QUANTITATIVE ANALYSIS OF BIOMASS, COMMUNITY STRUCTURE AND METABOLIC ACTIVITY OF ENVIRONMENTAL CONSORTIA.

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ABSTRACT: Specific microbes can often be quantitatively assayed in the mixed microbial consortia of environmental samples by determination of component patterns sufficiently unusual to serve as "signatures". Extraction and purification of the fatty acids ester-linked to the polar lipids (PLFA), derivatization with dimethyl disulfide to define the configuration and position of monoenoic components and assay by capillary GC/MS has been utilized to follow changes in bacteria and microeukaryotes involved in biofouling, corrosion, biodegradation, and infection. Measurement of rate of synthesis of poly beta-hydroxybutyrate relative to phospholipid, and the accumulation of trans monoenoic PLFA are indicators of nutritional stress. Rates of accumulation of 15-N or 13-C in specific component lipids are measures of specific metabolic activities.

By utilizing fatty acid patterns of bacterial monocultures, Myron Sasser of the University of Delaware in collaboration with Hewlett Packard has been able to distinguish between over 8000 strains of bacteria (1). This analysis requires both that the organisms be isolated from the environment and that they be cultured to a significant biomass prior to saponification, extraction and analysis by capillary gas chromatography.

Our laboratory has been involved in the development of assays to define microbial consortia in which the bias of cultural selection of the classical plate count is eliminated. Since the total community is examined in these procedures without the necessity of removing the microbes from surfaces, the microstructure of multi-species consortia is preserved. Our method eliminates the requirement of isolation of organisms and their subsequent growth prior to component analysis. In achieving sufficient selectivity, the specificity of the analysis is increased by purification of the extracts prior to analysis. This is further potentiated by increasing the sensitivity and information content of the terminal analysis by adding specific derivatization and MS analysis to the capillary gas chromatographic separation. Those components generally distributed in cells are utilized as measures of biomass. Components restricted to subsets of the microbial communities can be utilized to define the community structure. The concept of "signatures" for subsets of the community based on the limited distribution of specific components has been validated by using antibiotics and cultural conditions to manipulate the community structure. The resulting changes agreed both morphologically and biochemically with the expected results (2). Other validation experiments that involved isolation and analysis of specific organisms and finding the specific patterns in appropriate mixtures, utilization of specific inhibitors and noting the response, and changes in the local environment such as the light intensity are summarized in reviews (3, 4).

Phospholipids are found in the membranes of all cells. Under the conditions expected in natural communities the bacteria contain a relatively constant proportion of their biomass as phospholipids (5). Phospholipids are not found in storage lipids and have a relatively rapid turnover in some sediments so the assay of these lipids gives a measure of the "viable"

cellular biomass (6). The phosphate of the phospholipids or the glycerol-phosphate and acid-labile glycerol from phosphatidyl glycerol-like lipids that are indicators of bacterial lipids can be assayed to increase the specificity and sensitivity of the phospholipid assay (7).

Biomass and Community Structure: The ester-linked fatty acids in the phospholipids (PLFA) are presently both the most sensitive and the most useful chemical measures of microbial biomass and community structure thus far developed (8, 9). The restriction to fatty acids that are ester-linked in the phospholipid fraction (PLFA) of the total lipid extract greatly increases the selectivity of this assay as most of the anthropogenic contaminants as well as the endogenous storage lipids are found in the neutral or glycolipid fractions of the lipids. By isolating the phospholipid fraction for fatty acid analysis it proved possible to show bacteria in the sludge of crude oil tanks. The specificity and sensitivity of PLFA assay has been greatly increased by two specific derivatizations. The formation of dimethyl disulfide adducts for the determination of the configuration and position of double bonds in monoenoic fatty acids has proved essential in the definition of specific "signature" PLFA patterns (10-13). The formation of electron-withdrawing pentafluorobenzyl (PFB) esters, with separation by capillary GC can be detected as negative ions after chemical ionization MS at femtomolar sensitivities (14). This makes possible the detection of specific bacteria in the range of 10 to 100 organisms. Since many environments such as marine sediments often yield more than 150 PLFA, the addition of an inlet splitter with two independently controlled ovens allows fractionation on both polar and non-polar liquid phases prior to detection. This markedly increases the resolution of complex PLFA samples and requires a comprehensive data analysis system. Thus analysis of the PLFA can provide insight into the community structure of microbial consortia as well as an estimate of the biomass.

Although analysis of PLFA presently cannot provide an exact description of each species or physiologic type of microbes in a given environment, the analysis provides a quantitative description of the microbiota in the particular environment sampled. Organisms with sufficiently unusual "signature" patterns such as the potential BW agent F. tularensis and the type II methylotrophs important in halogenated hydrocarbon biodegradation are readily detectable in complex samples (10, 15). Shifts in the microbial community structure by PLFA analysis were detectable in the vadose zone of a subsurface aquifer sediment in response to contamination by creosote waste (16).

Potential problems with defining community structure by analysis of PLFA come with the shifts in fatty acid composition of some monocultures with changes in media composition or temperature (16) some of which were defined in this laboratory (17, 18). There is as yet little published evidence for such shifts in PLFA in nature where the growth conditions that allow survival in the highly competitive microbial consortia would be expected to severely restrict the survival of specific microbial strains to much narrower conditions of growth. The potential BW agent F. tularensis shows its "signature" PLFA pattern in samples derived from human infections, vaccines, soils and dead animals.

The sulfate-reducing bacteria contain lipids which can be utilized to identify at least a portion of this class. Some contain a unique profile of branched saturated and monounsaturated PLFA (13, 20-22) that allows differentiation between those utilizing lactate and those using acetate and higher fatty acids. Analysis of sulfate-reducing bacteria by N. Dowling of this

laboratory strongly suggests that the majority of sulfate-reducing bacteria found in marine sediments and in waters used in the secondary recovery of oil are the acetate-utilizing strains. These organisms are active even in fermentations in which there is no added sulfate as they can recycle organic sulfur in the feed-stock (23).

In addition to the PLFA gram-negative bacteria contain distinctive patterns of amide or ester linked aliphatic and hydroxy fatty acids in the lipid A of their lipopolysaccharide (LPS) wall polymers (24). This requires a second extraction of the lipid-extracted residue after saponification. By assay of LPS lipid A fatty acids with PFB esters it was possible to detect a few bacteria in mammalian tissue or secretions (14). Gram-positive bacteria often contain teichoic acid polymers as the substituted poly-glycerol or ribitol phosphate esters. Teichoic acid glycerol and ribitol can be released specifically by hydrolysis with cold concentrated hydrofluoric acid of the lipid-extracted residue (25). With this assay it proved possible to show that contamination of subsurface aquifer sediments induces a shift from predominantly gram-positive to gram-negative based on the ratios of teichoic acid glycerol to phospholipid (16).

Phospholipids and LPS lipid A fatty acids of dead bacteria are rapidly lost from marine sediments (6, 26-29). This indicates that these chemical markers provide good estimates for the standing viable or potentially viable microbiota and are not measures of fossil bacteria.

Nutritional Status: Certain bacteria form the endogenous lipid poly beta-hydroxybutyrate (PHB) under conditions when the organisms can accumulate carbon but have insufficient total nutrients to allow growth with cell division (30). The ratio of the rate of formation of PLFA to PHB from 14-C-acetate has been shown to be an extraordinarily sensitive measure of the nutrient environment in the bacterial habitat (31, 32). Recent studies indicate that the pathogen V. cholerae accumulates trans monoenoic fatty acids in the PLFA with starvation (33).

Metabolic Activity: The analyses described above all involve the isolation of components of microbial consortia. Since each of the components are isolated, the incorporation of mass-labeled isotopes from precursors can be utilized to provide rates of synthesis or turnover in properly designed experiments. Improvements in analytical techniques have increased the sensitivity of this analysis. Utilizing a chiral derivative and fused silica capillary GLC with chemical ionization and negative ion detection of selected ions, it proved possible to detect 8pg (90 femtomoles) of D-alanine from the bacterial cell wall (the equivalent of 1000 bacteria the size of E. coli (34). In this analysis it proved possible to reproducibly detect a 1% enrichment of 15-N D-alanine in the 14-N D-alanine.

CONCLUSIONS

The ability to measure the biomass, community structure, nutritional status, and metabolic activities of microbes at a sensitivity of a few bacteria (using lipid analysis with GC/MS) from essentially any source provides a method to quantitatively predict the consequences of their activity. For example the detection of contamination of fluids, foods, fuels, or surfaces such as semiconductors with a few specific microbes such as F. tularensis is possible. Odham et al. (14) have shown the detection and identification of a few bacteria in human urine with lipid extraction and

GC/MS analysis. With these methods it becomes possible not only to detect contamination but to establish whether repeated contaminations are from the same source. With continued increases in the information from the lipid patterns the identification of more and more specific organisms becomes possible. We feel that these purification methods if they can be automated and miniaturized will sufficiently increase the signal to noise ratio in the detection of particulate BW agents by MS techniques to provide the protection from these agents that is essential.

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