Phospholipids of Five Pseudomonad Archetypes for Different Toluene Degradation Pathways

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Abstract: Liquid chromatography/electrospray ionization/mass spectrometry (LC/ESI/MS) was used to determine intact phospholipid profiles for five reference pseudomonad strains harboring different (aerobic) toluene catabolic pathways: *Pseudomonas putida* mt-2, *Pseudomonas putida* F1, *Burkholderia cepacia* G4, *Burkholderia pickettii* PKO1, and *Pseudomonas mendocina* KR1. These five strains contained a predominant pool of phosphatidylethanolamines. Other phospholipids identified include phosphatidylglycerol, phosphatidylserine, phosphatidylmethylethanolamine, and phosphatidyldimethylethanolamine. There was a clear separation in phospholipid profiles that allows for the differentiation between the *Pseudomonas* and *Burkholderia* genera. Factor analysis of the phospholipid profiles showed that *B. cepacia* G4, *P. putida* mt-2, and *B. pickettii* PKO1 were clearly separated, while *P. putida* F1 and *P. mendocina* KR1 were clustered as a group. These results suggest that intact phospholipid profiling could be used to evaluate the relative abundance of specific degraders in bioreactors or in aquifer material. Nevertheless, the usefulness of this technique for taxonomic characterization of such complex samples remains to be demonstrated because of potential confounding effects of overlapping profiles and potential changes in phospholipid composition due to different growth conditions.

Introduction

The monoaromatic hydrocarbons, benzene, toluene, ethylbenzene, and xylenes (BTEX), are common environmental contaminants that represent a serious threat to groundwater resources and to public health (Tursman and Cork, 1992). Microbial degradation of these contaminants is widely regarded as a cost-effective approach to clean up BTEX-contaminated aquifers (National Research Council, 1994). Indeed, considerable progress has been made toward understanding and managing hydrogeochemical factors that influence the success of BTEX bioremediation. Nevertheless, process optimization is limited by our incomplete understanding of the diversity and prevalence of specific biochemical processes.

Microbial catabolic diversity is reflected in the fact that different pseudomonad strains can degrade toluene by five different oxygen-dependent pathways, which are named after the operons that code them (Zylstra, 1994). The TOL pathway, which was first discovered in *Pseudomonas putida* (*arvilla*) mt-2, is coded in the *tol* pWW0 plasmid. The TOD pathway is expressed by *P. putida* F1, which uses toluene dioxygenase to convert toluene into *cis*-toluene dihydrodiol (Gibson et al., 1968), which is subsequently dehydrogenated to 3-methylcatechol. The TOM pathway occurs in *B. cepacia* G4 (formerly known as *Pseudomonas cepacia* G4), which uses toluene *o*-monooxygenase in the initial attack to form *o*-cresol (Shields and Montgomery, 1989). The TBU pathway occurs in *B. pickettii* PK01 (formerly known as *Pseudomonas pickettii* PK01), which hydroxylates the ring using toluene *m*-monooxygenase to yield *m*-cresol (Kukor and Olson, 1996). Finally, the TMO pathway is expressed by *P. mendocina* KR1, which uses toluene *p*-monooxygenase to yield *p*-cresol (Whited and Gibson, 1991).

These five reference strains exhibit different substrate ranges and kinetic properties. For example, the

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TOD pathway has very broad substrate specificity, and toluene dioxygenase is capable of oxidizing more than 100 substrates (Gibson et al., 1995). This makes the TOD pathway desirable when dealing with complex contaminant mixtures. If trichloroethylene (TCE) is the main co-contaminant, however, toluene dioxygenase does not degrade TCE as fast as toluene *ortho*-monooxygenase (Ensley, 1991). Thus, the TOM pathway might be more desirable in this situation. The TOM pathway might also be desirable if hypoxic conditions prevail because strain G4 has been shown to outcompete strains mt-2, F1, and KR1 in toluene-fed chemostats under oxygen-limiting conditions (Duetz et al., 1994). The TBU pathway might also be desirable for hypoxic environments because strain PKO1 also exhibits enzyme kinetics favorable for low-oxygen conditions (Kukor and Olsen, 1996). Finally, KR1 has been shown to outcompete mt-2, F1, and G4 under toluene-limiting conditions (Duetz et al., 1994). Thus, the TMO pathway might be desirable for polishing purposes when BTEX are present at trace concentrations. This discussion illustrates that knowledge of which strain(s) prevails at a given site or bioreactor is important to understand BTEX bioremediation capabilities and limitations.

The purpose of this study was to characterize the phospholipid profiles of the archetypes that harbor the five known aerobic toluene degradation pathways. In doing so, information was obtained about the potential usefulness of intact phospholipids as biomarkers for taxonomic identification of specific BTEX degraders.

Materials and Methods

Bacterial Cultures

Five pseudomonad strains were selected for the application of phospholipid profiling in microbial characterization. All cells were grown on Trypticase Soy Agar (TSA) plates (100 mm × 15 mm). Each plate had the following formulation: 0.75 g of Bacto Tryptic Soy Broth, 0.375 g of DIFCO Bacter-Agar (DIFCO, Inc., Detroit, Michigan), and 25 mL of modified Hutner's mineral medium (Stanier et al., 1966).

Lipid Extraction

Total lipids were extracted with a modified Bligh and Dyer extraction method (Fang and Findlay, 1996). The total lipid extract was separated chromatographically on a silicic acid column (Fang and Findlay, 1996). The phospholipid fraction was dried under a gentle stream of nitrogen and was once again dissolved in methanol.

Liquid Chromatography/Electrospray Ionization/Mass Spectrometry (LC/ESI/MS)

The LC/ESI/MS analysis was performed on an HP 1090 liquid chromatography/HP 5989B mass spectrometer with an electrospray interface. Detailed procedures can be found in Fang and Barcelona (1998).

Phospholipids are designated as follows: C1:d1/C2:d2-PL (e.g., C16:0/C18:1-PS), where C1 and C2 are the number of carbon atoms in the fatty acyl chains on the sn-1 and sn-2 positions, respectively; d1 and d2 are the number of double bonds of the *sn*-1 and *sn*-2 fatty acyl chains, respectively; PL is the abbreviation for phospholipids (Table 1).

Results and Discussion

The phospholipid profiles and composition of the bacterial strains are shown in Figure 1 and Table 1. Major phospholipids identified in *P. putida* mt-2 were phosphotidylethanolamines. All except two phospholipids were unsymmetrical with two different fatty acids on the *sn*-1 and *sn*-2 positions (Table 1). The major phospholipid was 17:1/16:1-PE, which constituted 53.4% of the total phospholipids, whereas the symmetrical phospholipids 16:1/16:1-PE and 18:1/18:1-PE combined constituted less than 10% (Table 1). Phosphatidylethanolamine (PE) was the major component detected in *P. putida* F1 (Table 1). Two of the PEs have the same fatty acid distribution 18:1/16:1, probably with different double bond positions. These two phospholipids accounted for 76.1% of the total phospholipids.

The phospholipid composition of *B. cepacia* G4 was relatively simple with two PEs detected (Table 1). Fatty acids on the phospholipids include 16:0, 16:1, and 18:1. Three phospholipids were detected in *P. mendocina* KR1. This strain also contained phosphatidyldimethylethanolamine (PDME). 18:1/16:1-PE was predominant (82.2% of total phospholipids).

B. pickettii PKO1 contained more phospholipids than any other strains we analyzed. Sixteen different phospholipids in five different classes were detected: phosphatidylglycerol (PG), phosphatidylserine (PS), PE, phosphatidylmethylethanolamine (PME), and PDME (Table 1). This is the only strain that contained a phospholipid with a 15:1 fatty acid. The dominant phospholipids were the 18:1/16:1-PEs (24.1%) and 16:0/17:1-PE (10.9%).

The five pseudomonads contained a predominant pool of PEs. Phosphatidic acid, the precursor of phospholipid synthesis, and PS were not detected. The

Table 1. Phospholipids (%) identified in toluene-degrading bacteria

Peak #	RT[a] Bacterial strain[b] Phospholipid	(min)	G4	F1	mt-2	PKO1	KR1
1	16:1/16:0-PG[cd]	34.44				2.4	
2	18:1/16:1-PG	35.40				4.3	
3	16:1/16:1-PE	43.44		0.8	0.2	2.2	
4	16:0/17:0-PE	43.68				2.5	
5	16:1/17:0-PDME	43.87				3.3	
6	15:1/16:0-PE	44.89				9.4	
7	16:1/16:1-PME	45.03				0.8	
8	16:0/15:1-PS	45.25				0.8	
9	17:1/16:1-PE	45.32			53.4		
10	16:0/16:1-PE	46.05		15.8	9.4	9.8	6.1
11	18:1/16:1-PE	46.29		23.1		14.8	
12	18:1/16:1-PE	46.53		53.1		9.3	82.2
13	16:1/16:1-PDME	47.31					11.7
14	16:1/16:0-PE	47.40	57.1				
15	18:1/16:1-PE	48.18	42.9		7.2		
16	16:0/17:1-PE	48.47		1.0	6.3	10.9	
17	18:1/17:1-PE	49.92			10.5		
18	18:1/16:1-PE	50.02				10.4	
19	18:1/17:1-PE	50.31				9.7	
20	16:0/18:1-PE	51.13		4.5	3.9	4.9	
21	18:1/18:1-PE	51.66		1.7	9.2	4.6	

[a] RT = retention time (min).

[b] G4: *Burkholderia cepacia* G4; F1: *P. putida* F1; mt-2: *P. putida* mt-2; PKO1: *Burkholderia* pickettii PKO1; KR1: *P. mendocina* KR1.

[c] Position of double bonds was not determined.

[d] PG = phosphatidylglycerol; PS = phosphatidylserine; PE = phosphatidylethanolamine; PME = phosphatidylmethylethanolamine; PDME = phosphatidyldimethylethanolamine.

dominance of phophatidylethanolamine and the absence of PS can be explained by the fact that PEs are synthesized from decarboxylation of PS, and the methylated forms of PE are subsequently formed by stepwise methylation of PE (Goldfine, 1972). Interestingly, phospholipids isolated from the five pseudomonad strains were largely unsymmetrical. The symmetrical diacyl phospholipids constitute only small proportions of the total phospholipids.

Factor analysis (STATISTICA, Tulsa, OK) was conducted to compare phospholipid profiles among five pseudomonad strains. The factor loading plots for the first three factors are shown in Figure 2. The percentage of variance expressed by the first three factors was 50.8%, 19.0%, and 17.3%, respectively, with a cumulative total of 87.1%. It seems that there is a clear separation in phospholipid profiles that allows for the differentiation between the *Pseudomonas* and *Burkholderia* genera (Figure 2). There is also considerable variation within each genus for taxonomic identification of different strains. For example, factor analysis of phospholipid profiles showed clear separation of some pseudomonad strains (i.e., *B. cepacia* G4, *P. putida* mt-2, and *B. pickettii* PKO1), and clustering of others (i.e., *P. putida* F1 and *P. mendocina* KR1) (Figure 2). Yet, *B. cepacia* G4 is clearly separated from *P. putida* mt-2 on factors 2 and 3, while *P. putida* F1 and *P. mendocina* KR1 are separated from *B. cepacia* G4, *P. putida* mt-2, and *B. pickettii* PKO1 on factor 1.

Some phospholipids were present exclusively in some bacteria, suggesting their potential use as biomarkers. Specifically, *B. pickettii* PKO1 could be distinguished from the other strains by the presence of phospholipids 16:1/16:1-PME, 16:1/17:0-PDME, and 16:0/15:1-PS. Potential biomarkers for *P. mendocina* KR1 include 16:1/16:1-PDME. 16:1/16:0-PE was found exclusively in *B. cepacia* G4.

In summary, this research represents a first step toward delineating the usefulness of intact phospholipid profiling (IPP) for taxonomic identification of specific degraders in environmental samples. It should be kept in mind that these results correspond to pure cultures grown in well-defined media, and that phospholipid profiles can change as a result of growth conditions and exposure to BTEX (Heipieper and de Bont, 1994; Pinkart and White 1997; Ramos et al., 1997; Weber et al., 1994). In addition, potential confounding effects from lipid-pattern overlap when mixed

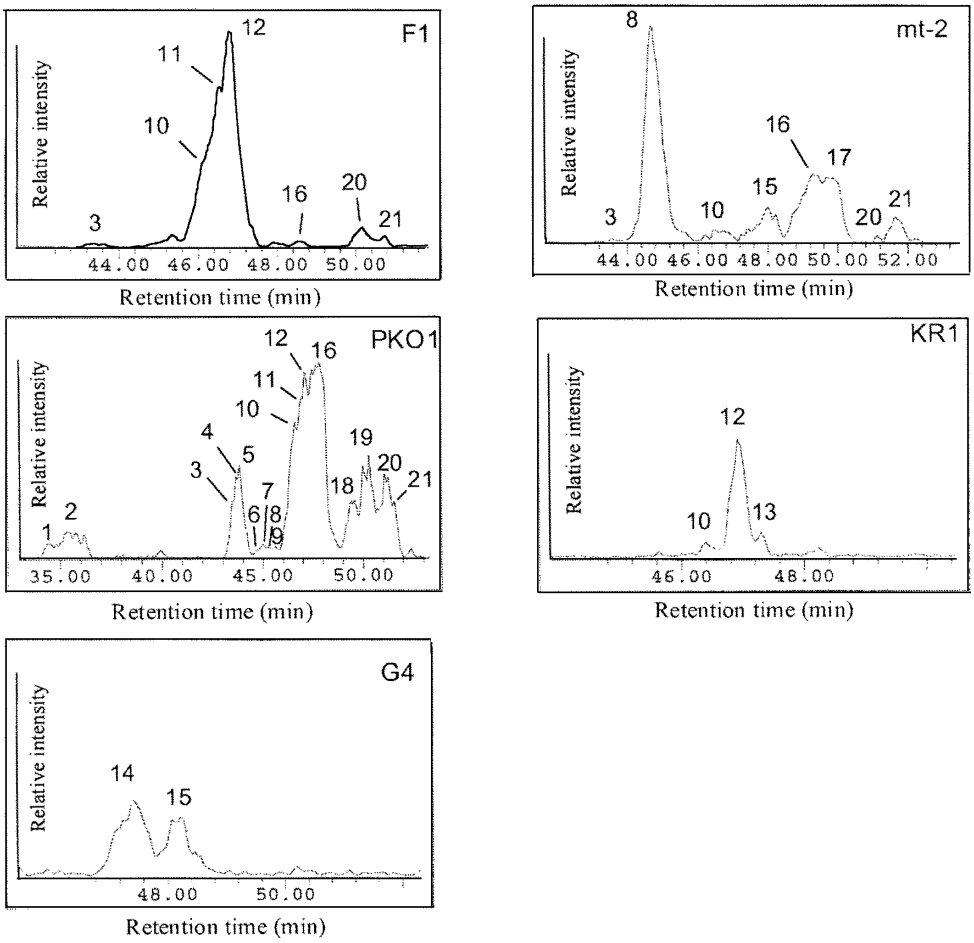

Figure 1. Total ion chromatogram of phospholipids isolated from five pseudomonad strains: G4 = *B. cepacia* G4; F1 = *P. putida* F1; mt-2 = *P. putida* mt-2; PKO1 = *B. pickettii* PKO1; KR1 = *P. mendocina* KR1. Peak numbers correspond to phospholipid identifications in Table 1.

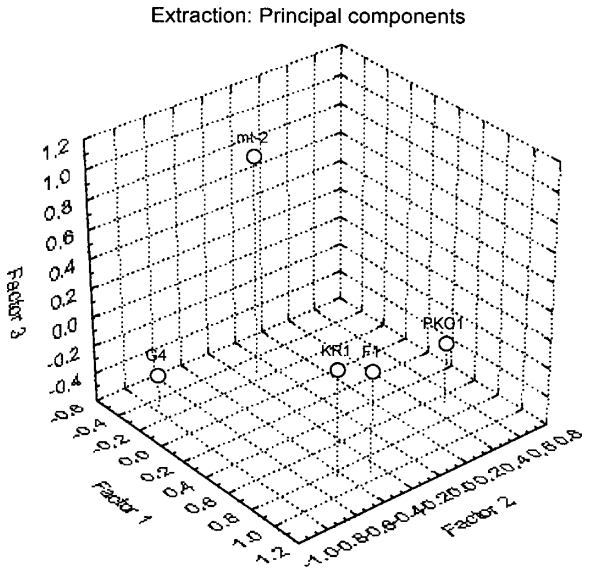

Figure 2. Factor loading plot showing variations in phospholipid profile among five pseudomonad strains

cultures are considered have not been investigated. Therefore, the usefulness of IPP as a taxonomic identification tool for field samples remains to be demonstrated. Nevertheless, the identification of some unique biomarkers and profile differentiation based on factor analysis suggests significant potential for differentiating closely related strains, even in complex environmental samples.

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