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Plasmid incidence and linear alkylbenzene sulfonate biodegradation in wastewater and pristine pond ecosystems

Alec Breen [1], Luis Jimenez [1], Gary S. Sayler [1] and Thomas W. Federle [2]

[1] *Center for Environmental Biotechnology, University of Tennessee, Suite 200, 10515 Research Dr., Knoxville, Tennessee, and* [2] *Environmental Safety Department, The Procter and Gamble Company, Ivorydale Technical Center, Cincinnati, Ohio, U.S.A.*

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SUMMARY

Linear alkylbenzene sulfonate (LAS) is a widely used anionic surfactant. Although approximately 1 million metric tons of LAS are produced annually, relatively little is known about the bacteria or the genetic factors that control LAS degradation in the environment. The objectives of this research were to: i) compare bacterial populations in wastewater and pristine pond systems; ii) determine the frequency of plasmids in bacteria from these sites; and iii) compare the frequency of DNA sequences coding for aromatic catabolism in isolates from these two sites. Plate counts indicated that exposure to wastewater resulted in higher levels of both heterotrophic bacteria and bacteria capable of growing on LAS containing medium (LAS/YEPG). In addition to higher numbers, a higher proportion of heterotrophs from the wastewater system were capable of growth on LAS/YEPG medium. Thus, the high levels of LAS in the wastewater system apparently selected for organisms that were able to tolerate and/or degrade it. Mineralization of ^{14}C-ring labelled LAS in any habitat related to the presence of organisms that grew on LAS/YEPG. Although many of these isolates could carry out primary degradation, no isolate could mineralize ^{14}C-ring LAS in pure culture. A higher incidence of plasmids was found in bacteria from the wastewater pond and among bacteria that grew on LAS containing medium. However, the presence of plasmid DNA did not necessarily confer the ability to degrade LAS nor was the ability to degrade LAS dependent on the presence of a plasmid. The incidence of selected genotypes for aromatic catabolism was similar among isolates on LAS/YEPG at both sites, suggesting that LAS ring degradation may be present in other populations or encoded by alternative sequences. In conclusion, LAS mineralization is mediated by a consortium and the evidence that initial attack of LAS is plasmid mediated is inconclusive.

INTRODUCTION

Approximately 3–4 million metric tons of synthetic surfactants are produced yearly in Western Europe, Japan and the United States [23]. Linear alkylbenzene sulfonate (LAS) is a commonly used anionic surfactant accounting for 28% of this total production. LAS is utilized in a wide range of cleaning products and is biodegradable under aerobic conditions. The biochemistry of its degradation has been investigated by a number of workers [4,22]. The initial attack involves oxygenase mediated oxidation of the terminal methyl group of the alkyl chain. Ring degradation occurs only after the side chain is largely removed by β-oxidation. Evidence indicates that ring cleavage involves desulfonation as result of monooxygenase activity and subsequent meta-cleavage of the resulting catechol [4]. Although it is commonly suggested in the literature that LAS degradation is plasmid-mediated, there is relatively little evidence for plasmid involvement [2,12]. Work on a plasmid bearing strain of *Pseudomonas testosteronii* and the existence of the OCT plasmid, which codes for the oxidation of straight chain alkanes, led Cain [4] to propose that LAS degradation may be plasmid-encoded. He also proposed that desulfonation and meta-cleavage of the aromatic ring are mediated by plasmid-encoded enzymes in a fashion analogous to pWWO, TOL plasmid-mediated toluene metabolism [4]. Furthermore, studies by Wittich et al. [25] demonstrate the ability of naphthalene-degrading populations to oxidize sulfonated naphtalenes. Naphthalene catabolism is commonly a plasmid-encoded phenotype [20].

Although the vast majority of LAS is discarded down the drain and subjected to sewage treatment, varying amounts of LAS occur in effluents discharged into aquatic environments. Under unusual circumstances, LAS is directly discharged into the environment without treatment [19]. An extreme example of such a practice occurs in rural settings, where undiluted wastewaters from laundromats are directly applied to agricultural soils and released to holding ponds or seepage pits without dilution or prior treatment. One such example exists in northern Wisconsin, where discharge of laundromat wastewater

Correspondence: G.S. Sayler, Center for Environmental Biotechnology, University of Tennessee, 10515 Research Dr., Suite 200, Knoxville, TN 37932, U.S.A.

since 1962 into a natural depression has resulted in the formation of a permanent pond and wetland system [5,6].

The focus of the present work was to determine plasmid abundance in bacteria from this LAS contaminated environment and to relate LAS degradation with the presence of specific plasmids. The incidence of plasmid DNA is various environments has been the subject of a number of investigations, since it has been theorized that a higher incidence of plasmid encoded phenotypes may be characteristic of chemically stressed microbial communities [7,13,15]. Bacteria with plasmids are ubiquitous in natural environments; however, recent reports indicate that the frequency of plasmid occurrence in bacteria may be significantly greater in environments that have a history of exposure to xenobiotic chemicals. Hada and Sizemore [9] demonstrated that the frequency of plasmid bearing organisms was higher in an oil contaminated field site relative to an uncontaminated control site. Ogunseitan et al. [17] observed a similar correlation when comparing chemically contaminated and pristine aquifers. Not all forms of chemical perturbation have been shown to elicit alterations in plasmid frequency. Wickham and Atlas [24] did not observe any selection for plasmid bearing organisms when soil communities were stressed with antibiotics or mercuric chloride. A study of the Campeche Bank by Leahy et al. [15] showed no correlation between plasmid incidence and proximity to an oil field.

The specific objectives of this study were: i) to quantitatively compare LAS degradation and microbial populations in LAS contaminated and nearby uncontaminated pond ecosystems; ii) to determine the relative frequency of plasmids in heterotrophic bacteria from these sites; and iii) to compare frequency of various selected catabolic genotypes in bacterial populations from the sites. The objectives relate to a long-term goal of developing molecular tools for predicting LAS biodegradation in various habitats.

MATERIALS AND METHODS

Test chemicals

A commercial mixture of LAS with an average alkyl chain-length of C_{12} was obtained from Stepan Company (Northfield, IL). [U-^{14}C ring] sodium tridecylbenzene sulfonate (LAS) with a specific activity of 8.69 mCi/mmol was obtained from New England Nuclear (Boston, MA). Purity was 98% based upon thin-layer chromatography (TLC) on silica gel G with chloroform/methanol/water/formic acid (80/25/3/1).

Study site

The laundromat wastewater pond system consists of two ponds that are located approximately 0.5 km north of the community of Summit Lake in north-central Wisconsin. It is formed by wastewater discharged into a natural depression since 1962. Average monthly discharge, based upon the number of loads, varies from 160 000 l in the winter to 468 000 l during the summer. One pond (Laundromat Pond) is formed by wastewater, which is discharged to a sluiceway and then into a distribution tank located in the pond's center. This pond is approximately 22 by 66 m with an average depth of 1.5 m and is characterized by low levels of dissolved oxygen (0.5–1 mg/l) and the presence of a floating cyanobacterial mat. Approximately one-third of the total area of the pond is colonized by emergent vascular plants. A second pond (Secondary Pond) is formed from Laundromat Pond overflow and is characterized by a continual algal bloom and supersaturated levels of oxygen. Both ponds are surrounded by large deciduous trees, and precipitation and non-point runoff are the only other sources of water to the ponds. The average LAS concentration as determined by interference limited methylene blue active substances (IL-MBAS) response was 32 mg/l in the Laundromat Pond and 7 mg/l in the Secondary Pond.

The Control Pond is naturally formed and located approximately 2 km south of the Laundromat Pond. It is comparable in size and shape to the Laundromat Pond (LMP) but is not impacted by laundromat operation or any other apparent point source of pollution. It also contains a wetland area and is surrounded by deciduous trees. No IL-MBAS were detected in this pond.

Bacterial enumeration and isolation

Pond water samples were serially diluted in phosphate buffered saline (1.2 g Na_2HPO_4, 0.8 g NaH_2PO_4 and 8.5 g NaCl per liter of distilled water, final pH 7.6), and dilutions were plated onto YEPG agar (dextrose 1.0 g/l, polypeptone 2.0 g/l, yeast extract 0.2 g/l ammonium nitrate 0.2 g/l and agar 16 g/l) [21] and YEPG agar supplemented with 0.01% LAS/YEPG. Sediment and detrital (1.0 g) material were placed in 20.0 ml of 1% sodium pyrophosphate and vortexed for 1 min prior to dilution and plating. Plates were incubated at 28 °C for 10 days.

Plasmid analysis

All bacterial isolates were tested for the presence of plasmids. Since the isolates were not characterized, two plasmid DNA isolation protocols were used to insure the detection of plasmid DNA. The methods of Kado and Liu [14], a rapid procedure for isolation of plasmid DNA from Gram-negative organisms, and of Anderson and McKay [1], a procedure optimized for Gram-positive organisms, were used. Plasmid DNA bands were electrophoresed in 0.7% agarose gels and visualized under ultraviolet light. Plasmid DNA for subsequent use in gene probe analyses

were prepared in large scale using the Anderson and McKay extraction method with cesium chloride/ethidium bromide equilibrium centrifugation [16].

Gene probe analysis

Isolates were screened for the presence of pertinent genotypic traits by colony hybridization with the gene described in Table 1 [10,21]. Gene probes were prepared by labelling the DNA of interest with ^{32}P-dCTP (ICN, Irvine, CA). Probe DNA was separated from unincorporated nucleotide on a G-50 Sephadex column. The isolates were grown on agar plates for five days and then transferred to nylon membranes (ICN Biomedicals, Irvine CA). The colonies were lysed and the nucleic acids were heat fixed (80 °C) for 1 h. The membranes were incubated with probe DNA for 14 h at 65 °C in a hybridization solution composed as follows: 0.02% (w/v) ficol, 0.02% (w/v) polyvinylpyrrolidone, 0.02% bovine serum albumin, 0.18 M NaCl, 0.01 M Na phosphate, pH 8.3, 0.1 mM EDTA, 0.2% SDS and 500 μg/ml non-homologous DNA. Unbound probe DNA was washed from the membranes with buffer composed of 10 mM NaCl, 20 mM Tris pH 7.5, 1 mM EDTA and 0.1% SDS. The 2-h washes were done at 65 °C. Autoradiography was then carried out on air dried filters for 24 h at − 80 °C using X-OMAT AR X-ray film (Eastman Kodak, Rochester, NY).

Methylene Blue active substance (MBAS) plate assay

Organisms that attack LAS were detected on plates with a Methylene Blue reagent (King, J.M.H. 1987. Ph.D. thesis, University of York). Methylene Blue is a cationic dye, which will complex with LAS. The reagent is prepared as follows: 120 ml of a 0.1% (w/v) aqueous solution of Methylene Blue is added to 500 ml of a 10.0% (w/v) solution of Na_2HPO_4 which contains 6.8 ml of concentrated H_2SO_4. The resulting mixture is brought up to 1 l with distilled H_2O. LAS containing agar plates were flooded with this mixture and stained for 10 min. The plates were then washed and incubated at 37 °C for 20 to 30 min. A clear zone surrounding a colony is indicative of LAS degradation.

TABLE 1

Gene probes used to screen bacteria isolated on LAS/YEPG agar

Probe DNA	Source	Phenotype
NAH7	*Pseudomonas putida*	Naphthalene degradation
pDTG113	*E. coli*	Upper pathway of naphthalene degradation
pSS50	*Alcaligenes* sp.	Chlorobiphenyl degradation
pP + G13	unknown	Cryptic

Mineralization assay

Mineralization of LAS by microbes present in water, aerobic sediments and associated with plant detritus was determined. Water was collected from the center of each pond using aseptic technique. Sediments were collected only from areas of the Laundromat Pond (LMP) and Control Pond, which were colonized by aquatic macrophytes and were aerobic. The Secondary Pond contained no such areas. Plant detritus (decaying oak leaves) was obtained from the margins of each pond. Water samples (5 ml), sediment samples (10 g), or pieces of leaf matter (1.9-cm diameter disks) in sterile water (1 ml) were placed into triplicate vials (40 ml) and amended with approximately 50 ng of the uniformly ring radiolabelled LAS. The vials were sealed with teflon-backed silicon septa equipped with plastic wells containing a fluted filter paper soaked with 0.2 ml of 1.5 N KOH to trap the evolved $^{14}CO_2$. The sealed vials were incubated statically for 7 days. Following acidification of the samples, the filter papers were removed and placed in a scintillation vial with cocktail, and radioactivity was determined using a liquid scintillation counter. Counts were corrected to 100% efficiency based upon external standardization.

Data were expressed as the percentage of the radiolabelled LAS recovered as $^{14}CO_2$ after 7 days. The data were corrected utilizing abiotic controls amended with 0.1 ml formalin and or with the detrital sample controls containing sterile water without a leaf disk recovered as $^{14}CO_2$.

RESULTS

Sampling was conducted in July and November 1987. Microbes were enumerated on YEPG agar plates (total heterotrophs) and on YEPG supplemented with LAS (LAS/YEPG). Overall, counts on both agars were higher in November than in July (Table 2). In addition, counts on both agars were consistently higher in the wastewater system than in the control system. With one exception, counts on LAS/YEPG expressed as a percentage of those on YEPG for sediment and detritus were four to five times higher in the wastewater than in the control system. Furthermore, while water from the control system harbored no organisms that grew on LAS/YEPG, water samples from the wastewater ponds contained 1.7×10^3 to 4.6×10^6 CFU/ml.

Table 3 shows the mineralization of LAS in various habitats of the two pond ecosystems. Only the benzene ring of the LAS molecule was labelled, providing a

TABLE 2

Enumeration of bacteria (CFU/g) on LAS/YEPG and YEPG agars in various habitats of the wastewater and control pond ecosystems in July and November

Habitat ecosystem	July 1988		November 1988	
	LAS/YEPG	YEPG	LAS/YEPG	YEPG
Pond water				
Laundromat pond	1.8×10^4	8.1×10^7	4.6×10^6	3.2×10^8
Secondary pond	1.7×10^3	2.1×10^6	1.3×10^5	5.0×10^7
Control pond	NG	1.2×10^4	NG	2.4×10^6
Sediment				
Laundromat pond	4.5×10^3	1.0×10^9	1.9×10^6	3.5×10^7
Control pond	2.6×10^3	6.5×10^6	2.0×10^4	1.4×10^7
Detritus				
Laundromat pond	1.0×10^4	2.6×10^5	2.8×10^6	4.3×10^8
Control pond	2.8×10^3	2.6×10^5	2.0×10^4	1.4×10^7

NG = no growth

TABLE 3

Mineralization of LAS in various habitats of the wastewater and control ecosystems in July

Habitat ecosystem	% label converted to CO_2
Pond water	
Laundromat pond	16.7 ± 2.7
Secondary pond	11.4 ± 2.2
Control pond	NM
Sediment	
Laundromat pond	21.7 ± 2.5
Control pond	19.3 ± 1.6
Detritus	
Laundromat pond	25.6 ± 3.0
Control pond	3.7 ± 0.2

NM = no mineralization after 7 days

measure of total LAS destruction, since alkyl side chain oxidation will not result in the production of $^{14}CO_2$. In all habitats, mineralization levels were greater in the wastewater system than in the control system. Mineralization data also were consistent with bacterial population data. Sediments from control and contaminated sites had similar levels of LAS mineralization (19.3 and 21.7%, respectively) and comparable numbers of organisms that grew on LAS/YEPG media (2.6 and 4.5×10^3) in July. Detritus from the control pond exhibited LAS mineralization levels approximately 6–7 times lower than those exhibited by detritus from the laundromat pond in July. Counts on LAS agar were likewise 5-fold lower with control pond detritus. No mineralization of LAS was observed in the control pond water and no microbes from this habitat grew on LAS/YEPG. In contrast, in the wastewater ponds there were 10^3–10^4 CFU/ml on LAS/YEPG and LAS was mineralized at a level of 41.9%.

Plasmid incidence in bacteria isolated on non-selective (YEPG) medium and LAS containing medium was compared in the LMP site and the control sites (Table 4). A significant fraction of the isolates from both sites contained plasmids. Plasmid density was higher in November than in July and was higher in bacteria isolated on LAS/YEPG agar than those isolated on YEPG agar. The incidence of plasmids among total heterotrophs was 18% in the control system and 36% in the wastewater system. Notably the incidence of plasmids in total isolates on LAS/YEPG was similar in both sites.

A subset of isolates from LAS medium from both the contaminated and control sites were characterized further. The MBAS plate assay was used to examine the ability of isolates to carry out primary or alkyl side chain oxidation of the LAS molecule. As Table 5 shows most microbes isolated on LAS media appear to be LAS tolerant rather than capable of LAS oxidation. Only 23% of selected isolates from the wastewater system that grew on LAS/YEPG were able to degrade LAS based upon the MBAS assay. A smaller percentage (8%) of the isolates from the control system were MBAS positive. Only a small percentage (8–23%) of plasmid bearing isolates on LAS/YEPG from the control and wastewater systems exhibited positive MBAS reactions. In the control site, all MBAS positive isolates contained plasmids. However, in the wastewater site, 12% of the MBAS positive isolates

TABLE 4

Percentage of bacteria isolated on LAS/YEPG and YEPG agars from wastewater and control pond ecosystems that contained plasmids

Ecosystem	Time	No of isolates tested		% plasmid bearing	
		LAS/YEPG	YEPG	LAS/YEPG	YEPG
Wastewater system	July	36	44	22	30
	November	177	173	51	37
	Total	213	217	46	36
Control system	July	20	26	35	12
	November	37	36	49	22
	Total	57	62	44	18

carried plasmids. Thus, many plasmid containing isolates did not metabolize LAS and not all isolates that metabolized LAS carried plasmids. Notably, none of the isolates were able to mineralize LAS in pure culture.

This same subset of isolates was hybridized with gene probes involved in aromatic hydrocarbon oxidation. Table 1 lists the probes and Table 5 presents the results. The NAH7 probe, which serves as a broad specificity indicator for aromatic hydrocarbon oxidation, exhibited some homology with isolates from the three sites surveyed. This was true a lesser extent for the pDTG113 probe, a more specific probe consisting of the catabolic genes for the upper portion of the canonical naphthalene degradative pathway. Both probes were used to test the hypothesis that NAH or related catabolic gene sequences mediate LAS biodegradation and would be more abundant at sites exposed to LAS. NAH7 homologous sequences were found in isolates from both control and contaminated sites as were the pDTG113 genes. No organisms hybridizing with these gene probes were capable of LAS ring mineralization in pure culture, and these genes were not more preponderant at the contaminated site relative to the control. Both contaminated and uncontaminated sites harbored similar levels of these genes. The whole pSS50 plasmid, which has been of use in the isolation of 4-chlorobiphenyl degrading organisms from aquatic environments, was also used as a probe [18]. The results with pSS50 showed 5% of the wastewater isolates hybridized with pSS50 but none of the control pond isolated shared any homology with this probe. A plasmid of a cryptic nature, in an uncharacterized isolate, from the contaminated site, designated pP + G13, was used to assess the abundance of this indigenous plasmid in these environments. The pP + G13 sequences were more abundant among control site LAS isolates, where 16% of the isolates hybridized, than in the LMP sediment, where

TABLE 5

Percentage of a subset of bacteria isolated on LAS/YEPG agar from wastewater and control systems that contained plasmids, exhibited positive MBAS reactions, were capable of mineralizing ^{14}C-ring labelled LAS and hybridized with specific gene probes

Characteristic	Wastewater system[1]	Control system[2]
Plasmid containing	46	68
MBAS positive	23	8
Mineralized ^{14}C-Ring LAS	0	0
MBAS positive with plasmid	12	8
Positive hybridizations		
NAH7	10	12
pDTG113	6	4
pSS50	5	0
pP + G13	1	16

[1] 142 Isolates tested
[2] 25 Isolates tested

1.0% of the isolates hybridized. In summary, none of the gene probe sequences chosen for this analysis were more common in the isolates from the contaminated site relative to the control site. Thus, the genetic potential for aromatic hydrocarbon potential as assessed with these gene probes is similar at both sites or the organisms that mineralize LAS do not grow on LAS/YEPG medium.

DISCUSSION

Although LAS degradation occurs in many habitats, relatively little is known about the bacteria that mediate the degradation process. Furthermore, the population and genetic factors that control LAS degradation in the environment have not been well investigated. This study examined LAS degradation in wastewater and pristine pond ecosystems and attempted to relate mineralization with the presence of bacteria that grew on LAS containing medium (LAS/YEPG). In addition, this work investigated the relationship between LAS contamination and the occurrence of bacteria, which harbour plasmid DNA and gene sequences coding for the catabolism of aromatic compounds.

Plate counts indicated that exposure in wastewater resulted in higher levels of both heterotrophic bacteria and bacteria capable of growing on LAS containing medium. In addition to higher numbers, a higher proportion of heterotrophs from the wastewater system were capable of growing on the LAS medium. Thus, chronic exposure to high levels of LAS selected for organisms that tolerate and/or utilize it as a carbon source. Mineralization of LAS related directly to the presence of these bacteria. Mineralization was observed in all habitats that contained bacteria that grew on LAS/YEPG. Conversely, no mineralization was observed in habitats from which no organisms could be cultured. In addition, high LAS/YEPG counts tended to correspond with low turnover times.

Although growth of organisms on LAS medium and mineralization were directly related, most bacteria that grew on LAS medium tolerated LAS rather than degraded it. Only 23% of the isolates on LAS/YEPG from the wastewater pond could degrade LAS. From the control system, 8% of the isolates were MBAS positive. While many isolates partially degraded LAS, no isolate from either system was able to degrade LAS in pure culture. Thus, it appears that complete mineralization of LAS involves multiple organisms acting in concert. One can hypothesize that one population mediates oxidation of the side chain and a second mediates oxidation of the sulfonated ring. LAS/YEPG medium likely would select for the first population but not the second population. The low incidence of catabolic genes for aromatic catabolism among bacteria isolated on this medium is consistent with this hypothesis.

Plasmid DNA has been shown to confer drug and heavy metal resistance and catabolic capabilities to bacterial populations. Although plasmids were virtually ubiquitous in bacteria from natural environments, several studies have shown that the proportion of bacteria harbouring plasmids is significantly greater in environments experiencing acute or chronic chemical contamination [3,8,9]. For example, more than 40% of the bacteria recovered from a chemically-contaminated aquifer contained plasmids compared to 0.9% from a pristine aquifer [17]. Such results suggest that plasmid strains are selected for and that the plasmids may confer resistance to the contaminants or the ability to biodegrade them. In the present study, heterotrophs from the wastewater system exhibited twice the incidence of plasmids (36%) as those from the control system (18%). Furthermore, isolates on LAS/YEPG from both systems had a higher incidence of plasmids (44–46%) than the overall heterotrophic populations. The exact benefit of these plasmids is unknown. There was no direct correspondence between presence of a plasmid and an ability to tolerate or biodegrade LAS. In the control system, 8% of the isolates on LAS/YEPG were MBAS positive. However, all MBAS positive strains contained a plasmid. In the wastewater system, 46% of the isolates on LAS/YEPG contained a plasmid but only 12% were MBAS positive and contained a plasmid. Whether plasmids confer any ability to degrade LAS remains an open question.

Gene probes have become a useful tool for the study of environmental microbiology both for isolating organisms and enumerating specific genotypes [18,21]. In this study the NAH7 plasmid was used as a broad specificity probe to detect aromatic catabolic genes. This plasmid is widely disseminated and commonly found at chemically impacted sites. Work by Cain suggested that TOL-like plasmids (which include NAH7) may be involved in LAS oxidation. Many of the aromatic catabolic pathways are evolutionarily related and share gene order and sequence similarities [11]. Gene probe analysis with NAH7 as well as with pDTG113 (a more specific probe containing the genes responsible for conversion of naphtalene to salicylate) indicated no significant difference in the frequency of these sequences between contaminated and control sites. Furthermore, LAS exposure did not result in an increase in the abundance of any of the sequences tested. This finding suggests that in this setting degradation of LAS likely is mediated by as yet uncharacterized catabolic genes or that the organisms that degrade the aromatic drug of LAS do not grow on LAS/YEPG.

In summary, the wastewater site supported higher numbers of heterotrophic bacteria as well as bacteria

capable of growing on a medium containing LAS. Mineralization of ^{14}C-ring LAS in any habitat was related to the presence of degraders in that habitat. Although a higher incidence of plasmids was found in bacteria from the wastewater pond and among bacteria that grew on LAS containing medium, an ability to degrade LAS is not solely dependent upon the presence of plasmid DNA.

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