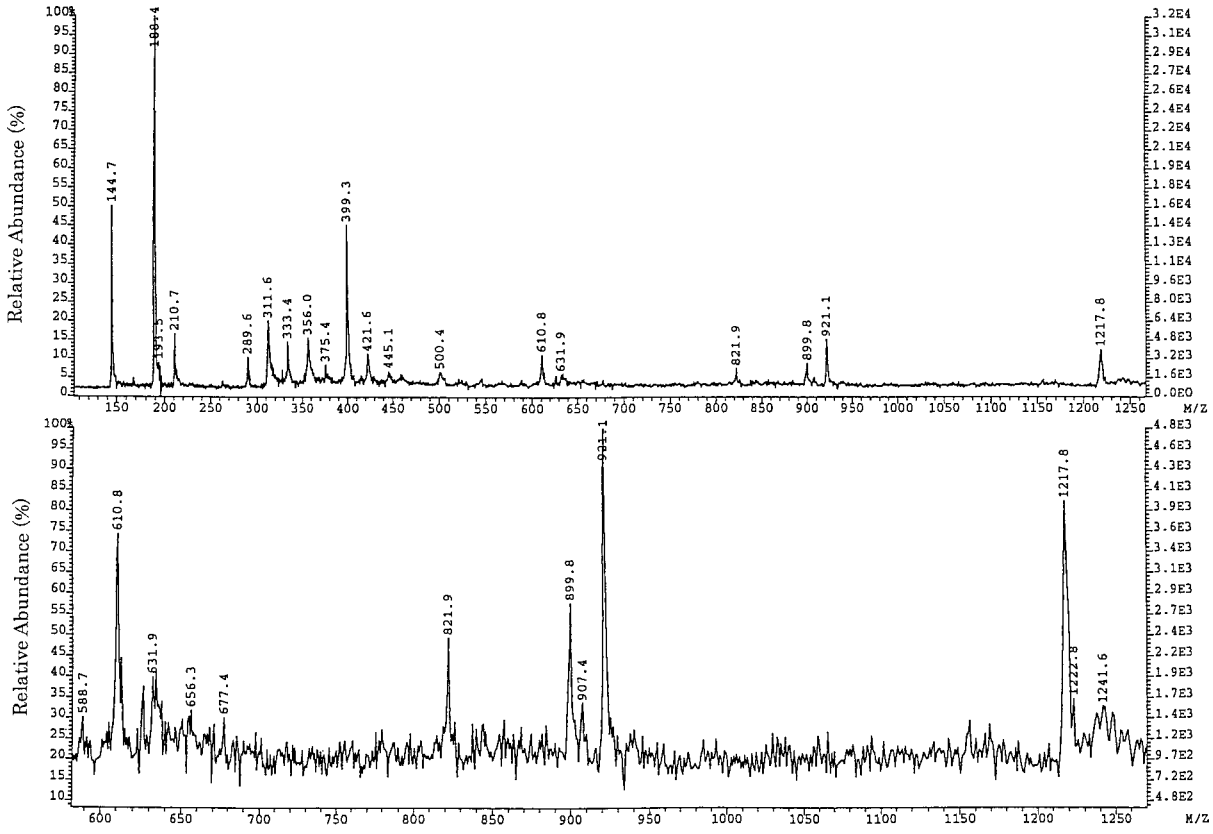


Fig. 8. Negative MALDI-MS of the polar lipids in *Hb. Salinarum*.

transferase catalyzes the transformation reaction from methionine to *S*-adenosylmethionine which cooperates in this process of the methylation.[15] By contrast, in *Escherichia coli*, the methyl-accepting protein of the chemotaxis is related to the adaptation to stimuli.[15] On the analogy of the case of *E. coli*, a halobacterial mutant, *ON-1*, which has no phototaxis seemed to have the unmethylated lipids. By the aforementioned method, however, the mixture of the polar lipids in *ON-1* was obtained with the extraction. The (−)-FAB-MS of the lipid-mixture fairly coincides with that of the native strain as shown in Fig. 7. On the other hand, the

Table 1. Semiquantitation of the Polar Lipids with ESI-MS

Strains	PG	PGPOMe	S-TGD	S-DGD	Protein involved
Hb. salinarum S-9	3%	77%	20%	0%	Bacteriorhodopsin
Hb. salinarum FlxR	22	51	27	0	Sensory rhodopsin
Hr. sp. *aus-1*	5	40	0	55	Archaerhodopsin
S-9 with TLC	5	75	20	0	—

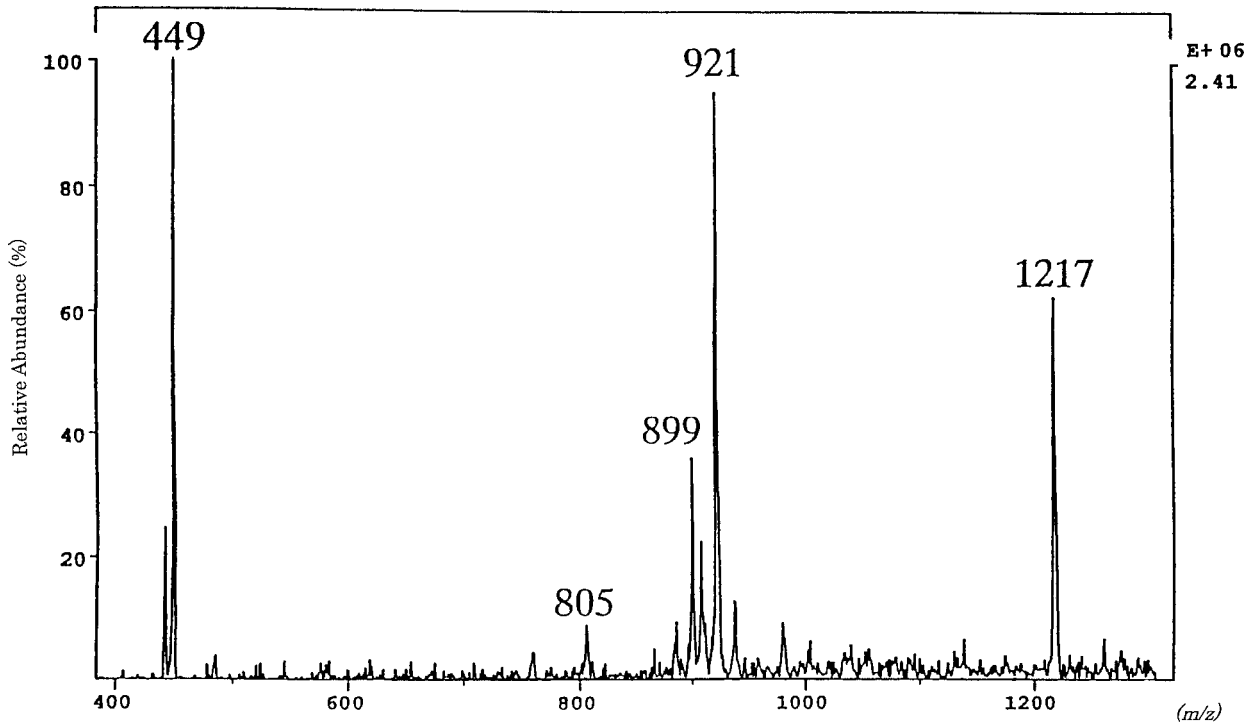


Fig. 9. Negative ESI-MS of the polar lipids in *Hb. Salinarum.*

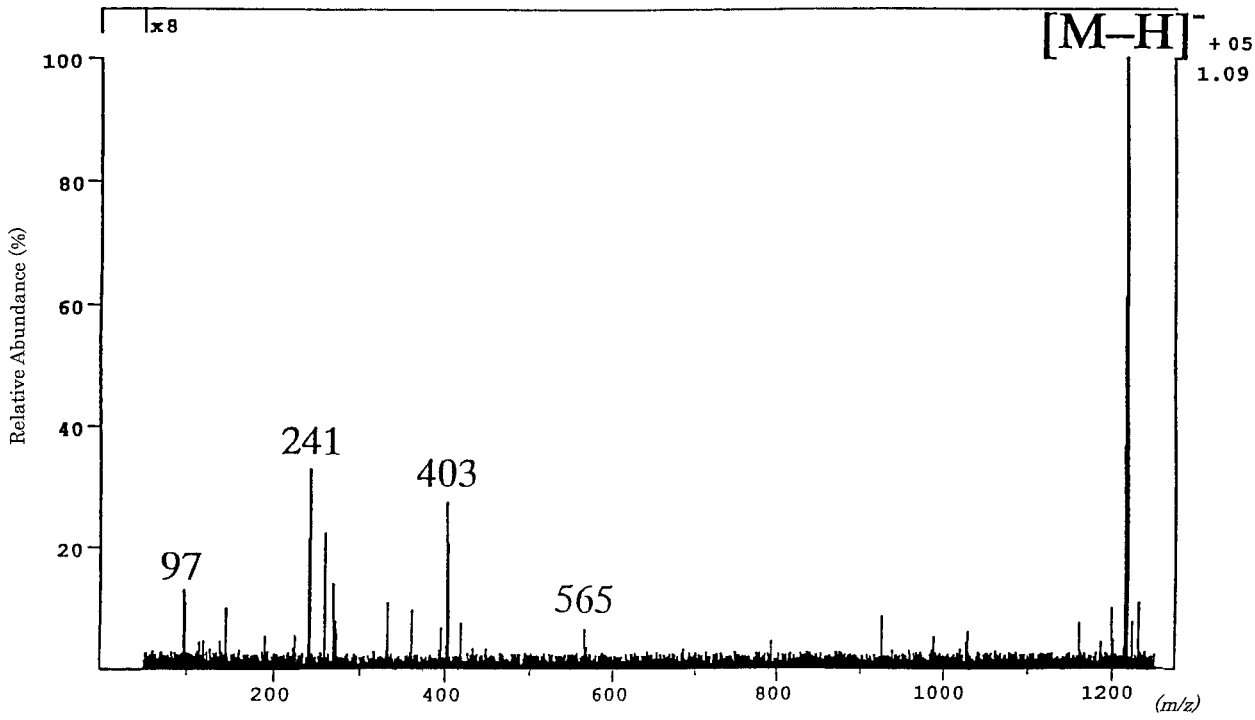


Fig. 10. MS/MS spectrum in the negative FAB-MS of the polar lipids with precursor ion at *m/z.*

mutant *ON-1* does not show the phototaxis by methylation.[16] These facts suggest that the methylation process in the lipids is not directly related to that in the protein.

3.4 MALDI-MS of polar lipid-mixture

The advantages of FAB-MS in the analyses of the

lipids as mentioned above are comparably similar to these of MALDI-MS in Fig. 8. However, the several additional peaks were revealed in the MALDI mass spectra; *m/z* 399, 610, and 821. These peaks are assignable to the deprotonated oligomeric sodium salts of the matrix (M for the molecular mass), 2(M−1)+Na, 3(M−1)+2Na, and 4(M−1)+3Na, respectively. The appearance of these ions was definitely dependent on the used matrices (DHB, SA), inorganic additives (K, Cs), and the measured compounds (phosphatidyl choline). As the result, all the peaks of the lipids in MALDI were exhibited as the identical masses in the high mass-region on FAB ionization but with the different intensities to some extent. The difference of the intensities between the spectra on MALDI and FAB ionizations chiefly depended on time-averaging. As a result, the FAB ionization time-dependently produced the abundant ions in the low mass region, although the MALDI provided the constant abundance of the ions.[17] This phenomenon has an affect on the preferable availability of MALDI for the quantitation of a mixture.

3.5 Composition analyses of phospholipids by ESI-MS

The quantitation of the polar lipids has been performed by means of TLC or HPLC so far.[11] However, the semi-quantitation of the lipid-components is frequently needed for rapid visualization of the outline in the composition-analysis of the polar lipids. Therefore, the MS analysis is efficient for evaluating the amount of the lipid composition. Although we have determined that a large percentage of phosphatidyl glycerophosphate is methylated on the terminal phosphoric acid,[13] the amount of the methylate has not been quantified with FAB-MS because of the time-dependent abundance of the ions. Averaging method in FAB mass spectra also involved large error of measurement. On the other hand, the ESI-MS gave the comparatively constant profiles in the spectra. Thus, the steady appearance of the ions deserves a promising method in determining the relative amounts of the components by measuring the intensities of the peaks in the ESI-mass spectra. Prior to the quantitation, linearity be-

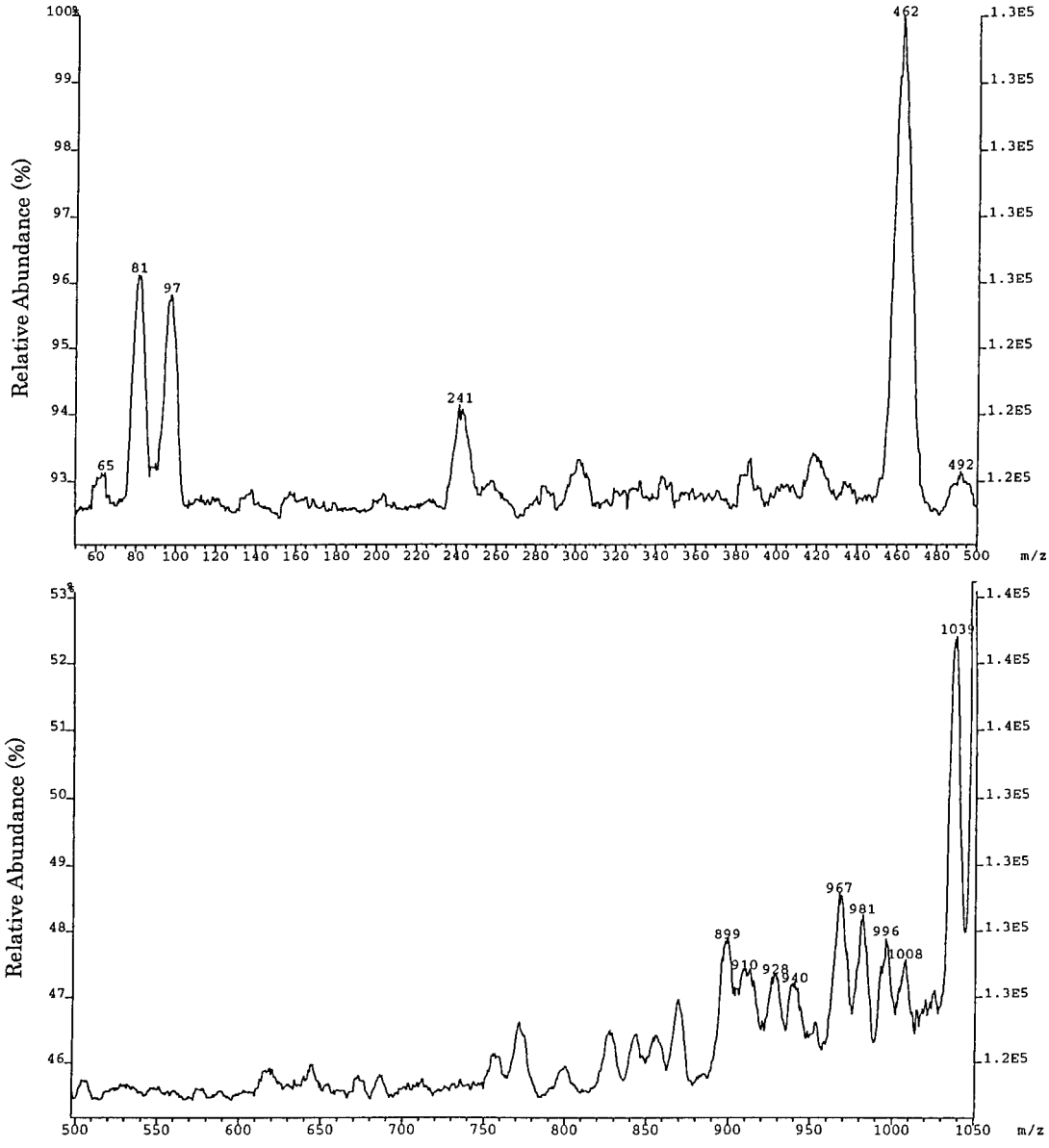


Fig. 11. MIKE spectrum of S-DGD from *m/z* 1055 in the negative LSIMS.

tween the abundance of the ions and the amount of the lipids was checked for estimation. Under the linearity for semi-quantitation, the relative percentages of the polar lipids in *S-9* strain were estimated with the ESI-MS as S-TGD 20%, PGP-OMe 77%, and PG 3%, respectively. These values are in fair agreement with the quantities determined by the TLC-isolation.[11]

On the basis of the quantitation, the composition of the mutant, FlxR, was also estimated as shown in Table 1. It is noticeable that FlxR mutant contains five times or more amounts of PG as large as the mutant *S-9*. The differences in the abundance of PG seem to be related to photosesory function of the proteins.[16] These results are summarized in Table 1.

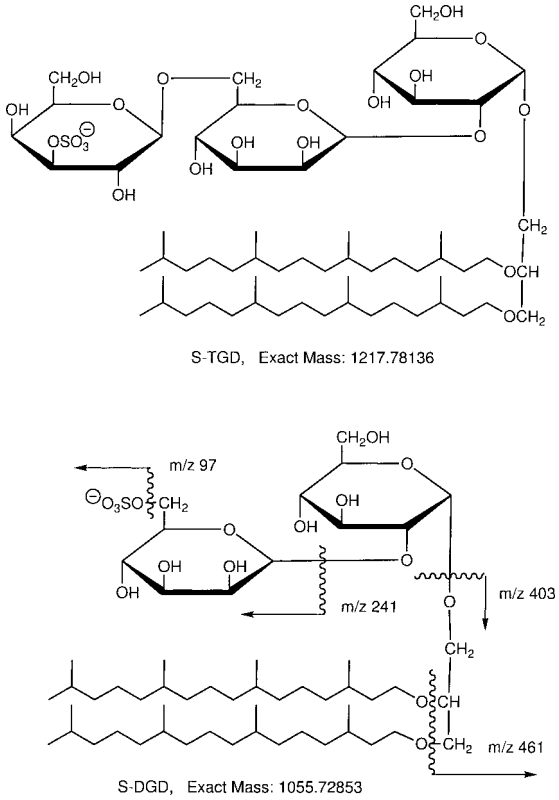
The ESI spectra involve another information on molecular structure in Fig. 9. The appearance of the abundant ions in the spectra can be explained as follows; the intensive peaks at *m/z* 1217, 921, 907, 899, 885, 805, and 449 correspond to the negative ions, $\{GLS-H\}^-$, $\{PGPOMe-2H+Na\}^-$, $\{PGP-2H+Na\}^-$, $\{PGPOMe-H\}^-$, $\{PGP-H\}^-$, $\{PG-H\}^-$, and $\{PGPOMe-2H\}^{2-}$, respectively. The latter peak at *m/z* 449 informed us that the formation of the doubly charged ion is distinguished from the case of the FAB ionization. Neither of the doubly charged ion of PGP-OMe was found in the MALDI yet.

3.6 Fragmentation analyses of S-DGD with MS/MS by CID in negative FAB-MS

More information was indicated that in the strain, *Hr.* sp. *aus-1*, the peak for diglycolipid sulfate (S-DGD) was observed as a major component, whereas S-TGD was found in *S-9* mutant. Fragmentation of the glycolipid in FAB ionization was similarly shown as a single fission in Fig. 10. The fragment ions generated from the precursor ion of S-TGD at *m/z* 1217 in the MS/MS spectrum with Ar-CID have been detected at *m/z* 565, 403, 241, and 97. Similarly, the fragment ions from the ion of the S-DGD at *m/z* 1055 appeared at *m/z* 241, 97, and 81 in the MS/MS spectrum. Moreover, the mass analyzed ion kinetic energy spectrum (MIKE spectrum) of S-DGD also gave the fragment ions at *m/z* 461, 241, 97, and 81 as shown in Fig. 11. The peaks at *m/z* 97 and 81 strongly suggest the sulfate anion but not the phosphate anion. The structure of the ion at *m/z* 461 can be derived from elimination of phytanols as shown in Scheme 2. However, the ion at *m/z* 461 in the MS/MS spectrum with CID could not be observed. The appearance of the ion was facile on the configuration of the apparatus (EBE) for short path. As a result, these fragmentation processes consistently suggest that S-DGD is composed of two glucopyranoses, a sulfate, and two phytanols as glyceroether.

3.7 Chemitaxonomy in halobacteria with MS-elucidation

The difference in the production of the S-TGD apparently correlated to the strain as shown in Table 1. It is seemed that the presence of either diglucosyl or triglucosyl sulfate in the polar lipids of the bacteria is characteristic of the strains. Recently, Kamekura *et al.* classified the family of halobacteriaceae and correlated genetic classification to sulfated glucoside contents by sequences of 16S rRNA encoding genes.[7] The species, *Hb. salinarum* (including old classified species, *Hb. halobium* and *Hb. cutirubrum*), contain S-TGD but not S-DGD. In contrast, the strain, *Hr.* sp. *aus-1*, has the S-DGD instead of the S-TGD. Thus, the genetic classification is related to the semi-quantitative analysis of the sulfates by mass spectrometry. Therefore, sulfate-analysis with mass spectrometry suggests the possibility of chemitaxonomy as described by Kamekura.



Scheme 2. The structures of the fragment ions produced in fragmentation of S-DGD.

In contrast, the detailed composition-analysis of the phospholipids also provided the other characteristics. The composition of phosphatidyl glycerol (PG) in *Hr.* sp. *aus-1* relatively increased in comparison with that of *Hb. sarinarum* corresponding to the decrease of the methylated phosphate (PGP-Me). From this tendency, another taxonomy is appeared in the methylation of the phosphate. In fact, it is likely that the halobacteria are capable of mutation in the methionine-deficient conditions. As a result, the abundant PG was observed in the polar lipids of the mutant in *Hb. sarinarum*. Moreover, this hypothesis is not necessarily generalized since the other mutant *ON-1* (containing unmethylated protein) showed no difference in the phospholipid contents in comparison with the *S-9* strain (containing methylated protein) as shown in Table 1. This classification with the lipid-methylation is non-expandable for mass spectrometric taxonomy of the halobacteria.

Acknowledgments

We thank Professor K. Mukouhata in Kochi University of Technology for the helpful discussion on the mutants.

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Keywords: Halobacteria, Phospholipid, Glycolipid sulfate, ESI-MS, FAB-MS, Negative ion, Chemotaxonomy, MS/MS analysis