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Characterization of microbial traits associated with glyphosate biodegradation in industrial activated sludge

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SUMMARY

The microorganisms from two industrial (I1, I2) activated sludges that treat glyphosate (*N*-phosphonomethyl glycine) wastes and one domestic (D1) sludge were enumerated by microscopic examination and by the use of eight selective media. I1 and I2 had higher total counts but fewer pseudomonads and no yeasts. The enumerations correlated directly with traditional biological performance measurements. A total of 393 microbial strains were isolated from the sludges to correlate the occurrence and relationship of glyphosate-degrading activity (GDA) to 155 biochemical and morphological characteristics. Each activated sludge contained unique bacterial populations with the microbes treating industrial wastes, capable of utilizing a wide range of carbohydrates. Numerical taxonomy (arithmetic average linkage) using simple matching and Jaccard coefficients confirmed that there were five (D1), three (I1), and 12 (I2) clusters. GDA was found in only a small portion of the industrial clusters and did not correlate with any other characteristic tested, even though the GDA strains had a large phenotypic diversity. This suggests that GDA is not a universal trait and its expression requires enrichment through specific selective pressures.

INTRODUCTION

Glyphosate is a widely used broad-spectrum herbicide that is formulated under several trade names by Monsanto Agricultural Company (St. Louis, MO). The manufacturing facilities that produce glyphosate use activated sludge to treat the process wastes and achieve federal and state discharge permit levels. Thus, it is important that the biosystems maintain a large, stable microbial population with glyphosate-degrading activity (GDA). Glyphosate-degrading bacteria have only recently been isolated and characterized in pure culture [1,9,14] although their presence in soils and waters had already been established [11].

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The activated sludge process has long been used as a secondary treatment of wastewaters; yet, relatively few studies have been done on the microbiology of activated sludge. Dias and Bhat [2] and Prakasam and Dondero [10] found that (i) the numbers and types of microbes varied with the sewage composition, and (ii) enumerations on synthetic nutrient media did not represent the actual microbial diversity. Apparently, only two studies have been done on industrial activated sludge. Liao and Dawson [6] and Takii [17] monitored sludge that treated industrial carbohydrates. They noted population shifts as the feed composition changed. A literature review by Pipes [8] listed several bacterial species associated with activated sludge, including *Acinetobacter, Alcaligenes, Brevibacterium, Flavobacterium, Pseudomonas, Zooglea*, and several budding bacteria. Lighthart and Oglesby [7] described several methodologies which provided for extensive taxonomic characterization. However, only Takii [17] has considered the numerical taxonomy of industrial activated sludge. While he used synthetic media instead of sewage extracts, the results suggested that good biosystem performance was a function of the microbial community structure.

The purpose of this study was to enumerate and characterize microorganisms from industrial and domestic activated sludges. The distribution and diversity of microbes with GDA was also considered. Ultimately, the goal was to enhance and stabilize GDA for maintaining high rates of glyphosate removal in the industrial biosystems.

MATERIALS AND METHODS

Sampling

Activated sludge samples were taken weekly between August and November from a domestic plant (D1) and the two Monsanto plants currently manufacturing glyphosate in the U.S.A. (I1, I2). They were stored at 0–4°C until processed. Three separate samples from each site were processed (Waring blender, high speed, 4 min) prior to dilution. All platings were done in triplicate.

Media and culture conditions

Table 1 presents the composition of the eight media used to enumerate microbial groups. Amendments were added in order to select for specific microorganisms [3]. The influent to the aeration basin (IAB) for D1 was a synthetic blend of 100 mg/l glucose and 10 mg/l yeast extract. The industrial IAB was taken from an I1 composite sampler that was then centrifuged, filtered, sterilized, and the pH adjusted to 7.5. It was then added to the heat-sterilized enumeration media in a 1:1 dilution (final pH 6.8–7.2). Any amendments were then added (Table 1) and the plates poured. Samples from each site were plated on the media using standard enumerative techniques. They were incubated for 3 days at 25°C, except for the methylotroph, formalotroph, and yeast plates (5 days).

For microbial isolation and characterization, sludge samples were plated on an isolated medium. This consisted of glucose (0.1%, w/v), yeast extract (0.01%, w/v), and IAB (1–2%, v/v) from either I1 or I2, except for D1 which had no IAB amendment. Isolates were picked from plates containing 30–300 colonies. They were streaked twice for purity on a modified rapid CH medium (DMS Laboratories,

Table 1

Media used to enumerate different microbial groups

Medium[a]	Amendment[b]
Total	None
Gram$^+$	25 mg/l phenethyl alcohol
Gram$^-$	1.25 mg/l Brilliant green
Pseudomonas	2.5 mg/l Irgasan
Yeasts	Tartaric acid (to pH 4)
Bacillus	Heat dilution blanks, 5 min at 60°C
Methylotrophs	0.1% methanol on filters in plate lids
Formalotrophs	0.01% formaldehyde on filters in plate lids

[a] Each medium was made up in glass-distilled, deionized water with L-salts [8] and purified agar (1.5%, w/v).

[b] An aeration basin influent dilution and an organics stock (glucose, 0.1% w/v; and yeast extract, 0.01%, w/v) were used in all media but the methylotrophs and formalotroph media.

Flemington, NJ) using L-salts [5] and lyophilized in CH (carbohydrate) broth amended with 10% sucrose.

Test descriptions and characterization protocol

In the enumeration study, acridine orange direct counts (AODC) were done in addition to the plate counts. For AODCs, the procedure of Hobbie et al. [4] was done using nucleopore filters stained with sudan black (0.06% in 50% ethanol). Microbes with GDA were enumerated on agar plates amended with analytical glyphosate. The accumulation of aminomethylphosphonic acid (AMPA) was assayed as described by Balthazor and Hallas [1] except that tubes were used instead of flasks.

For microbial characterizations, lyophilized cultures were inoculated into CH broth and incubated for 48 h. Each isolate was then washed, resuspended in saline (0.85%), and inoculated into various media and test strips. The ZYM and AN-IDENT (Analytab products, Plainview, NY) contained 19 and 20 chromogenic enzyme tests, respectively. $Gram^+$ cocci of the streptococcus groups were characterized on API20s strips (Analytab Products, Plainview, NY). API20e strips (Analytab Products, Plainview, NY) were used to identify glucose-fermenting, $gram^-$ rods of the Enterobacteriaceae group. Test strips contained 21 and 27 carbohydrate fermentation tests and enzyme tests, respectively. Glucose-nonfermenting microorganisms were characterized on a Rapid NFT (DMS Laboratories, Flemington, NJ) strip. The strip contains eight enzymatic tests, and twelve carbohydrate assimilation tests which are based on microbial growth in the presence of a single carbohydrate source. The Rapid CH tests (DMS Laboratories, Flemington, NJ) have 50 single-carbon sources which were used to determine the assimilative and fermentative capacities of the microbes.

All of the test strips were incubated at 25°C except the NFT and CH strips which were incubated at 30°C. In general, procedures and interpretations followed methods previously described [3]. Results of API20e, API20s, NFT, ZYM, OF test, and agar medium tests were recorded after 4 days of incubation. The strains picked from chocolate agar were tested for DNAse activity by the RIM DNAse test (Austin Biological Laboratory, Austin, TX). Aerobic and anaerobic CH tests were incubated at 30°C for 8–10 days before results were interpreted. The AN-IDENT strip was recorded after 48 h incubation. The growth on glyphosate agar was recorded after 14 days incubation.

DATA BASE MANAGEMENT AND NUMERICAL TAXONOMY

In total, 226 traits were determined on 442 microbial strains. All characterization data were stored on a computer using several index editing programs. Binary test reactions were coded as zero for negative and 1 for positive. Continuous tests were transformed to binary codes where zero was negative and 1–5 were positive. AMPA generation was coded as 1 since no strains in the collection mineralized glyphosate. The characterization data on all isolates were summarized by Editmat [19].

Tests which were positive or negative for all strains, or tests not completed for every isolate were deleted from the data matrix. The result was a complete data matrix, with no missing values, containing 155 tests for a total of 393 isolates (54 D1, 165 I1, and 175 I2) strains.

Clustering analysis was executed by the CLUSTAN program package (Wishart Program, Edinburgh University, Version 2, Release 1, 1982 and Monsanto modifications of September 1983). Phenome diagnostic tests for assigned groups were determined by DIACHAR [15]. This is a basic program that determines the most diagnostic tests of each cluster in the data matrix as well as giving an indication of how strongly diagnostic they are in comparison to other groups. However, this program does not determine the most representative tests separating the clusters from one another in the matrix. Analysis for distinguishing tests between similar strain groups was done using the program CHARSEP [16].

RESULTS AND DISCUSSION

Enumerations of biosystems

Three subsamples from each biosystem were enumerated in triplicate for 3 months. The data were log-transformed prior to analysis; standard errors ranged from 5 to 15%. While all cells (viable and nonviable) were enumerated by microscopic direct counts, any microbe which could grow (i) on agar plates containing glucose and yeast extracts, and (ii) in the presence of various amendments was enumerated by plate counts. I1 IAB was used as a selective pressure in all industrial media, except methylotroph and formalotroph media. It consisted of unidentified components as well as formaldehyde, formic acid, and various phosphonates which are present at I1 and I2. It was assumed that this general amendment would inhibit the growth of transients while letting microbes grow which thrive in these biosystems. The results are summarized below:

Direct. The industrial counts were significantly higher than the domestic counts throughout the study. I1 and I2 counts ranged between 10^9 and $10^{9.5}$ while D1 ranged between 10^8 and $10^{8.75}$.

Total. I1 counts ($10^{7.6}$–$10^{8.2}$) were higher than I2 and D1 counts. I2 trended upwards from 10^7 to $10^{7.5}$ while D1 trended downwards from $10^{7.5}$ to $10^{7.1}$.

Gram$^+$. I1 counts ($10^{6.5}$–$10^{8.5}$) were slightly higher than I2 ($10^{6.5}$–$10^{7.7}$) and D1 ($10^{6.5}$–$10^{7.6}$) counts, though the I1 range was wider (over 2 logs).

Gram$^-$. There were no consistent differences between I1 ($10^{6.5}$–$10^{7.7}$), I2 (10^7–$10^{7.8}$) and D1 ($10^{6.1}$–$10^{7.6}$) counts.

Pseudomonads. D1 counts began higher (10^6) but ended lower ($10^{5.5}$) with counts comparable to I1. I1 counts trended upward ($10^{3.7}$—10^5) while I2 trended downward (10^5–10^4).

Bacillus. I1 ($10^{6.5}$) began with higher counts, but ranged to 10^5 before ending at 10^6. D1 counts ranged between $10^{3.7}$ and $10^{6.2}$, but I2 counts were reasonably consistent at 10^5.

Yeast. D1 counts ranged from $10^{3.6}$ to $10^{5.6}$; I1 and I2 counts were virtually nonexistent.

Methylotrophs. No clear differences between counts of I1 ($10^{6.8}$–$10^{7.8}$), I2 ($10^{6.8}$–$10^{7.3}$) and D1 (10^6–$10^{7.5}$).

Formalotrophs. I1 ($10^{6.5}$–$10^{7.5}$) and D1 ($10^{5.7}$–$10^{6.8}$) were higher than I2 ($10^{4.5}$–$10^{5.9}$).

In general, the industrial biosystems had higher total counts (Total and Direct) than the domestic biosystem, but no yeasts and fewer pseudomonads. All biosystems had large populations of gram$^+$ and gram$^-$ bacteria as well as methylotrophs, formalotrophs, and *Bacillus* spp. However, care should be taken in comparing counts between plates since a particular cell could grow on more than one medium (e.g., pseudomonads would grow on gram$^-$ medium and also might use methanol and formaldehyde).

Only four known references in the literature have considered broad groups of activated sludge microbes. Dias and Bhat [2] found that gram$^-$ microbes predominated in their studies and Unz and Dondero [18] found low fungi (yeast) counts. Both studies were done with domestic activated sludge. Takii [17] studied industrial carbohydrate waste biosystems and found that gram$^-$ predominated in low-carbohydrate wastes and gram$^+$ predominated in high-carbohydrate wastes. Seiler and Blain [12] also looked at industrial activated sludge treating chemical wastes and found that the predominant species were *Pseudomonas, Alcaligenes*, and *Zoogloea.*

Correlations of enumerations with other measurements

I1 and I2 waste treatment engineers routinely collect numerous data for monitoring their biosystems. The transformed enumeration means for each sampling date were used to correlate the engineering data with the microbial enumerations. Table 2 lists the significant I1 and I2 correlations with the aeration basin analyses. Statistical significance from zero was defined by Fisher's test at the 0.05 significance level.

Several enumerations correlated with traditional measurements for optimizing biosystem operation. Gram, total, *Pseudomonas, Bacillus*, and methylotroph plate counts appeared most often in the correlations. In general, the analyses were positively

Table 2

Significant correlations of I1 and I2 enumerations with aeration basin analyses[a]

Analyses[a]	Correlation		
	I1	I2	Enumeration
Oxygen uptake rate	0.65	n.a.[b]	Direct
	−0.67		Total
	0.64		*Pseudomonas*
	0.67		*Bacillus*
	0.74		Methylotroph
	−0.64		Formalotroph
Dissolved oxygen	−0.72		$Gram^-$
	−0.61		*Bacillus*
	−0.6		Methylotrophs
Temperature	0.74		*Pseudomonas*
Mixed liquor	0.64		Direct
Suspended solids	0.61	0.70	Total
		0.61	$Gram^+$
	0.61		$Gram^-$
	−0.63	−0.80	*Pseudomonas*
	0.74		*Bacillus*
	0.69	−0.74	Methylotroph
NH_3-N	−0.7		Direct
		−0.7	Total
	0.82	0.82	*Pseudomonas*
	0.73	0.73	Methylotroph

[a] Only statistically significant correlations ($P < 0.05$) are indicated as defined by Fisher's test.

[b] n.a. = not analyzed.

correlated except for dissolved oxygen. BOD and TOC treatment efficiencies were also compared to the enumerations (data not shown) with the latter analyses showing the more consistent correlations. Glyphosate-degrading microorganisms were also tracked. However, no significant correlations were found with the described analyses or with glyphosate-treatment efficiencies. A literature review found no references to studies which compared microbial counts to biosystem operation. Optimizing a biosystem that has been successfully enriched for GDA would require some measure of performance. Nutrient agar plates amended with IAB and glyphosate do not seem sensitive enough to provide that critical measure.

MICROBIAL CHARACTERIZATIONS

In this study, the microbial diversity (numbers and types of bacteria) within the three biosystems was considered. Special attention was placed on the isolation medium formulation and the plates that colonies were picked from for two reasons: (i) native microbes – rather than transients – would be isolated from the activated sludge samples, and (ii) a representative and random group of microbes from each biosystem sample would be selected. The 393 isolates were then tested against 155 traits. In general, the most useful tests for differentiating the isolates from another were based on carbohydrate utilization. The Rapid CH was also the most versatile to use for this characterization application.

Several trends were noted in reviewing the compiled cellular and colonial morphology data. $Gram^-$ rods predominated in all biosystems. The I1 and I2 isolates were opaque (67%, 75%), white (41%, 50%), or off-white (58%, 23%), smooth and circular (70%, 60%) with entire margins (99%, 93%), respectively. The domestics differed only in the size of the colony shape (pin point 80%). In addition, DOM strains were largely flagellated (63%) whereas isolates from I1 and I2 showed a greater percentage of non-motile forms (66%, 61%, respectively).

Table 3 presents the predominant characteristics found in the microorganisms. It is clear that the microbes of I1 were capable of exploiting the most carbon sources. Industrial microbes were also more competent than D1. Enzymatic tests were less definitive but followed the same trend.

These data are developed to a greater extent in the frequency distribution of all tests presented in Fig. 1. The I1 strains reacted positively to > 35% of the characterizations and had two groupings (36% and 72%). The distribution of I2 strains was much broader (14% to 71%) and grouped in three different areas. The widest distribution appeared with D1

Table 3

Predominant metabolic characteristics in activated sludge microorganisms[a]

Trait	I1	I2	D1
Fermentation[b]			
D-arabitol	93	82	30
D-turanose	96	48	38
trehalose	96	44	38
saccharose	96	52	37
maltose	96	51	44
mannitol	95	83	35
inositol	95	84	53
L-fructose	99	90	43
D-glucose	99	84	75
adonitol	99	42	27
arginine	94	94	78
glycerol	100	98	42
N-acetylglucosamine	95	64	52
Enzymatic[c]			
α-glucosidase	97	44	35
Gly aminopeptidase	100	99	42
glucosaminidase	96	65	**50**
Arg aminopeptidase	100	98	82
Leu aminopeptidase	100	97	68
alkaline phosphatase	99	85	90

[a] A predominant characteristic was defined as occurring in at least one biosystem at >95%.

[b] Histidine, alanine, and tyrosine were utilized by >99% of all microorganisms.

[c] Acid phosphatase and ester lyase were utilized by >99% of all microorganisms.

strains (0–65%), though most of the strains were positive for only 20–35% of the tests. None of the strain frequencies congregated at either end of the graphs (i.e., all positive or all negative). Thus, the selected traits should differentiate between isolates of different biosystems as well as isolates within the same system.

The absence of a characteristic activity can also be of significance as a potential selectable marker gene. Several fermentative (sorbose, raffinose, lactose) and enzymatic (α-fucosibase, ornithine decarboxylase) capabilities were absent as well as sporeformers, acid fast bacteria, and fluorescent pseudomonads.

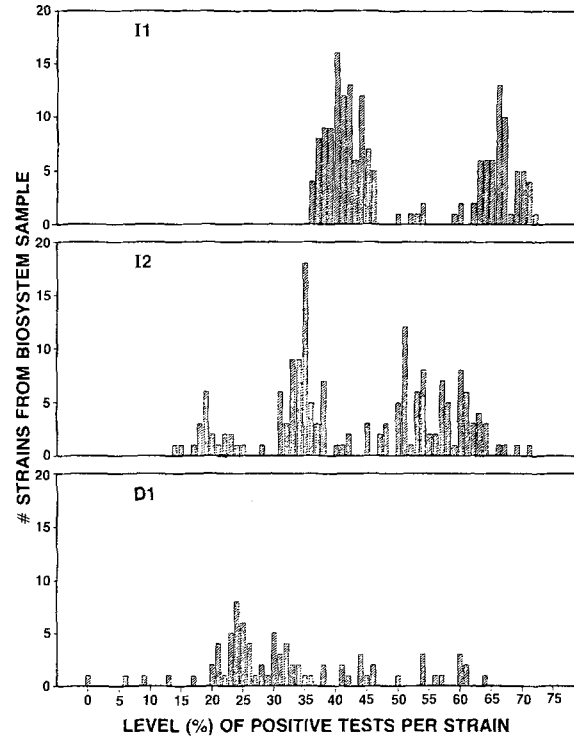

Fig. 1. The number of strains from each activated sludge at each positive percentage level.

NUMERICAL TAXONOMY

A combined cluster analysis of I1, I2, and D1 isolates using the Unweighted Average Linkage Algorithm (UPGMA) on simple matching coefficients is shown in Fig. 2. A 1.0 similarity coefficient would indicate identical matches of all tests for the linked strains; the higher the strains are linked, the less similar they are.

The dendogram indicates that, in general, the isolates grouped by biosystem. When these clusters were identified, an 83% similarity level was achieved. This level yielded a total of 81 clusters in which 316 strains comprised 19 major groups containing three or more members (80% of the total). The remaining isolates appeared as 47 singlets and in 15 doublet sets. The major clusters did not change notably when the data were analyzed by average linkage of Jaccard coefficients. At the similarity coefficient 0.554, activated sludge isolates

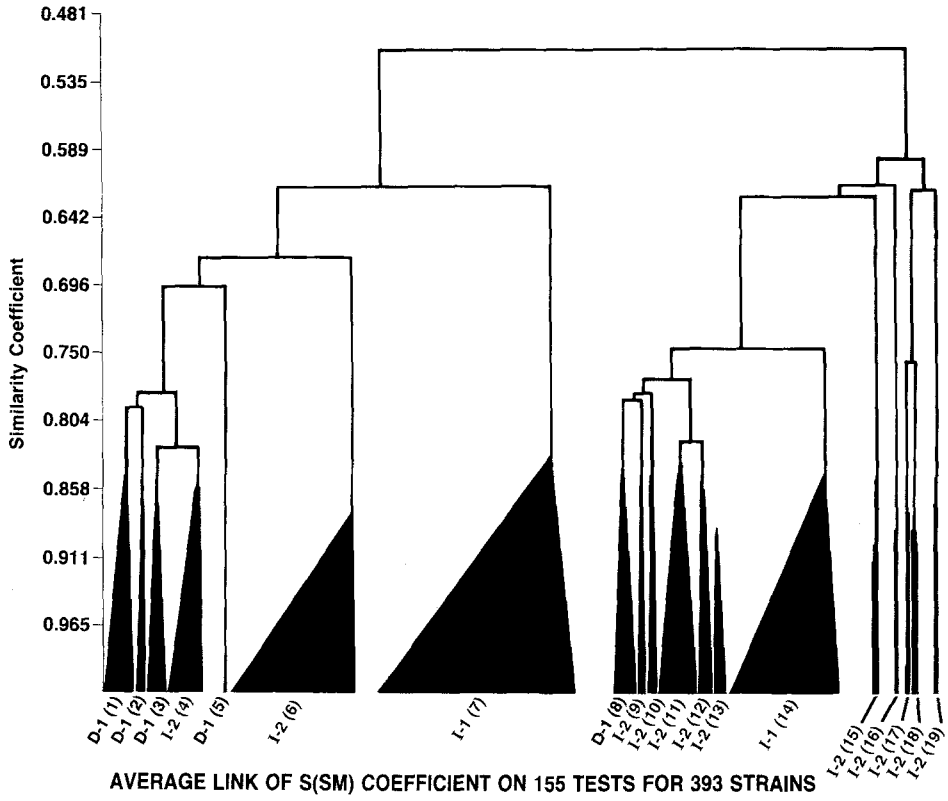

Fig. 2. Average linkage of S(SM) coefficient on 155 tests for 393 strains.

from the three biosystems could be categorized into two divisions. Isolates that appeared on the left side of the dendogram (division 1) were more similar to one another on the basis of negative test reaction matches, while the strains on the right were matched because of positive tests. Division 1 is comprised of four D1 (1, 2, 3, 5), one I1 (7), and two I2 (4, 6) strain clusters. Division 2 includes a greater number of clusters: one I1 (14), one D1 (8), and ten I2 (0–13, 15–19). Table 4 lists the best diagnostic tests for each cluster as determined by CHARSEP. With these in mind, each biosystem is summarized below.

I1 divided into predominantly two large groups separated by the major division. In general, the clusters in both divisions were consistently more similar to I2 than D1 strains. Tests separating the two clusters generally reflected the differences between divisions (e.g., the assimilation of carbohydrates versus selected enzymatic activities).

I2 clustered into the greatest number of groups. The two largest clusters contained over 50 members, and were separated by the two major divisions. All clusters containing three or four members were in division 2. These groups linked at very low coefficient values and therefore indicate that I2 has a very diverse microbial consortium. The clusters are also linked to D1 at higher similarity coefficients than the I1. I2 had a small domestic waste contribution. This may explain its similarity to D1.

Approximately 75% of all D1 isolates clustered into groups at the 0.825 similarity coefficient. Strains from D1 clusters 1–4 had the most common test reactions. They did not ferment carbohydrates and did not have α-glucosidase. However, they were positive for Voges-Proskauer and certain amidases. Outlying D1 strains fall evenly between the two divisions. These strains tend to link to small I2 clusters at very low similarity coefficient values.

None of the biosystem strains were identifiable

Table 4

The best diagnostic tests for clusters differentiated by numerical taxonomy[a]

Biosystem	Cluster	Test	Reaction
I1	7	Melezitose	+
		Galactose	−
	14	α-Galactosidase	+
		β-Galactosidase	+
I2	4	Mannitol	−
		Inositol	−
	6	α-Glucosidase	−
		Saccharose	−
	9	Amygdalin	+
		Voges-Proskauer	+
	10	Acetyl-aminidase	+
		Voges-Proskaubt	+
	11	Acetyl-aminidase	+
		Nitrate reduction	+
	12	D-Tagaraose	−
		Dulcitol	−
	13	Acetyl-aminidase	−
		L-Malate	−
	15	Arabinose	+
		Amygdaline	+
	16	β-Galactosidase	+
		ONPG	+
	17	Melibiose	+
		Citrate	+
	18	Melibiose	+
		Amygdalin	+
	19	Gram reaction	+
		Dulcitol	+
D1	1	Mannitol	−
		Nitrate reduction	+
	2	Citrate	+
		Mannitol	−
	3	Fructose	−
		Ribose	−
	5	Fructose	−
		Mannose	−
	8	L-Xylose A	+
		Erythritol A	+

[a] Tests distinguished by CHARSEP [16].

species despite the use of commercially available identification keys. These keys are commonly used for medical speciation; environmental schemes with large data bases are not yet available.

GDA CHARACTERIZATIONS

GDA did not correlate with any enumeration counts even though both I1 and I2 biosystems lost GDA during the sampling period (I1 for 10 days in September and I2 from October 15 onwards). Balthazor and Hallas [1] prepared plates containing a defined medium with glyphosate as the sole phosphorus source; they found high counts on plates streaked with I1 (where GDA was present) and low counts on plates streaked with D1 (which had not been exposed to glyphosate wastes). However, the results presented here showed that GDA could not be accurately followed or predicted by enumerating general microflora.

In the numerical taxonomy studies, it should be noted that the ability to degrade glyphosate was not a selection criterium during the initial isolations. Nevertheless, there were 62 microbes isolated with GDA. Twenty-two were from I1 (13%) and 40 were from I2 (23%). All the GDA isolates were AMPA generators; none mineralized the compound under the flask assay conditions.

The GDA trait was entirely absent in the D1 strains. Thus, GDA seems to be a trait that requires enrichment through selective pressure. Isolates with GDA did not cluster into a separate group. Instead, they (80%) were distributed among seven clusters; all species grouped in their own biosystem. I1 (1) cluster had twice as many microbes with GDA as I1 (2). I2 (5) cluster had 25% of the biosystem's GDA microbes. Thus, GDA is not a trait universally possessed by all microorganisms in biosystems treating glyphosate wastes. A variety of microbes have this trait, but these comprise only a small portion of the total population. The characterizations also suggest that these isolates comprise many bacterial genera. The only common trait among the organisms is the mode of action on the glyphosate molecule, as all are AMPA generators.

Attempts were made to determine whether any positive test reactions in the characterization protocol could be correlated with GDA. The data matrix was transposed and a cluster analysis done using the Jaccard coefficient. Thus, two tests reacting positively for a large number of the isolates link at a

high similarity coefficient. It was found that GDA does not correlate to any other tests (data not shown); it linked at a coefficient of <0.15. Thus, none of the tests in the characterization protocol could be used as a quicker assay to determine whether glyphosate-degrading bacteria were present in a particular biosystem.

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