Lipidomics of intact mitochondria by MALDI-TOF/MS

Roberto Angelini,* Rita Vitale,† Vinay A. Patil,§ Tiziana Cocco,* Bernd Ludwig, Miriam L. Greenberg,§ and Angela Corcelli[1],*,††**

Department of Basic Medical Sciences,* University of Bari "Aldo Moro," Bari, Italy; Institute for Microelectronics and Microsystems,† National Research Council (IMM-CNR), Lecce, Italy; Department of Biological Sciences,§ Wayne State University, Detroit, MI; Biocenter,** Institute of Biochemistry, Goethe University, Frankfurt, Germany; and Institute for Chemical-Physical Processes, National Research Council (IPCF-CNR),†† Bari, Italy

Abstract A simple and fast method of lipid analysis of isolated intact mitochondria by means of MALDI-TOF mass spectrometry is described. Mitochondria isolated from bovine heart and yeast have been employed to set up and validate the new method of lipid analysis. The mitochondrial suspension is directly applied over the target and, after drying, covered by a thin layer of the 9-aminoacridine matrix solution. The lipid profiles acquired with this procedure contain all peaks previously obtained by analyzing the lipid extracts of isolated mitochondria by TLC and/or mass spectrometry. The novel procedure allows the quick, simple, precise, and accurate analysis of membrane lipids, utilizing only a tiny amount of isolated organelle; it has also been tested with intact membranes of the bacterium *Paracoccus denitrificans* for its evolutionary link to present-day mitochondria. The method is of general validity for the lipid analysis of other cell fractions and isolated organelles.— Angelini, R., R. Vitale, V. A. Patil, T. Cocco, B. Ludwig, M. L. Greenberg, and A. Corcelli. **Lipidomics of intact mitochondria by MALDI-TOF/MS. *J. Lipid Res.* 2012.** 53: **1417–1425.**

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Historically lipid profiling of biological samples has been achieved with preliminary isolation of tissues, cells, membranes or organelles, followed by lipid extraction and purification with chromatographic techniques, such as HPLC or (HP)TLC (1, 2), and by lipid characterization and/or quantitation with gas chromatography (GC) (3), GC-mass spectrometry (MS) (4), fast atom bombardment (FAB)-MS (5–7), ESI-MS (8–12), and/or NMR analyses (13).

In the last decade, the capabilities of MALDI time-of-flight (TOF)/MS in lipid analysis have been largely demonstrated for the analysis of lipid extracts from different biological materials (14–18), but the most promising advantage of the MALDI-TOF/MS technique turned out to be the possibility to perform lipid analysis avoiding extraction and/or separation steps.

Recent advances in MALDI-TOF/MS techniques of lipid analysis have led to the direct coupling of HPTLC-MALDI (19–22), as well as to the direct analysis of tissue slices with the MALDI-MSI (23–25). In both cases, a plate containing the sample is coated with the matrix solution (silica plate or tissue on microscope glass plate, respectively), attached onto the MALDI target, and directly scanned with the laser to obtain a fast MS analysis of the lipids on the plate. Both these methods allow a fast and convenient lipid analysis, skipping extraction steps that are usually required for these kinds of analyses.

In 2010 we developed a protocol that allows the direct lipid analysis by MALDI mass spectrometry in intact archaeal membranes without prior extraction/separation steps: lyophilized archaeal membranes were ground with 9-aminoacridine (9-AA) as dry matrix, and the powder mixture was crushed in a mechanical die press to form a thin pellet; small pieces of the pellet were then attached onto the MALDI target, and the archaeal membrane lipid profile was directly achieved (26).

Another new protocol for the direct lipid fingerprinting with MALDI-TOF/MS was published by Ferreira et al. in 2010 (27). They report a direct lipid analysis of a single embryo and oocyte by MALDI-TOF/MS: the biological

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Abbreviations: BHM, bovine heart mitochondria; CL, cardiolipin; IPC, inositolphosphoceramide; IS, internal standard; MALDI-TOF/MS, MALDI time-of-flight mass spectrometry; MIPC, mannosyl-inositolphosphoceramide; $M(IP)_2C$, mannosyl-diinositolphosphoceramide; MLCL, monolysocardiolipin; PA, phosphatidic acid; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PI, phosphatidylinositol; PL, phospholipid; PS, phosphatidylserine; PSD, post-source decay; WT, wild-type; 9-AA, 9-aminoacridine.

[1] To whom correspondence should be addressed.
e-mail: a.corcelli@biologia.uniba.it

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sample is moisturized with the matrix solution, and lipid profiles are directly obtained.

Results contained in the above reports suggest that MALDI-TOF/MS can be successfully used for the direct analysis of intact biological samples, allowing a fast and detailed lipidomic analysis without artifacts due to the extraction/separation steps.

Here we show that the direct MALDI-TOF/MS lipid analysis of intact mitochondria, isolated from heart tissue, and yeasts give rise to lipid profiles similar to those previously obtained from the analyses of the corresponding mitochondrial lipid extracts. In addition, we have also analyzed intact bacterial membranes of *Paracoccus denitrificans*, whose components and functional organization closely resemble the electron transport chain of the inner mitochondrial membrane. We focus on the presence of cardiolipin (CL), the signature lipid of mitochondria, and show the great potential of our technique for the qualitative and quantitative detection of a lipid that is tightly bound to membrane proteins and might be difficult to completely recover in lipid extracts.

Preliminary results of this investigation have been presented at the 52nd International Conference on the Bioscience of Lipids (ICBL), Warsaw, Poland (28).

EXPERIMENTAL PROCEDURES

Materials

9-Aminoacridine hemihydrate was purchased from Acros Organics (Morris Plains, NJ). The following commercial glycerophospholipids (used as standards) were purchased from Avanti Polar Lipids Inc. (Alabaster, AL): 1,1',2,2'-tetratetradecanoyl cardiolipin; 1,1',2,2'-tetra-(9Z-octadecenoyl) cardiolipin; 1,2-ditetradecanoyl–*sn*-glycero-3-phosphate; 1,2-ditetradecanoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol); 1,2-ditetradecanoyl-*sn*-glycero-3-phospho-L-serine; and 1,2-di-(9Z-hexadecenoyl)-*sn*-glycero-3-phosphoethanolamine. All organic solvents used in extraction and MS analysis were commercially distilled and of the highest available purity and were purchased from Sigma Aldrich, J. T. Baker (Avantor Performance Materials, Center Valley, PA), or C. Erba, Reagenti S.p.A. [Arese (MI), IT].

Yeast strains, growth conditions, and mitochondria isolation

The following yeast *S. cerevisiae* strains were used for this study: FGY3 (Mat a, *ura3–52*, *lys2–801*, *ade2–101*, *trp1Δ 1*, *his3Δ 200*, *leu2Δ 1*), which served as wild-type, and strains carrying null mutations in the genes *CRD1* (29) and *TAZ1* (30). Cells were grown in YPGE (3% glycerol, 1% ethanol) at 30°C, and mitochondria were isolated as described by Daum et al. (31). Total mitochondrial protein was determined by Bradford reagent (Bio-Rad) with BSA as the standard.

Isolation of mitochondria from bovine heart

Mitochondria were prepared from bovine heart as previously described (32).

Paracoccus denitrificans growth conditions and membrane isolation

Cells of *Paracoccus denitrificans* were grown on succinate medium, and its membranes were isolated as previously described (33).

Preparation of mitochondria samples for MALDI-TOF/MS lipid analysis

The procedure for sample preparation is analogous to the sandwich method, commonly used in MALDI/TOF-MS. The different mitochondrial suspensions were all diluted to 0.1 μg/μl of total mitochondrial protein concentration, determined by Bradford assay. Then 1 μl of mitochondrial suspension was spotted onto the MALDI target. After water evaporation, a thin layer (0.25 μl) of matrix solution (9-AA, 30 mg/ml in 2-propanol/acetonitrile, 60/40, v/v) is then spotted on the dried sample. After the evaporation of the matrix solvent, the samples are ready to be directly analyzed with MALDI-TOF/MS.

To quantify the CL content in the samples, 10 μl of solution of internal standard (IS), tetra-myristoyl-CL $(14:0)_4$, 64 μM in chloroform, were mixed with 90 μl of the matrix solution. Finally 0.25 μl of the mixed matrix solution, containing 1.6 pmol of the internal standard, were directly spotted on the dried sample as above.

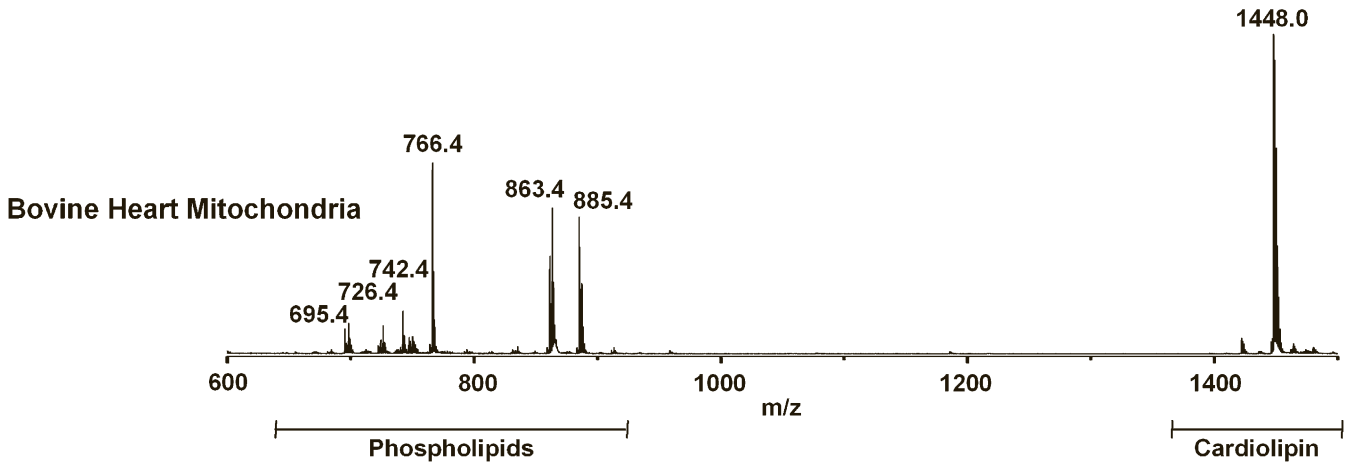

Fig. 1. Negative MALDI-TOF/MS lipid profile of intact bovine heart mitochondria acquired using 9-AA as matrix. The *m/z* signals corresponding to the different lipid components are (from the left): PA 36:4, $[M-H]^-$ at 695.4; PS 32:4, $[M-H]^-$ at 726.4; PE 36:2, $[M-H]^-$ at 742.4; PE 38:4, $[M-H]^-$ at 766.4; PI 36:1, $[M-H]^-$ at 863.4; PI 38:4, $[M-H]^-$ at 885.4; CL $(18:2)_4$, $[M-H]^-$ at 1448.0. A detailed list of detected peaks is shown in Table 1.

TABLE 1. Assignments of *m/z* values detected in the negative ion mode MALDI-TOF mass spectra of intact bovine heart mitochondria using 9-AA as matrix

m/z Value	Assignment $[M-H]^-$
695.4	PA 36:4
698.4	PS 30:4
722.4	PS 32:6
724.4	PS 32:5
726.4	PS 32:4
738.4	PE 36:4
740.4	PE 36:3
742.4	PE 36:2
747.4	PG 34:1
750.4	PS 34:6
764.4	PE 38:5
766.4	PE 38:4
792.4	PE 40:5
794.4	PE 40:4
796.4	PE 40:3
798.4	PE 40:2
831.4	PI 34:3
833.4	PI 34:2
835.4	PI 34:1
859.4	PI 36:3
861.4	PI 36:2
863.4	PI 36:1
883.4	PI 38:5
885.4	PI 38:4
887.4	PI 38:3
911.4	PI 40:5
913.4	PI 40:4
1185.6	MLCL 54:6
1422.0	CL 70:7
1436.0	CL 70:0
1446.0	CL 72:9
1448.0	CL 72:8[a]
1450.0	CL 72:7
1452.0	CL 72:6
1462.0	CL 72:1
1464.0	CL 72:0
1474.0	CL 74:9
1476.0	CL 74:8
1478.0	CL 74:7
1480.0	CL 74:6
1482.0	CL 74:5
1494.0	CL 75:6
1496.0	CL 75:5
1510.1	CL 76:5
1512.1	CL 76:4

[a]The chain length of the cardiolipin peak at m/z 1448.0 has been assigned by PSD analysis of present study (see spectrum in supplementary Fig. I).

Assuming that all the CL species have the same capability to ionize, the picomoles of CL species present in the sample were calculated by determining the ratio of the peak area of the CL molecular species of interest to the internal standard; this ratio was then multiplied by the picomoles of internal standard present on the target.

Preparation of mitochondrial lipid extracts for MALDI-TOF/MS lipid analysis

Total lipids were extracted using the Bligh and Dyer method as previously described (34); the extracts were carefully dried under N_2 before weighing and then dissolved in chloroform at the final concentration of 10 mg/ml. From a wild-type (WT) yeast mitochondrial suspension, containing 1 mg of total protein, 0.3 mg of total lipids were extracted.

To quantify the CL content, the lipid extract solution (10 mg/ml in $CHCl_3$) was first mixed 1:1 with a solution of IS CL $(14:0)_4$ 0.4 mM in $CHCl_3$, obtaining a solution that was 5 mg/ml of total yeast mitochondrial lipids and 0.2 mM of IS CL $(14:0)_4$. Then, as previously described by Sun et al. (14), 20 µl of the resulting solution were diluted in 180 µl of 2-propanol/acetoneitrile (60/40, v/v), then 10 µl of the diluted solution was mixed with 10 µl of matrix solution (9-AA, 10 mg/ml in 2-propanol/acetonitrile, 60/40, v/v). The resulting lipids-matrix solution was then spotted onto the MALDI target in droplets of 0.35 µl and analyzed as described below.

MALDI-TOF mass spectrometry

MALDI-TOF mass spectra of "intact" samples were generally acquired on a Bruker Microflex LRF mass spectrometer, whereas post-source decay (PSD) spectra were acquired on a Bruker Autoflex mass spectrometer (Bruker Daltonics, Bremen, Germany). The systems utilize a pulsed nitrogen laser, emitting at 337 nm, the extraction voltage was 20 kV, and gated matrix suppression was applied to prevent detector saturation. For each mass spectrum, 999 single laser shots (sum of 3 × 333) were averaged. The laser fluence was kept about 5% above threshold to have a good signal-to-noise ratio. All spectra were acquired in reflector mode using the delayed pulsed extraction; only spectra acquired in negative ion mode are shown in this study. Peaks areas, spectral mass resolutions, and signal-to-noise ratios were determined by the software for the instrument "Flex Analysis 3.3.65" (Bruker Daltonics).

A mix containing 1,1',2,2'-tetratetradecanoyl cardiolipin; 1,1',2,2'-tetra-(9Z-octadecenoyl) cardiolipin; 1,2-ditetradecanoyl-*sn*-glycero-3-phosphate; 1,2-ditetradecanoyl-*sn*-glycero-3-phospho-(1'-rac-glycerol); 1,2-ditetradecanoyl-*sn*-glycero-3-phospho-L-serine; and 1,2-di-(9Z-hexadecenoyl)-*sn*-glycero-3-phosphoethanolamine was always spotted next to the sample as external standard, and an external calibration was performed before each measurement. The mass range of the authentic standards is 590–1450 amu.

RESULTS

MALDI-TOF/MS lipid profiles of intact mitochondria, isolated from bovine heart and yeast, and of intact membranes of the bacterium *Paracoccus denitrificans* were recorded.

Mitochondria isolated from bovine heart

The mass spectrum of lipids of intact bovine heart mitochondria (BHM), acquired in negative ion mode and using 9-AA as matrix is shown in **Fig. 1**. The signals in the spectra attributable to the negative $[M-H]^-$ molecular ions of phospholipids, sphingolipids, and glycolipids are collected in **Table 1**. The peaks can be grouped into three main *m/z* ranges: *i*) *m/z* 650–1000, attributable to major phospholipids; *ii*) *m/z* 800–1400, to glycosphingolipids and lysocardiolipins; and *iii*) *m/z* 1350–1500, to CLs.

In the range of major phospholipids (Fig. 1), the peak at *m/z* 695.4 can be attributed to phosphatidic acid (PA) 36:4; the peaks at *m/z* 742.4 and 766.4 are attributable to phosphatidylethanolamine (PE) 36:2 and 38:4; the peak at *m/z* 747.4 is assigned to phosphatidylglycerol (PG) 34:1; the peaks at *m/z* 698.4, 726.4, and 750.4 are assigned to phosphatidylserine (PS) 30:4, 32:4, and 34:6; and various species of phosphatidylinositol (PI) are present at *m/z* 835.4 (34:1), 861.4 (36:2), 863.5

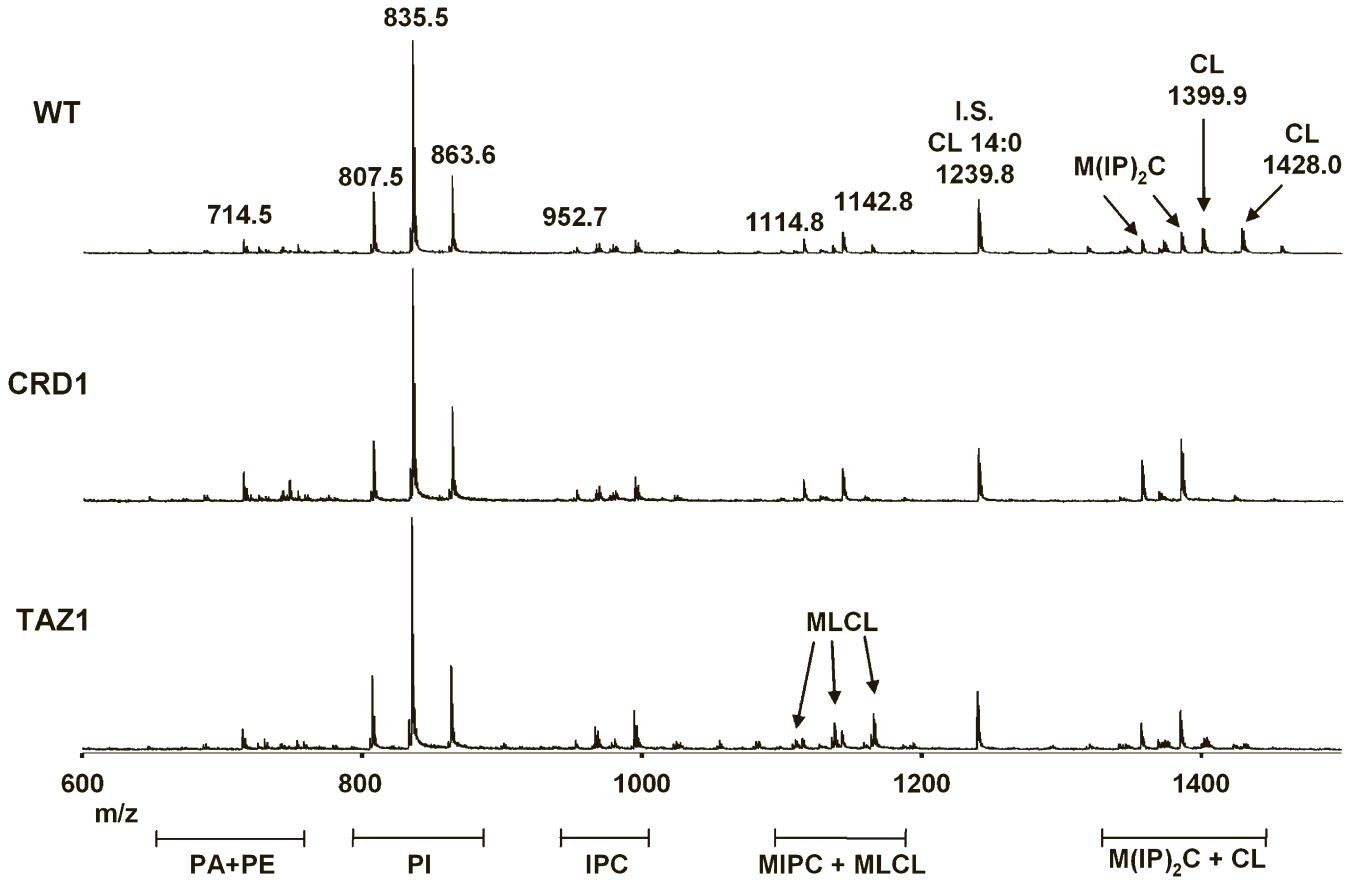

Fig. 2. Negative MALDI-TOF/MS lipid profiles of intact yeast mitochondria (WT, *crd1*Δ, and *taz1*Δ null mutants) acquired using 9-AA as matrix. The *m/z* signals corresponding to the different lipid components are (from the left): PE 34:2, $[M-H]^-$ at 714.5; PI 32:1, $[M-H]^-$ at 807.5; PI 34:1, $[M-H]^-$ at 835.5; PI 36:1, $[M-H]^-$ at 863.6; IPC (t18:0/26:0-C), $[M-H]^-$ at 952.7; MIPC (t18:0/26:0-C), $[M-H]^-$ at 1114.8; MIPC (t20:0/26:0-C), $[M-H]^-$ at 1142.8; CL 68:4, $[M-H]^-$ at 1399.9; CL 70:4, $[M-H]^-$ at 1428.0. A detailed list of detected peaks is shown in Tables 2 and 3. Tetra-myristoyl-CL $(14:0)_4$, peak at *m/z* 1239.8, was used as internal standard (IS) to quantify the CL content in yeast mitochondria.

(36:3), 885.4 (38:4), 887.7 (38:3), 911.3 (40:5), and 913.4 (40:4). A detailed list, including minor peaks, is available in Table 1.

In the cardiolipin range, a major peak at *m/z* 1448.0 is present, which corresponds to the CL having four 18:2 chains, as confirmed by PSD analysis (see supplementary Fig. I). In addition to this major CL 72:8, other CLs are present (see details in Table 1).

In the lyso-CL range, only a very small peak at *m/z* 1185.6 can be attributed to monolysocardiolipin (MLCL) 54:6, arising from the CL 72:8.

Mitochondria isolated from WT yeast and CL mutants

Mitochondria isolated from yeast WT cells and CL null mutants (*crd1*Δ and *taz1*Δ) were analyzed with the new method.

The MALDI-TOF/MS profiles of the three different mitochondrial preparations are shown in **Fig. 2**.

The signals in the spectra attributable to the negative $[M-H]^-$ molecular ions of yeast mitochondrial lipids are collected in **Table 2** (phospholipids) and **Table 3** (glycosphingolipids).

The peaks can be grouped in three main *m/z* ranges: *i*) *m/z* 650–1000, attributable to major phospholipids; *ii*) *m/z* 800–1400, to glycosphingolipids and lysocardiolipins; and *iii*) *m/z* 1350–1500, to CLs.

In the range of major phospholipids (Fig. 2), the small peaks at *m/z* 647.3 and 671.5 can be attributed to phosphatidic acid (PA) 32:0 and 34:2; the peak at *m/z* 714.5 is attributable to phosphatidylethanolamine (PE) 34:2; and various species of phosphatidylinositol (PI) are present at *m/z* 807.6, 835.6 and 863.6 corresponding to PI 32:1, 34:1 and 36:1 respectively. The peak of PI at *m/z* 835.6 was also investigated by PSD analysis; results indicated the presence of 18:1 and 16:0 chains (data not shown).

In the range of glycosphingolipids, a number of peaks are present. The peaks *m/z* 900–1000 are attributable to the inositolphosphoceramides (IPC), with a long chain base of 18 or 20 carbons, different level of hydroxylation, and linked fatty acyl residues with chain length of 26 carbons. The peaks at *m/z* 1114.8 and 1142.8 corresponds to mannosyl-inositolphosphoceramides (MIPC); larger mannosyl-diinositolphosphoceramide $[M(IP)_2C]$ molecules can be also seen from *m/z* 1340 to 1420 mixed with the cardiolipin peaks.. Notably, due to the lack of cardiolipin peaks in the lipid profile of the *crd1*Δ mutant, the glycopshingolipid peaks can be easily distinguished by comparing lipids of wild-type and mutant strains. However, the peaks

TABLE 2. Assignments of *m/z* values detected in the negative ion mode MALDI-TOF mass spectra of intact yeast mitochondria (WT, *crd1*Δ and *taz1*Δ mutants) using 9-AA as matrix, attributable to phospholipids

m/z Value	Assignment $[M-H]^-$
619.2	PA 30:0
643.4	PA 32:2
645.4	PA 32:1
647.3	PA 32:0
663.1	PG 28:1
671.5	PA 34:2
673.5	PA 34:1
686.5	PE 32:2
688.5	PE 32:1
701.1	PA 36:1
703.1	PA 36:0
714.5	PE 34:2
716.5	PE 34:1
730.4	PS 32:2
732.5	PS 32:1
742.5	PE 36:2
745.5	PG 34:2 (only *crd1*Δ)
747.5	PG 34:1 (only *crd1*Δ)
758.5	PS 34:1
760.5	PS 34:0
775.6	PG 36:1 (only *crd1*Δ)
777.6	PG 36:0 (only *crd1*Δ)
779.5	PI 30:1
781.5	PI 30:0
784.1	PE 36:3
786.1	PE 36:2
805.5	PI 32:2
807.5	PI 32:1
821.5	PI 33:1
833.5	PI 34:2
835.5	PI 34:1[a]
854.5	PE 44:2
861.5	PI 36:2
863.5	PI 36:1
1107.7	MLCL 48:3
1109.8	MLCL 48:2
1135.8	MLCL 50:3 only WT and *taz1*Δ
1137.8	MLCL 50:2 only *taz1*Δ
1163.8	MLCL 52:3 only WT and *taz1*Δ
1165.8	MLCL 52:2 only *taz1*Δ
1191.8	MLCL 54:3 only WT and *taz1*Δ
1193.8	MLCL 54:2 only *taz1*Δ
1343.9	CL 64:4 only WT and *taz1*Δ (more in WT)
1345.9	CL 64:3 only *taz1*Δ
1347.9	CL 64:2 only *taz1*Δ
1371.9	CL 66:4 only WT and *taz1*Δ (more in WT)
1373.9	CL 66:3 only *taz1*Δ
1375.9	CL 66:2 only *taz1*Δ
1399.9	CL 68:4[a] only WT and *taz1*Δ (more in WT)
1401.9	CL 68: 3 only *taz1*Δ
1403.9	CL 68: 2 only *taz1*Δ
1428.0	CL 70:4[a] only WT and *taz1*Δ (more in WT)
1430.0	CL 70:3 only *taz1*Δ
1432.9	CL 70:2 only *taz1*Δ
1456.0	CL 72:4

[a] Chains of peaks at *m/z* 835.5, 1399.9, and 1428.0 were assigned by PSD analyses.

of two MIPCs and two $M(IP)_2C$s were analyzed by PSD, confirming their structural identification (only two examples of these analyses are shown in spectra in supplementary Fig. II-A, B). A detailed list of glycosphingolipid assignments can be seen in Table 3.

Fig. 3 shows two enlargements in the MLCLs and CLs ranges, respectively.

TABLE 3. Assignments of *m/z* values detected in the negative ion mode MALDI-TOF mass spectra of intact yeast mitochondria (WT, *crd1*Δ and *taz1*Δ mutants) using 9-AA as matrix, attributable to glycosphingolipids

m/z Value	Assignment $[M-H]^-$	Reference
936.5	IPC (t18:0/26:0-B)	45-46[b], 43[c]
952.7	IPC (t18:0/26:0-C)	45-46[b], 43[c]
964.5	IPC (t20:0/26:0-B)	45-46[b], 43[c]
968.5	IPC (t18:0/26:0-D)	45-46[b], 43[c]
980.5	IPC (t20:0/26:0-C)	45-46[b], 43[c]
1098.6	MIPC (t18:0/26:0-B)	45[b], 43[c]
1114.8	MIPC (t18:0/26:0-C)[a]	45[b], 43[c]
1126.8	MIPC (t20:0/26:0-B)	45[b], 43[c]
1130.8	MIPC (t18:0/26:0-D)	45[b]
1142.8	MIPC (t20:0/26:0-C)[a]	45[b], 43[c]
1340.8	$M(IP)_2C$ (t18:0/26:0-B)	43[d], 45[d]
1356.8	$M(IP)_2C$ (t18:0/26:0-C)[a]	43[d], 45[d]
1368.9	$M(IP)_2C$ (t20:0/26:0-B)	45[d]
1384.9	$M(IP)_2C$ (t20:0/26:0-C)[a]	45[d]

Glycosphingolipids peaks have been assigned to their molecular species according to the past literature (43-46). Number of hydroxyl groups in the fatty-acyl moiety as in Guan and Wenk (43): B = 0, C = 1, D = 2.

[a] PSD analyses of the peaks at *m/z* 1384.9, 1356.8, 1142.8, 1114.8 were performed.

[b] Based on precursor ion scanning (PIS) or multiple reaction monitoring (MRM) data achieved on the identical $[M-H]^-$ observed in yeast *Saccharomyces cerevisiae*.

[c] PIS or MRM data achieved on the correspondent $[M+H]^+$ observed in yeast *Saccharomyces cerevisiae*.

[d] PIS or MRM data achieved on the correspondent $[M-2H]^{2-}$ observed in yeast *Saccharomyces cerevisiae*.

In the lyso-CL range, peaks at *m/z* 1107.7, 1135.8, 1163.8, and 1191.8, can be attributed to MLCL 48:3, 50:3, 52:3, and 54:3, respectively.

In the CLs range, a large number of peaks are present, attributable to CLs of different chain lengths. Major peaks at *m/z* 1343.9, 1371.9, 1399.9, 1428.0. and 1456.0 are attributed to CL 64:4, 66:4, 68:4, 70:4, and 72:4, respectively. Again, PSD analyses of the two peaks at 1399.9 and 1428.0 confirmed assignments based on literature (spectra are available in supplementary Fig. III).

As previously reported in *taz1*Δ mutant, an aberrant CL-acyl composition occurs (35, 36). Circles in the *taz1*Δ mutant mass spectrum in Fig. 3 show the presence of CL molecular species containing saturated fatty acids, probably 16:0 and 18:0 instead of 16:1 and 18:1, as reported in the past literature (35, 36). CL molecular species 64:4 at *m/z* 1343.9, 66:4 at *m/z* 1371.9, 68:4 at *m/z* 1399.9, 70:4 at *m/z* 1428.0, and 72:4 at *m/z* 1456.0 are the most abundant in the wild-type. In contrast, CL molecular species with unsaturated fatty acids markedly decrease in the *taz1*Δ mutant, whereas the more saturated CLs, indicated by circles in the Fig. 3, are predominantly present. As a consequence, the acyl composition of the MLCLs species changes similarly in the *taz1*Δ mutant; peaks at *m/z* 1109.8, 1137.8, 1165.8 and 1193.8 are attributable to MLCL 48:2, 50:2, 52:2 and 54:2 respectively. For details see Table 2.

These data confirm the accumulation of MLCL and the decrease in unsaturated fatty acyl CL species in the *taz1*Δ yeast mutant (35).

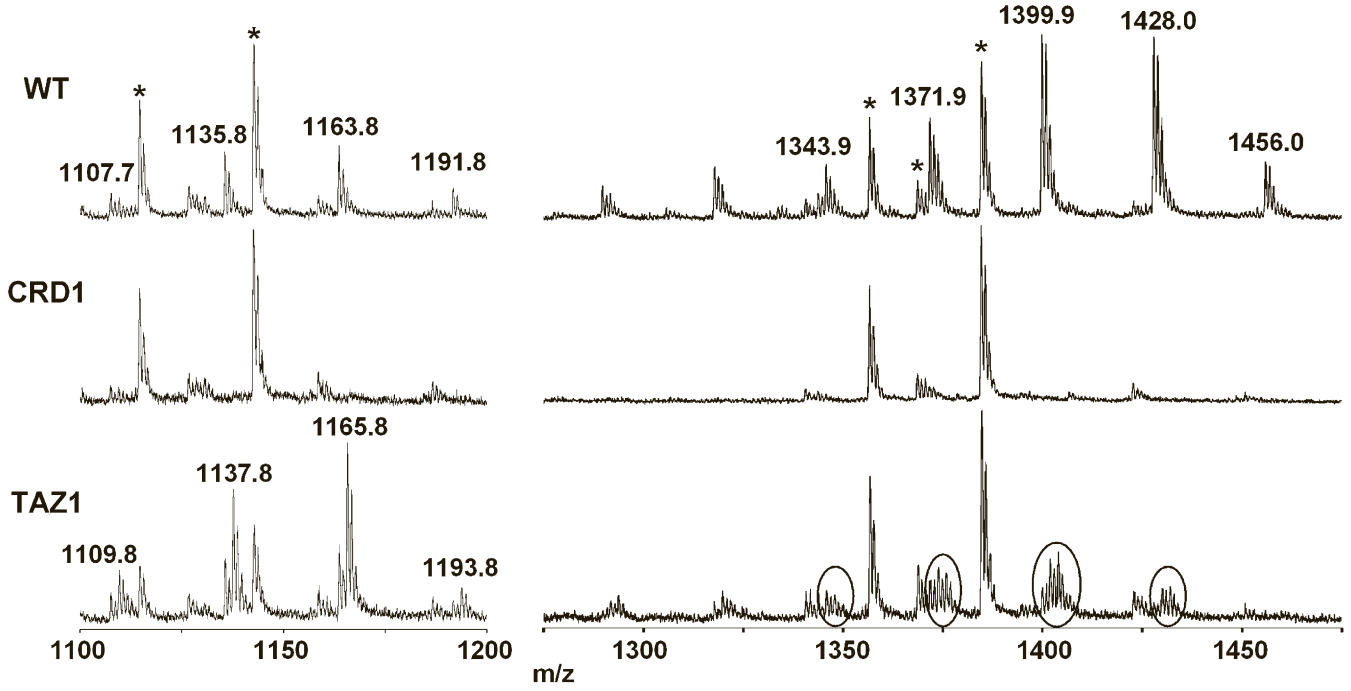

Fig. 3. Enlargement of the negative MALDI-TOF/MS lipid profiles of intact yeast mitochondria (WT, *crd1*Δ and *taz1*Δ mutants) acquired using 9-AA as matrix, focus on yeast mitochondrial MLCLs and CLs. Peaks marked with an asterisk are attributable to inositolceramides (glycosphingolipids). The *m/z* signals corresponding to MLCLs and CLs with different chain lengths are (from the left): MLCL 48:3, $[M\text{-}H]^-$ at 1107.7; MLCL 50:3, $[M\text{-}H]^-$ at 1135.8; MLCL 52:3, $[M\text{-}H]^-$ at 1163.8; MLCL 54:3, $[M\text{-}H]^-$ at 1191.8; CL 64:4, $[M\text{-}H]^-$ at 1343.9; CL 66:4, $[M\text{-}H]^-$ at 1371.9; CL 68:4, $[M\text{-}H]^-$ at 1399.9; CL 70:4, $[M\text{-}H]^-$ at 1428.0; CL 72:4, $[M\text{-}H]^-$ at 1456.0. In the mitochondria from the *crd1*Δ mutant, neither MLCL nor CL is present. In the mitochondria from the *taz1*Δ mutant, a lower amount of CL and a higher amount of MLCL are present compared with the wild-type, and both MLCL and CL are present with unusual saturated fatty acyl chains.

We quantified the CL content in the mitochondria of wild-type and *taz1*Δ mutant yeast, using the tetra-myristoyl-CL as an internal standard (**Fig. 4**). At the same time, one aliquot of wild-type mitochondria was extracted, and the internal standard was mixed to equilibrium to quantitate CL in the lipid extract and compare results. The CL content in the "intact" wild-type mitochondria was 26.0 (±3.8 SD) picomoles per microgram of total mitochondrial proteins, whereas CL in the lipid extract of the same mitochondrial preparation was 22.1 (±2.6 SD) picomoles per microgram of total mitochondrial proteins, indicating that results obtained with the two different methods are clearly comparable. In addition, the CL content in the "intact" *taz1*Δ mutant mitochondria was 8.8 (±1.7 SD) picomoles per microgram of total mitochondrial proteins. Therefore, using the "intact" method, the CL content in *taz1*Δ mutant was found to be 33.8% (±11.5 SD) of the wild-type. In the *crd1*Δ mutant, the CL is absent or present in traces, but notably some minor peaks attributable to molecular species of its precursor PG are present at *m/z* 745.5 (34:2), 747.5 (34:1), 775.6 (36:1), and 777.6 (36:0). For details, see Table 2; in addition, a scheme of the CL biosynthesis pathways in yeast is shown in supplementary Fig. IV.

A comparison of lipid profiles of the WT yeast intact mitochondria and the WT yeast mitochondrial lipid extract is shown in supplementary Fig. V. The peaks of cardiolipins, lysocardiolipins, and complex glycosphingolipids are smaller in the lipid extract than in the lipid profile of intact membranes, suggesting that these components, as expected, are partially lost during the extraction process. Further analysis of lipids associated with the remaining heterogeneous protein pellet after the lipid extraction revealed that the reduced and/or missing peaks in the lipid extract are better visible and/or enriched in this material. All together, above results show that the complete extraction of all lipid species is difficult to obtain.

Membranes isolated from *Paracoccus denitrificans*

The validity of the new method was finally tested with intact membranes of *Paracoccus denitrificans*, a bacterium of close evolutionary relationship to mitochondria. **Fig. 5** shows the MALDI-TOF mass spectrum of the intact membranes of *Paracoccus denitrificans*, acquired in the negative ion mode using 9-AA as matrix. A detailed list of signals in the spectra attributable to the negative $[M\text{-}H]^-$ molecular ions of phospholipids (PL) is presented in **Table 4**. The peaks can be grouped into two main *m/z* ranges: *i*) *m/z* 600–900, attributable to PLs and *ii*) *m/z* 1350–1450, to CLs. The *m/z* range 900–1350 was omitted because only some unidentified peaks were present. The CL *m/z* range was enlarged four times in the *y* axis to emphasize the CL signals pattern.

In the range of PLs, the peak at *m/z* 699.5 can be attributed to PA 36:2; the peak at *m/z* 742.6 is attributable to PE

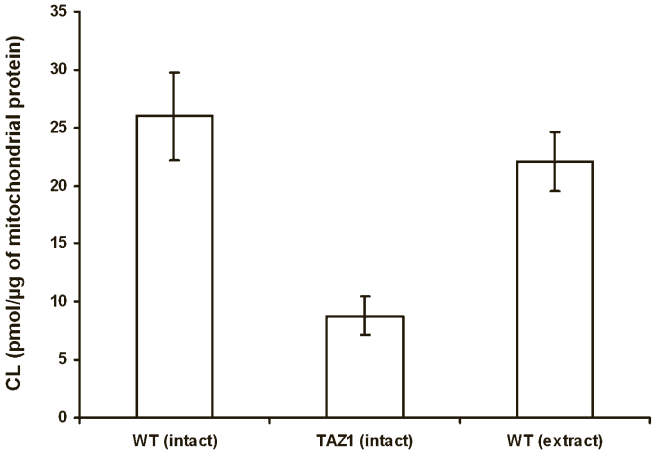

Fig. 4. Quantitation of CL content in yeast mitochondria. To quantify the CL content, the ratio of the peak area of the CL molecular species of interest to the internal standard CL $(14:0)_4$ was determined and multiplied by the picomoles of internal standard present on the target. We have analyzed one preparation of each strain and collected 5–7 spectra per strain. Error bars represent SD. The CL content in the "intact" wild-type yeast mitochondria is 26.0 (±3.8 SD) picomoles per microgram of total mitochondrial proteins. The CL content in the "intact" *taz1*Δ mutant yeast mitochondria is 8.8 (±1.7 SD) picomoles per microgram of total mitochondrial proteins. The CL content in the lipid extract of wild-type yeast mitochondria is 22.1 (±2.6 SD) picomoles per microgram of total mitochondrial proteins.

36:2; and various species of PG are present at *m/z* 747.6 (34:1) and 773.6 (36:2).

In the CL range, a large number of peaks are present, attributable to CLs of different chain lengths. Major peaks at *m/z* 1404.2, 1430.2, and 1456.2 are attributed to CL 68:2, 70:3, and 72:4, respectively. A detailed list, including minor peaks, is available in Table 4. Results are comparable with and update the past literature (37).

DISCUSSION

Lipid isolation is, beyond doubt, a big and controversial chapter in lipidomics: the manipulation processes of biological samples used to perform lipid extraction and lipid separation techniques can introduce artifacts and hamper the accurate determination of the proportion of various lipid components in the biomembranes. For this reason, a two-step extraction method is often needed to recover almost all the lipid (80–99% of relatively apolar and 74–95% of polar lipids) that are present in a biological sample (38). If preparative TLC is used for lipid purification, a likely source of error can be represented by partial loss of sample during the extraction of lipids from the silica bands (39).

It has already pointed out that MALDI-TOF/MS is an important analytical technique in the emerging field of the single-cell metabolomics (40–43): the need to preserve the original metabolome during sample processing is a crucial aspect in studies of biological dynamics, and it is often a very difficult task because of the presence of enzymes in the sample and the fast metabolic turnover rates.

Here, we have presented a method of direct MALDI-TOF/MS analysis of lipids in intact mitochondria and bacterial membranes. A small drop of suspension of mitochondrial membranes or bacterial cells was deposited on the MALDI target and dried at room temperature, and then the sample was covered with a droplet of matrix (9-AA) solution and directly scanned with the MALDI laser to desorb and analyze the lipid content.

This study offers additional evidence that, as previously demonstrated by Schiller et al. (15–18), MALDI-TOF/MS can provide data comparable to other modern MS methods, but in a faster and more convenient way.

Our method promises not only fast and quick lipid analysis but also the possibility to extend the knowledge on mitochondrial lipidome previously studied by ESI-MS lipid extract analyses in many publications (38, 44–47).

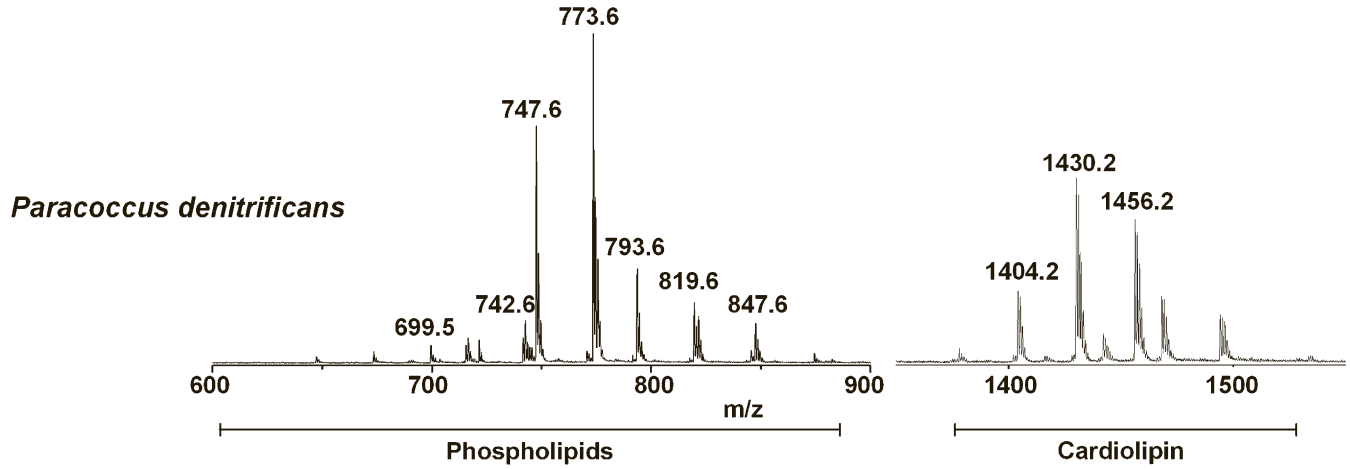

Fig. 5. Negative MALDI-TOF/MS lipid profiles of intact membranes of *Paracoccus denitrificans* acquired using 9-AA as matrix. The *m/z* signals corresponding to the different lipid components are (from the left): PA 36:2, $[M-H]^-$ at 699.5; PE 36:2, $[M-H]^-$ at 742.6; PG 34:1, $[M-H]^-$ at 747.6; PG 36:2, $[M-H]^-$ at 773.6; PG 38:6, $[M-H]^-$ at 793.6; PG 40:5, $[M-H]^-$ at 819.6; PG 42:7, $[M-H]^-$ at 847.6; CL 68:2, $[M-H]^-$ at 1404.2; CL 70:3, $[M-H]^-$ at 1430.2; CL 72:4, $[M-H]^-$ at 1456.2. A detailed list of detected peaks is shown in Table 4.

TABLE 4. Assignments of *m/z* values detected in the negative ion mode MALDI-TOF mass spectra of intact membranes of *Paracoccus denitrificans* using 9-AA as matrix

m/z Value	Assignment $[M-H]^-$
647.3	PA 32:0
673.5	PA 34:1
699.5	PA 36:2
715.6	PG 32:3
716.6	PE 34:1
721.6	PG 32:0
741.6	PG 34:4
742.6	PE 36:2
744.6	PE 36:1
745.6	PG 34:2
747.6	PG 34:1
773.6	PG 36:2
1404.2	CL 68:2
1430.2	CL 70:3
1456.2	CL 72:4

It is indeed in line with a recent study in which ESI-MS, another "soft ionization" MS technique, has been used for direct analysis of intact membrane proteins: ATPases/synthases from *T. termophilus* and *Enterococcus hirae* have been analyzed as intact membrane domains and with soluble subunit interactions preserved in the vacuum during the MS analysis. This approach has brought about new insights in the regulatory effect of lipids bound to these transmembrane proteins (48).

Data reported show that all negatively charged membrane lipids can be detected by means of this method; phosphatidylcholine species can also be detected in intact membranes with 9-AA as matrix in the positive ion mode (not shown). Worthy of note, yeast inositol-containing ceramides, including inositolphosphoceramide, mannosyl-inositolphosphoceramide, and mannosyl-diinositolphosphoceramide, can be detected also.

Finally, the great potential of our technique is that it easily allows focusing on the signature lipid of mitochondria cardiolipin, a lipid component that is tightly bound to membrane proteins and that is often difficult to recover in lipid extracts.

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