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Phospholipid Composition and Metabolism of *Micrococcus denitrificans*

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The phospholipid composition of *Micrococcus denitrificans* was unusual in that phosphatidyl choline (PC) was a major phospholipid (30.9%). Other phospholipids were phosphatidyl glycerol (PG, 52.4%), phosphatidyl ethanolamine (PE, 5.8%), an unknown phospholipid (5.3%), cardiolipin (CL, 3.2%), phosphatidyl dimethylethanolamine (PDME, 0.9%), phosphatidyl monomethylethanolamine (PMME, 0.6%), phosphatidyl serine (PS, 0.5%), and phosphatidic acid (0.4%). Kinetics of ^{32}P incorporation suggested that PC was formed by the successive methylations of PE. Pulse-chase experiments with pulses of ^{32}P or acetate-*1*-^{14}C to exponentially growing cells showed loss of isotopes from PMME, PDME, PS, and CL with biphasic kinetics suggesting the same type of multiple pools of these lipids as proposed in other bacteria. The major phospholipids, PC, PG, and PE, were metabolically stable under these conditions. The fatty acids isolated from the complex lipids were also unusual in being a simple mixture of seven fatty acids with oleic acid representing 86% of the total. Few free fatty acids and no non-extractable fatty acids associated with the cell wall or membrane were found.

Micrococcus denitrificans is an unusual, gram-negative *Micrococcus* that has a membrane-bound electron transport system with a composition remarkably similar to that of beef heart mitochondria (32, 33). The cytochrome oxidase prepared from membranes of this organism reacts with mammalian cytochrome *c*, which is unusual for most bacterial cytochrome oxidases (21, 38). Despite the large amount of work with the electron transport system in this organism, there has been little work reported on the lipid composition (37).

In this study, the lipids of the membrane containing the electron transport system will be shown to contain phosphatidyl choline (PC) as a major phospholipid and to have oleic acid as the principal fatty acid, properties again atypical for bacteria but much like mammalian mitochondria.

MATERIALS AND METHODS

Materials. Materials were obtained from the supplier noted previously (46, 48). $H_3{}^{32}PO_4$, $H_3{}^{33}PO_4$, and acetate-*1*-^{14}C were purchased from New England Nuclear, Boston, Mass.

Organism and growth conditions. *Micrococcus denitrificans* (ATCC 13543) was obtained from L. Smith. The cells were maintained on slants of medium supplemented with 1.5% agar. The medium used in the experiments consisted of 34.4 mM K_2HPO_4, 29.3 mM KH_2PO_4, 18.6 mM NH_4Cl, 0.81 mM $MgSO_4 \cdot 7H_2O$, 0.005 mM $MnSO_4 \cdot H_2O$, 0.36 mM $CaCl_2$, 0.62 mM sodium molybdate $\cdot 2H_2O$, 0.017 mM $FeSO_4 \cdot 7H_2O$, 0.023 mM citric acid $\cdot H_2O$, 55.5 mM glucose, and 5 g of Casamino Acids (Difco) per liter of medium at *p*H 6.8. For experiments in which $H_3{}^{32}PO_4$ was used, the phosphate concentrations were reduced to 3.4 mM K_2HPO_4 and 2.9 mM KH_2PO_4. The organism was grown in low-form Erlenmeyer flasks with vigorous gyratory motion at 30 C. Cells were harvested by centrifugation at 15,000 $\times$ *g* for 15 min, the medium was decanted, and the cells were resuspended in 50 mM phosphate buffer, *p*H 7.6.

Lipid extraction and analysis. The lipids were extracted from the cells by the procedure of Bligh and Dyer (5). The lipids were deacylated by mild alkaline methanolysis (43) at 0 C. The deacylation was complete in 60 min. The glycerol phosphate esters were separated on cellulose thin-layer plates (Eastman 6064) by using the solvents described (34). Authentic standards were utilized for identification of the glycerol phosphate esters (34, 35, 48).

The glycerol phosphate esters derived from the phospholipids by mild alkaline methanolysis were glycerol phosphoryl serine (GPS) derived from phosphatidyl serine (PS), glycerol phosphoryl ethanolamine (GPE) derived from phosphatidyl ethanolamine (PE), glycerol phosphoryl monomethylethanolamine (GPMME) derived from phosphatidyl monomethyl-

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ethanolamine (PMME), glycerol phosphoryl dimethylethanolamine (GPDME) derived from phosphatidyl dimethylethanolamine (PDME), glycerol phosphoryl choline (GPC) derived from PC, glycerol phosphoryl glycerol (GPG) derived from phosphatidyl glycerol (PG), bis-glycerol phosphoryl glycerol (GPGPG) derived from cardiolipin (CL), glycerol phosphate (GP) derived from phosphatidic acid (PA).

For lipid analyses, the lipids from cells grown in the presence of radioisotope were deacylated and separated by chromatography on Eastman cellulose thin-layer plates, detected by autoradiography on Kodak No-Screen X-ray film, and the radioactivity in the individual glycerol phosphate esters was determined in the scintillation spectrometer (35, 43). Lipid phosphorus was determined by the method described previously (46).

Fatty acids were recovered from the complex lipids after mild alkaline methanolysis (43, 47). Free fatty acids were extracted from the lipid extract with Na_2CO_3 (43, 45). The residue after extraction was saponified and the fatty acids were extracted and then methylated (45). Methyl esters of the fatty acids were identified by their retention times on polar (EGS) and nonpolar (SE-30) gas chromatographic columns under the conditions described previously (45). Monoenoic fatty acid esters were hydrogenated in methanol and identified by gas chromatography (45). The double bond in the monoenoic C_{18} fatty acid was cleaved with periodate-permanganate and the fragments were identified by their retention times on a gas chromatograph (7, 31, 45).

RESULTS

Extraction of the lipids. A total of 57.7 mg of lipid was extracted from 370 mg (dry weight) of *M. denitrificans* by using the Bligh and Dyer (5) method. This indicated that the bacteria contained about 15.4% lipid. From a total of 112.5 mg (dry weight) of cells, the lipids were extracted, and the residue was collected, washed with water by centrifugation, and saponified in 3 N KOH containing 50% ethyl alcohol for 2 hr at 100 C. After cooling, the mixture was extracted with 0.2 volumes of petroleum ether, acidified to *p*H 2.0 with hydrochloric acid, and extracted three times with diethyl ether. After drying with anhydrous sodium sulfate, the residue was methylated in anhydrous methanolic hydrochloric acid containing dimethoxypropane. Analysis by gas-liquid chromatography indicated that no fatty acids could be released from the residue by saponification after Bligh and Dyer extraction. A trace amount of a free fatty acid which was a C_{18} monoenoic fatty acid could be detected by extraction of the lipid extract in petroleum ether with 0.47 M Na_2CO_3 (45).

Identification of the fatty acids. Fatty acid methyl esters recovered after mild alkaline methanolysis of the lipid extracted from 112.5 mg (dry weight) of cells were separated by gas-liquid chromatography on polar (EGS) and nonpolar (SE-30) columns. A James (16) plot of the logarithm of the retention times versus the number of carbon atoms in the fatty acid when compared with authentic standards showed fatty acids with 13, 14, 16, and 18 carbon atoms as well as monoenoic fatty acids of 13, 16, and 18 carbon atoms. Hydrogenation with one atmosphere of H_2 of the fatty acid esters (dissolved in anhydrous methanol in the presence of 5% platinum on charcoal at 25 C) removed the peaks corresponding to the monoenoic fatty acids and quantitatively increased the detector response for the 13, 16, and 18 carbon atom saturated esters. The proportions of the fatty acid esters revealed a strikingly high concentration of the monoenoic C_{18} fatty acid (Table 1). The monoenoic fatty acid was cleaved by periodate permanganate (31). The hydrolysis mixture yielded a C_9 monoenoic fatty acid which was identified by its gas-chromatographic retention on a Poropak column (45). This C_9 monocarboxylic fatty acid was derived from the omega end of the monoenoic C_{18} acid. The other fragment of the C_{18} monoenoic fatty acid yielded a C_9 dicarboxylic acid determined by its gas-chromatographic retention time after methylation and analysis on an SE-30 column (45). This indicated that the monoenoic C_{18} fatty acid was oleic acid.

Identification of the phospholipids. *M. denitrificans*, when extracted during exponential growth, contained 50 μmoles of lipid phosphate per gram (dry weight). Lipids were extracted from cells grown with $H_3{}^{32}PO_4$ for the identification of the phospholipids. The lipids were separated by chromatography in two dimensions on silica gel-impregnated paper (SG-81) and were detected by their chromatographic mobilities corresponding to authentic

TABLE 1. *Composition of the fatty acid methyl esters derived from the complex lipids of Micrococcus denitrificans*

Fatty acid (no. of carbon atoms)	EGS[a]	SE-30[a]
13	0.4	<0.05
Monoenoic 13	0.8	0.12
14	<0.1	0.16
16	8.3	9.1
Monoenoic 16	0.6	0.41
18	3.8	3.7
Monoenoic 18	86.1	86.5

[a] Percentages calculated from the total response after gas chromatography (45) on polar (EGS) and nonpolar (SE-30) columns.

PE, CL, PG, and, surprisingly, PC. A phospholipid with chromatographic mobility slightly greater than CL in both dimensions, representing 5.3% of the ^{32}P, was also detected. A chromatogram was dipped into 0.25% (w/v) ninhydrin dissolved in acetone and then heated at 100 C for 5 min. A single red spot corresponding to the PE was detected. A second chromatogram and the ninhydrin-treated chromatogram were dipped into freshly prepared 2% phosphomolybdic acid, and then washed in water for 45 min. The chromatograms were dipped in freshly prepared 2% $SnCl_2$ dissolved in 2.5 N hydrochloric acid. The non-ninhydrin-treated chromatogram revealed blue spots with the chromatographic mobilities of PE and PC. The ninhydrin-treated chromatogram showed only one blue spot corresponding to the PC position, indicating that the PE had been deaminated by the ninhydrin. PC is a ninhydrin-negative, phosphomolybdate-positive lipid. In the absence of strong acid hydrolysis, only the nitrogen-containing lipids react with phosphomolybdate (30).

The total phospholipid extract was deacylated by mild alkaline methanolysis (43). The methanolysis was complete in 60 min at 0 C, and the ^{32}P was quantitatively recovered in the water phase. The quantitative recovery of ^{32}P in the water phase precluded the presence of phosphosphingolipids, phosphonolipids, alkenyl phospholipids, or alkylether phospholipids (23). The glycerol phosphate esters recovered after mild alkaline methanolysis were separated by chromatography in two dimensions on acid-washed aminocellulose papers and on Eastman cellulose thin-layer plates as illustrated in Fig. 1. In these solvent systems, esters were found which moved with the chromatographic mobility of GPE, GPG, GPS, GP, GPGPG, GPC, GPMME, and GPDME. The unknown phospholipid PX yielded GPG on mild alkaline methanolysis (A. Tucker, *personal communication*). The phospholipid composition of exponentially growing *M. denitrificans* was: PG, 52.4%; PC, 30.9%; PE, 5.8%; PX, 5.3%; CL, 3.2%; PDME, 0.9%; PMME, 0.6%; PS, 0.5%; and PA, 0.4%, assuming all the phospholipids had the same specific activity. This was very likely as the organisms had been growing with $H_3{}^{32}PO_4$ for at least eight doublings.

Cellular location of the phospholipids. Cells grown with $H_3{}^{32}PO_4$ were harvested in the exponential phase of growth, and half the culture was extracted by the Bligh and Dyer method (5). The other half was treated with lysozyme, the spheroplasts were lysed by passage through a hypodermic needle, and the membranes were recovered (33). The membranes have been shown to contain the respiratory pigments found in the whole cells (reference 33; confirmed in our laboratory). The membranes contained 88% of the lipid ^{32}P found in the intact cells.

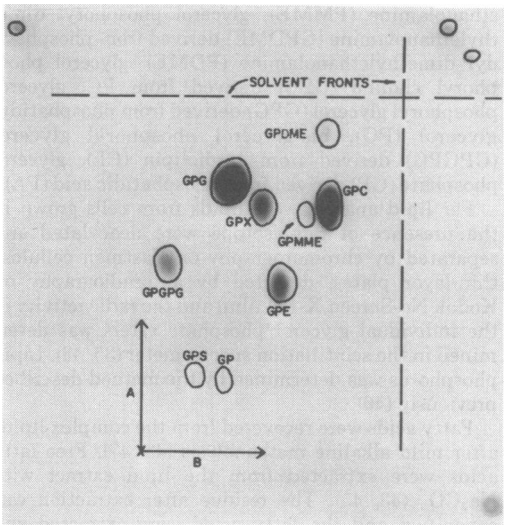

FIG. 1. *Autoradiogram of the glycerol phosphate esters of the phospholipids. The organism was grown from a small inoculum to the logarithmic phase of growth in the presence of* $H_3{}^{32}PO_4$, *the cells were harvested by centrifugation, and the lipid was extracted by the Bligh and Dyer procedure (5). The lipid extract was subjected to mild alkaline methanolysis (43), and the glycerol phosphate esters were recovered. The glycerol phosphate esters derived from* ^{32}P*-labeled phospholipids were chromatographed on Eastman cellulose chromatograms (6064) in two dimensions. The solvents were: A, 3.8 mM ethylenediaminetetraacetic acid, 0.7 M ammonium bicarbonate in 90 mM ammonium hydroxide containing 67% ethyl alcohol, and B, isobutyric acid-water-13 M ammonium hydroxide (66:33:1). The esters were located by autoradiography and identified by co-chromatography with authentic standards. The abbreviations used are GPG, glycerol phosphoryl glycerol derived from phosphatidylglycerol; GPGPG, bis-phosphatidylglycerol from cardiolipin; GPS, glycerol phosphoryl serine from phosphatidylserine; GP, glycerol phosphate from phosphatidic acid; GPE, glycerol phosphoryl ethanolamine from phosphatidylethanolamine; GPMME, glycerol phosphoryl monomethylethanolamine from phosphatidylmonomethylethanolamine; GPDME, glycerol phosphoryl dimethylethanolamine from phosphatidyldimethylethanolamine; GPC, glycerol phosphoryl choline derived from phosphatidylcholine; and GPX, a phosphate ester derived from an unknown phospholipid.*

Metabolism of the phospholipids. The loss of radioactivity from the phospholipids after a short period of growth with the isotopes followed by growth in the absence of the isotopes is a measure of the minimal rate of decrease in specific radioactivity (48). During exponential growth, the proportions of each phospholipid and the total amount per cell remained constant, so changes reflect the metabolism of each lipid. In such a pulse-chase experiment, there was no loss of ^{14}C (from acetate-*1*-^{14}C) or ^{32}P from GPG, GPE, or GPC (Fig. 2 and 3). The complex nature of the lipid metabolism of the whole cells was indicated by the turnover of GP derived from PA. PA is the presumptive precursor of all the phospholipids. In GP, half the ^{14}C was lost from the glycerol in 85 min, but half the ^{32}P was lost in 440 min. The loss of both ^{14}C and ^{32}P from GPGPG, GPMME, GPDME, and GPS was distinctly biphasic. The most rapid initial rate of metabolism showed the loss of half the ^{14}C and ^{32}P from the CL in about 14 min. This rapid rate continued for a period of about 25 min, after which the rate slowed to where half the ^{14}C from the glycerols was lost in 80 min and half the ^{32}P was lost in 110 min. The rapid turnover of GPDME, GPMME, and GPS required between 20 and 30 min for the loss of half the radioactivity. In this experiment, the bacteria doubled in 80 min.

Incorporation of ^{32}P into the phospholipids. If $H_3{}^{32}PO_4$ was added to cells grown with $H_3{}^{33}PO_4$, the ratio of the $^{32}P/^{33}P$ was a measure of the specific activity of the lipids. GPGPG (derived from CL), which showed the most rapid initial turnover, was also rapidly labeled (Fig. 4). GP showed an initial specific activity lower than either GPS or GPGPG. GPG, a precursor of GPGPG (35), showed a lower specific initial activity than the other lipids. These data suggest that only small portions of both GP and GPG were involved in the synthesis of the rapidly metabolizing CL fraction. The nitrogen-containing phospholipid with the highest initial specific activity was GPS, followed, in order of highest initial specific activities, by GPE, GPMME, GPDME, and GPC which is compatible with the biosynthetic sequence PS → PE → PMME → PDME → PC.

DISCUSSION

M. denitrificans has an unusual lipid composition for a *Micrococcus*. Most species are gram-positive and contain from 1.0 to 10% of the dry weight as lipid (49) compared to up to 15% for *M. denitrificans*. Most micrococci contain between 9 and 30 μmoles of lipid phosphate per gram (dry weight) compared to 50 μmoles of lipid phosphate for *M. denitrificans*. However, *M. cerificans* grown with hexadecane contains up to 123 μmoles of lipid phosphate per gram dry weight (26). The fatty acids of the gram-variable micrococcal species usually contain saturated and branched C_{14} to C_{17} fatty acids (49) much like *S. aureus* (46). The gram-negative

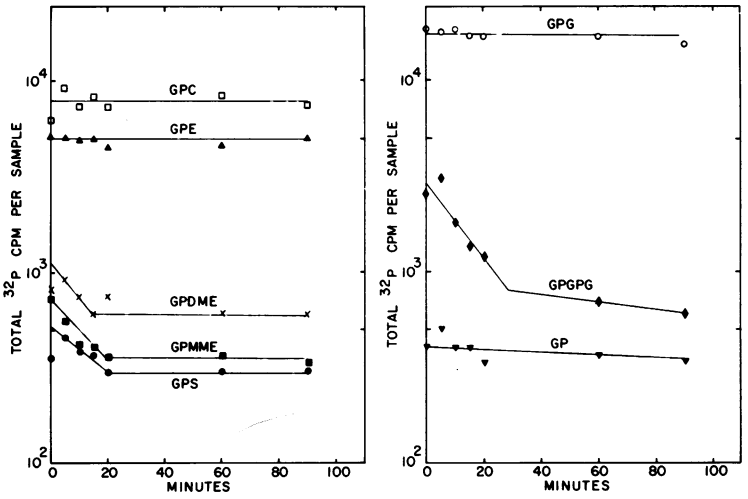

FIG. 2. *Turnover of the ^{32}P in the phospholipids. Cells were grown to mid-logarithmic phase in 250 ml of unlabeled medium, and a pulse of 150 μCi of sodium acetate-1-^{14}C and 150 μCi of $H_3{}^{32}PO_4$ was added. The culture was grown for an additional 2 hr, centrifuged at 30 C, and the pellet was resuspended in 250 ml of nonlabeled, prewarmed medium. There was no lag in the bacterial doubling time. Samples (25 ml) were removed at the times indicated and treated as in Fig. 1.*

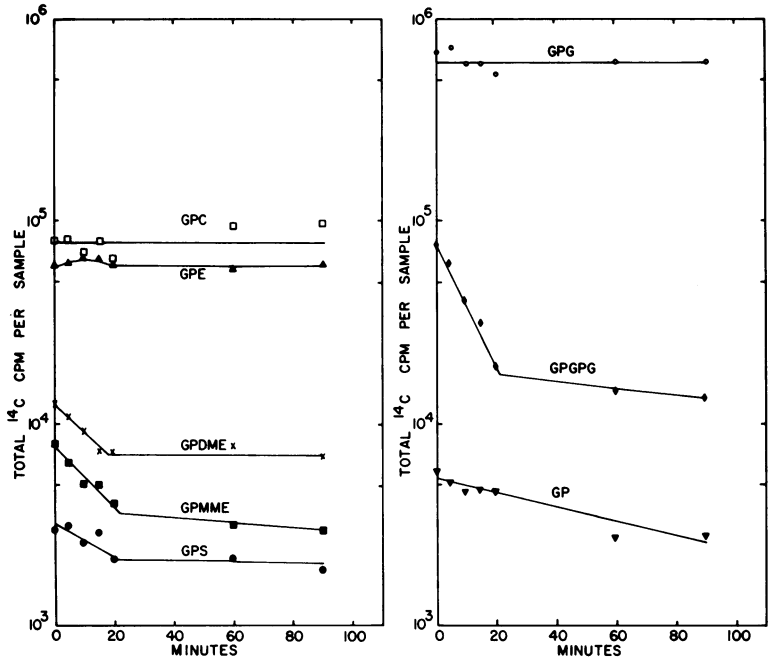

FIG. 3. *Turnover of ^{14}C in the phospholipids. See Fig. 2 for details.*

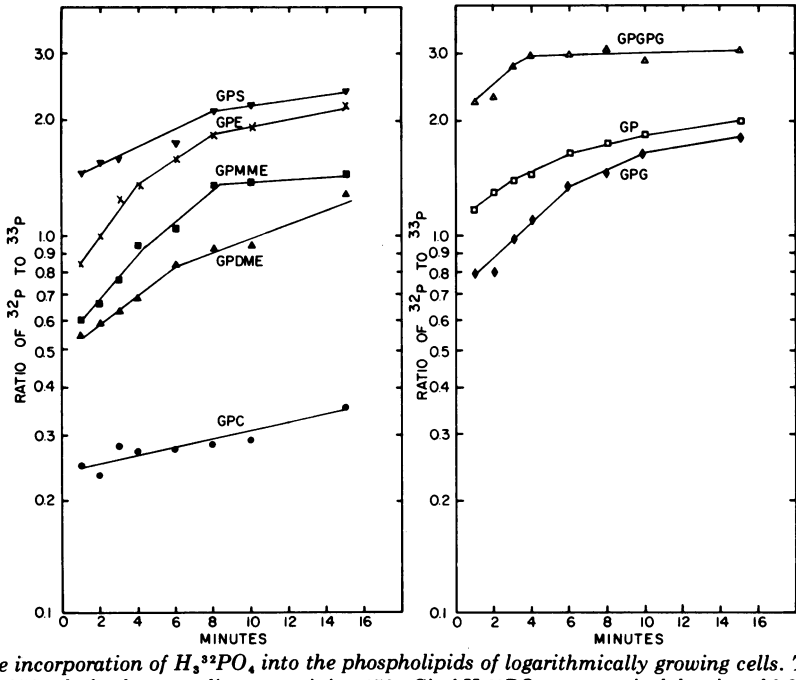

FIG. 4. *The incorporation of $H_3{}^{32}PO_4$ into the phospholipids of logarithmically growing cells. The organism was grown in 250 ml of culture medium containing 150 μCi of $H_3{}^{33}PO_4$ to an optical density of 0.8 measured at 750 nm. At this time, 1.0 mCi of $H_3{}^{32}PO_4$ was added, and 25-ml samples were removed at the times indicated. The lipid was extracted and analyzed as in Fig. 2, and the specific activities of the individual lipids were determined as the $^{32}P/^{33}P$ ratios.*

M. rhodochrous, *M. caseolyticus*, *M. cerificans*, *M. diversus*, *M. halodenitrificans*, and *M. denitrificans* have been reported to contain 23%, 28%, 47%, 62%, 62%, and 78% monoenoic C_{18} fatty acids, respectively (9, 49). The C_{18} monoenoic fatty acid has been shown to be oleic acid and to comprise 86% of the total fatty acids under the growth conditions used in this study (Table 1). *Hyphomicrobium* and *Nitrobacter* strains show an unusually simple fatty acid composition like *M. denitrificans*. In addition, these two bacterial species contain 92% monoenoic 18-carbon fatty acid but the isomer is *cis*-vaccenic (1) and not oleic acid as found in *M. denitrificans*.

The phospholipid composition of *M. denitrificans* was different from other micrococci, which show large amounts of CL and PG, but no PC (49). Nearly all contain glucolipids. *M. denitrifians* joins the select group of eubacteria containing PC as a major phospholipid (Table 2). The high PC level reported in *Lactobacillus casei* (40) has not been confirmed in a more recent study that included eight other species of *Lactobacillus* (8). Similarly, the PC in *Bacillus cereus* reported by Kates et al. (22) has not been found in other strains (15, 25).

Studies of the metabolism of the lipids indicated that there was no detectable turnover of the ^{32}P- or ^{14}C-glycerol in the GPC, GPG, or GPE (Fig. 2 and 3). The biphasic turnover seen with GPGPG, GPS, PGMME, and GPDME (Fig. 2 and 3) was similar to that detected in *E. coli* (2), *S. aureus* (35), and *Haemophilus parainfluenzae* (44, 48). In the latter organism, there is evidence that multiple pools of CL and PG with different metabolisms account for the complex kinetics (41, 42). It appears that CL hydrolysis contributes to the PA pool just as in *H. parainfluenzae*. This dilutes the PA pool so that it does not show the highest initial specific activity after a pulse of ^{32}P as would be expected for its role as the primary phospholipid precursor (42). Further evidence of the metabolic complexity of PA is the slower turnover of ^{32}P- and ^{14}C-glycerol in the GP (Fig. 2 and 3). In all bacteria thus far examined in detail, the PC is formed by the decarboxylation of PS to PE followed by successive methylations of PE to PMME, PDME, and PC (11, 13, 19, 26). The data of Fig. 4 suggest that such a pathway also exists in *M. denitrificans*, although the specific activity of PMME appears to reach a steady-state level before attaining the level in the PE. This, plus the fact that none of the ^{32}P lost from the PS, PMME, or PDME can be detected in the PC after a pulse-chase experiment (Fig. 2), makes it appear that multiple precursor pools exist in the PC pathway just as in the CL pathway.

TABLE 2. *Bacteria containing phosphatidyl choline (PC) as a major phospholipid*

Bacterial species	% PC	Reference
Lactobacillus casei	45	39
Rhizobium japonicum	35	6
Brucella abortus	34	38
Treponema pallidum, *T. zuelerae*	33	16, 27
Nocardia coeliaca	30	50
Rhodospirillum rubrum	30	4
Hyphomicrobium vulgare	29	11, 14
Micrococcus denitrificans	31	This study
Rhodomicrobium vannielii	25	28
Rhodopseudomonas spheroides	23	12, 23, 49
Thiobacillus novellus, *T. thiooxidans*	20	3, 17
Agrobacterium radiobacter, *A. tumefaciens*, *A. rhizogenes*	15	10, 14, 19
Bacillus cereus	6	21

It is hoped that further studies of the lipids in this organism will help clarify its taxonomic position as well as shed some light on the possible control reactions in the formation of its membrane lipids.

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