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Rapid screening for bacteria capable of degrading toxic organic compounds

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Summary

Routine procedures for isolating and characterizing microorganisms capable of degrading toxic chemicals are time consuming and labor intensive. The objective of this paper is to describe a new method for screening aerobic bacterial isolates and consortia that will rapidly determine metabolic capacity for various toxic chemicals and access the use of various substrates as inducers in the degradation process. This method uses the Biolog® multiwell plate technology – a four-step method that includes inoculation followed by incubation of a uniform suspension of cells into a microtiter plate – to test more than 40 bacterial isolates and mixtures against 30 target toxic chemicals. Several bacteria and consortia have been shown to degrade toxic chemicals at concentrations of 10–500 ppm. These results indicate that Biolog® GN and MT plates are useful tools for screening bacterial isolates and consortia for their ability to survive metabolize, and potentially degrade selected organic chemicals.

Key words:

Introduction

Bacteria capable of degrading toxic organic chemicals have been isolated from aquatic systems, deep wells, and terrestrial subsurface sites [1–4]. Procedures previously used to determine the ability of microorganisms to degrade or mineralize toxic contaminants may require repeated enrichments and exposure of organisms to various concentrations of toxic substances for weeks or months [5,6]. Because the use of heterotrophic aerobic bacteria in the bioremediation of contaminated sites is increasing [7,8], the capacity to quickly characterize potential degraders will enhance bioremediation screening procedures.

The use of inducers to enhance bioremediation has received increasing emphasis. Typically, inducers stimulate the microbial degradation of a toxic substance. Thus, the identification and subsequent use of the most effective inducer(s) may increase the

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degradation rate and reduce the time required for bioremediation.

This manuscript describes a rapid screening procedure that can be used to test bacterial isolates, consortia, or natural samples to determine their ability to degrade toxic chemicals, and to use specific substrates as inducers in the degradative process. Screening procedures, using Biolog® multiwell plates (Biolog®, Hayward, CA), are versatile, flexible, and suitable for testing a wide range of mixtures and concentrations of toxic chemicals and selected microorganisms.

Materials and Methods

Methodology

The following is the four-step Biolog® methodology: (1) Prepare within the standardized optical density, a uniform suspension of cells in sterile, normal saline. (2) Inoculate the cell suspension into the microtiter plate at a level of 150 μl per well. (3) Incubate the plate under appropriate conditions. (4) Analyze the colorimetric changes.

Modifications

The following modifications were made to the standardized Biolog® procedures: (1) Cultures were grown on either nutrient agar (Difco) or PTYG agar depending on the growth requirements of the bacteria, then incubated for a period of 24–48 h, time sufficient to obtain adequate numbers of bacteria. (2) Cells were carefully lifted from the agar using a sterile Q-tip® suspended in sterile saline, and washed by centrifugation 2–3 times to remove extraneous agar or nutrients from the cells. (3) Washed cells were resuspended in sterile saline and adjusted to an optical density at 590 μm that corresponded to 3×10^8 cells/ml.

Bacterial strains

Bacterial isolates were obtained from the U.S. Department of Energy's Subsurface Microbiological Culture Collection [2] curated by Dr. David Balkwill, University of Florida at Tallahassee. Additional bacterial isolates were obtained from water and sediment samples collected from ponds, lakes, and soils in Illinois, Georgia and South Carolina. Pure cultures, consortia, and whole water samples have been tested using the procedures described below.

Bacteria were routinely isolated and maintained on nutrient agar or protease-trypticase-yeast extract-glucose agar (PTYG) with the following composition: glucose, 10 g/l; yeast extract, 10 g/l; peptone, 5 g/l; trypticase, 5 g/l; $MgSO_4$, 0.6 g/l; $CaCl_2$, 0.07 g/l; agar, 15 g/l.

Biolog® procedures

Biolog® redox technology is the basic component of the rapid screening procedure [9–11]. Although originally designed to identify clinical bacterial isolates, the technology has been extended by Biolog® to identify environmental isolates. The basic described procedures use Biolog® GN, GP, and MT plates. Biolog® GN (Gram+) and GP (Gram−) plates contain the same minimal nutritional factors along with a different organic substrate (Table 1) in each of the 95 wells in a microtiter plate. MT

TABLE 1

Sole carbon sources in Biolog® GN microtiter plates

Carbohydrates	**Carboxylic acids**	**Amino acids**
N-Acetyl-D-galactosamine	Acetic acid	D-Alanine
N-Acetyl-D-glucosamine	*cis*-Aconitic acid	L-Alanine
Adonitol	Citric acid	L-Alanyl-glycine
L-Arabinose	Formic acid	L-Asparagine
D-Arabitol	D-Galactonic acid lactone	L-Aspartic acid
Cellobiose	D-Galacturonic acid	L-Glutamic acid
i-Erythritol	D-Gluconic acid	Glycyl-L-aspartic acid
D-Fructose	D-Glucosaminic acid	Glycyl-L-glutamic acid
L-Fucose	D-Glucuronic acid	L-Histidine
D-Galactose	α-Hydroxybutyric acid	Hydroxy-L-proline
Gentiobiose	β-Hydroxybutyric acid	L-Leucine L-Ornithine
α-D-Glucose	γ-Hydroxybutyric acid	L-Phenylalanine
m-Inositol	*p*-Hydroxyphenylacetic acid	L-Proline
α-Lactose	Itaconic acid	L-Pyroglutamic acid
Lactulose	α-Ketobutyric acid	D-Serine
Maltose	α-Ketoglutaric acid	L-Serine
D-Mannitol	α-Ketovalenic acid	L-Threonine
D-Mannose	D,L-Lactic acid	D,L-Carnitine
D-Melibiose	Malonic acid	γ-Amino butyric acid
β-Methyl D-glucoside	Propionic acid	
Psicose	Quinic acid	**Aromatic chemicals**
D-Raffinose	D-Saccharic acid	
L-Rhamnose	Sebacic acid	Inosine
D-Sorbitol	Succinic acid	Urocanic acid
Sucrose		Thymidine
D-Trehalose	**Alcohols**	Uridine
Turanose		
Xylitol	2,3-Butanediol	**Brominated chemicals**
	Glycerol	
Esters		Bromosuccinic acid
	Amides	
Mono-methylsuccinate		**Amines**
Methylpyruvate	Succinamic acid	
	Glucuronamide	Phenyl ethylamine
Polymers	Alaninamide	2-Aminoethanol
		Putrescine
Glycogen	**Phosphorylated chemicals**	
α-Cyclodextrin		
Dextrin	D,L-α-Glycerol phosphate	
Tween 80	Glucose-1-phosphate	
Tween 40	Glucose-6-phosphate	

plates differ in that they have no organic substrates in any wells. Minimal nutritional factors and tetrazolium dye are present in each well. As a response to bacterial metabolic activity the tetrazolium dye is oxidized and turns each active well a shade of purple.

Standardized densities of bacterial suspensions, as described by Biolog®, were inoculated into each well, the plates incubated, and the color changes indicative of the

characteristic metabolic patterns were recorded. Although the 95 different carbon sources in Biolog® GN plates were pre-selected specifically for characterizing and differentiating Gram-negative aerobic bacteria, they were also useful in demonstrating metabolic patterns for mixed cultures. Carbon sources in the Biolog® plates are dominated by carbohydrates [28], carboxylic acids [24], and amino acids [20] plus various amides, aromatic chemicals, and other compounds (Table 1). The A-1 well of the microtiter plate has no substrate, and thus no nutrient source with which to initiate a metabolic reaction with the indicator dye, and consequently serve as a negative control. Replicate plates without the test compounds were established for each set of analyses. Volatile chemicals were added to each well and individual volatile chemicals were kept separate from each other during the course of the investigation so that cross contamination of chemicals did not occur.

The Biolog® automated plate reader was factory programmed to sense a threshold optical density value for each plate based on the color of the A-1 reference control well. Thus, the optical density readings that determined each inoculum pattern, were significantly above the threshold level. Approximately 100 bacterial strains and combinations have been screened with up to 30 different chemicals in more than 1000 Biolog® GN and MT plates.

Preparation of toxic chemicals

All tested chemicals were prepared from reagent grade chemicals as aqueous solutions or in an appropriate solvent (Table 2). Chemicals were prepared in solutions as parts per million (ppm) v/v, while concentrations of slightly soluble chemicals were estimated from the literature [12,13]. Organic compounds of specific interest were prepared in concentrations ranging from 10–1000 ppm, usually 250–500 ppm depending upon their solubility in water or organic solvents. If the tested chemical required a solvent, either ethanol or dimethyl sulfoxide was used. Solvent control plate were used to determine whether the solvent alone caused tetrazolium dye reduction in the wells.

Preparation of test and reference plates

Control plates received 150 μl per well of bacterial suspension, and test plates

TABLE 2

Organic compounds tested

Acetone	Dibenzofuran	Phenanthrene
Acridine	Dibenzothiol	Phenyl
Aminoanthracene	Dimethyl sulfoxide	Pyrene
Aminonaphthalene	Ethanol	Pyridine
Aniline	Fluorene	Quinoline
Benzene	Indole	Tetrachloroethylene
Benzofuran	Methanol	1,1,1-Trichloroethane
Benzo-naphtholthiophene	Naphthalene	Trichloroethylene
Carbazole	1-Naphthol	Toluene
Carbon tetrachloride	Nitrobenzene	Xylene
Chrysene	Nitronaphthalene	

received 75 μl per well of the tested organic compound along with 75 μl of the bacterial isolate suspension. In a few cases the bacteria and tested chemical were mixed together and inoculated at the same time. Usually two concentrations of each tested chemical were used. Replicate plates were prepared for each chemical concentration and each bacterial suspension. Other reference control plates included the following: tested chemical alone, formalin-killed bacteria, autoclaved bacteria, sterile saline solution, and sterile distilled water.

Inoculation design

During the initial portion of this study, a single bacterial isolate was inoculated into Biolog® GN plates. Subsequently, both axenic and mixed bacterial cultures were added to both GN and MT plates. Each Biolog® GN plate received a single concentration of the tested chemical and the appropriate bacterial suspension. The data presented in Table 3 are primarily for concentrations of 250 and 500 ppm. In several tests, duplicate GN plates were incubated at 13° and 23°C to determine differences in metabolic activity, to reduce the loss of volatile chemicals, and to provide the optima temperature for subsurface bacteria.

To test their degradative capacity, bacteria were inoculated into Biolog® GN plates to which a known concentration of the target chemical had been added to each well. Inoculated plates were then incubated for 24 h and the optical density recorded. Control Biolog® GN plates were inoculated without the tested chemical. Following incubation, any change in the metabolic pattern as compared to the characteristic pattern observed on the reference plates for that bacterial culture alone was considered a response to the chemical tested.

All color changes on the plates were read using a Biolog® multiwell reader, and data were stored and analyzed using Biolog® software. The color intensity of the tetrazolium indicator dye in each well was a measure of the metabolic activity of the bacteria present in the well. Bacteria capable of using a particular compound as a sole carbon or energy source caused a color change in the MT plates. Bacteria requiring specific inducers to degrade the tested compound showed an altered color pattern in the GN plates when compared with the pattern of the reference control GN plates.

Results and Discussion

Biolog® MT plates contain a minimal salts solution plus an indicator dye with no additional carbon and energy source. Therefore, an appropriate carbon and energy source must be added by the investigator to cause a metabolic reaction and subsequent color change. Biolog® MT plates offer as many as 96 replicate wells where the same substrate can be added to each well. In this study one concentration of each tested organic compound was added to adjacent wells for a total of 16 replicate wells for each compound; therefore, Biolog® MT plates were used to simultaneously test as many as six different chemicals in a microtiter plate. Volatile compounds were located on separate plates. Plates receiving tested chemicals were then inoculated with the appropriate bacterial solution and incubated at 13° and 23°C.

All bacterial strains and mixtures, when inoculated into Biolog® GN plates in the absence of tested chemicals, produced metabolic patterns that were easily read by eye

or the plate reader. Two general types of metabolic patterns observed were as follows:

- Most isolates produced a distinct pattern in which wells were colored and the remainder were clear and without a color change.
- Other isolates produced nondistinct patterns in which nearly all of the wells showed a slight color change from clear to a light pink or faint purple while some wells were dark purple.

Patterns changed when isolates were exposed to the different tested chemicals.

Several types of reactions were observed when the tested chemicals and active bacteria were added to Biolog® GN plates. In some cases, the lower concentration of the chemical led to an enhanced reaction, while the higher concentration led to inhibition in several wells. With other chemicals the higher concentrations showed an enhanced reaction. Patterns were fairly consistent for duplicate plates both in the presence and absence of tested chemicals. All control wells in both the reference GN and MT plates were colorless. None of the tested chemicals alone caused a reaction in

TABLE 3

The numbers of wells on Biolog® GN 96 well plate that registered positive when isolate B0388 was exposed to different toxic organic chemicals and their solvents (Concentrations were 250 or 500 ppm v/v unless otherwise noted.)

Organic chemical	Concentration	
	250 ppm	500 ppm
Bacteria alone	38	38
Acetone	50	53
Acridine	7	5
Aminoanthracene	0	1
Aminonaphthalene	17	0
Aniline	42	39
Benzene	28	58
Benzofuran	12	13
Benzo-naphtholthiophene	10	6
Carbazol	3	5
Carbon tetrachloride	30	26
DMSO	8 (15%)	0 (25%)
Dibenzofuran	0	4
Dibenzothiol	15	0
Ethanol	32 (2.5%)	22 (5%)
Indole	4	0
Methanol	80	85
Naphthalene	5	17
1-Naphthol	6	0
Nitrobenzene	38	73
Nitronaphthalene	23	5
Phenol	12	28
Pyridine	28	49
Quinoline	10	15
Tetrachloroethylene	2	3
1,1,1-Trichloroethane	30	24
Toluene	51	61
Trichloroethylene	12	31
Xylene	12	5

any well. Incubations at 23°C led to more rapid development of the color pattern due to biological activity, but the overall reaction patterns did not change with respect to temperature.

Results with various bacterial isolates incubated in the presence of different chemicals indicated that specific substrates may serve as inducers thereby enhancing the degradation of specific chemicals. For example, isolate C0679 showed an enhanced reaction to ethanol in the presence of α-keto glutaric acid but not with other tested chemicals. In all plates containing tested chemicals, isolate C0679 showed an enhanced reaction in the presence of amino butyric acid. In the presence of α-keto glutaric acid, isolate A0735 was inhibited by most of the tested chemicals, while the activity of isolate C0593 was highly variable.

The data in Table 3 show an example (isolate B0388) of the results with the bacterial isolate when exposed to 28 tested chemicals in a Biolog® GN plate. Duplicate control plates registered 38 positive wells out of a possible 96 wells. Seven chemicals at either 250 or 500 ppm – acetone, aniline, benzene, methanol, nitrobenzene, pyridine, and toluene – caused an increased metabolic reaction when compared to the control plates containing the isolate alone. Twenty-one of the tested chemicals caused a reduction in the number of positive wells for at least one of the tested concentrations. Several of the tested chemicals caused extensive inhibition of bacterial activity. These data indicate that different chemicals caused different metabolic reactions with respect to isolate B0388. Such data are valuable in determining the response of bacterial populations to organic compounds that may stimulate the degradation of the targeted organic to be degraded either in situ or ex situ.

The data in Table 4 demonstrate the activity of selected bacteria cultures and their mixtures against specific organic compounds at a single concentration. The numbers reflect the average change in optical density as determined from the plate reader when comparing the control plates to the test plates. For example, isolate A0371 gave high optical density readings in all wells when exposed to benzene, ethanol, tetrachloroethane, trichloroethylene, toluene, and xylene on a single MT plate as compared to

TABLE 4

Average percent change, when compared to control plates, for each isolate tested against a specific concentration of a compound in replication of either 16 or 32 tests

Bacteria	Chemicals					
	Benzene (125 μg/l)	Ethanol (125 μg/l)	PCE (40 μg/l)	TCE (40 μg/l)	Toluene (150 μg/l)	Xylene (10 μg/l)
A0371	520	445	765	492	843	413
A0735	65	592	65	43	39	4184
B0694	24	84	10	46	29	43
B0725	0	90	ND	10	8	71
C0593	28	97	65	70	55	0
C0679	55	253	67	8	15	−9
C0593 + C0679	40	64	64	42	39	29
A0735 + C0679	20	279	35	22	31	7
B0694 + B0725	9	10	7	4	4	8
Mixture	45	132	56	1	0	−12

the control plates. The data are then expressed as a percentage of the optical density obtained both on the test plate when compared to the control plates. Isolates A0735 and C0679, gave positive readings in most wells when exposed to the same chemicals. In every test, the 16 replicate wells (MT plates) had similar color responses for each chemical, and these responses indicated that the consortia and bacterial mixtures were capable of reducing the indicator dye in the presence of each of the tested chemicals. In a few cases, the consortia demonstrated greater activity than either single isolate. The data are not to be interpreted solely as destruction of the introduced compounds, but rather that these compounds can be acted upon by the microbial components in such a way that the indicator dye is oxidized and gives information as to the metabolic activity of each of the isolates with regard to a specific compound. If one isolate were more active against certain compounds than another isolate, the Biolog® technology provides such a screening tool. Thus the results are to be interpreted in the light that the tested compounds can both stimulate metabolic activity and that the compounds themselves may be degraded by the measured metabolic activity. Those two parameters are not separated with this technique.

The two Biolog® plates, GN and MT, provide different approaches for analyzing the ability of bacterial isolates and consortia to metabolize selected organic compounds. When toxic chemicals were added to Biolog® GN plates and the plates were inoculated with isolates, consortia, or natural samples, the resulting patterns indicated whether the microorganisms present in the sample were capable of metabolizing the substrates in the presence of the chemicals. Such substrates may be appropriate inducers for enhancing the degradation of the chemicals. Likewise, if no color change occurred in wells that were positive in the control reference plates, this indicated that the chemicals inhibited the microbial metabolic activity in that well, and that the substrate would not be an effective inducer.

Further analyses of the various amino acids, carbohydrates, and other substrates ma reveal additional information regarding the effect of the chemicals on the bacterial activity. For example, increasing concentrations of target chemicals may be added to a series of GN plates to determine those concentrations that enhance or inhibit the test bacteria or consortium.

Conclusions

The use of Biolog® MT plates provides the capability of selecting microbial systems adapted to metabolize conscripted chemicals as their sole carbon and/or energy source. Several concentrations of the target chemical may be added to the same plate so that a range of concentrations can be tested simultaneously. Also, based on the results from the GN screening, the best inducers may be added to MT plates for further analyses. Thus, the appropriate combination of target chemicals and inducers can provide information on the ability of the test organism to use the target chemical as a sole carbon and/or energy source and whether an inducer is useful in enhancing the degradation of the chemical.

These results indicate that Biolog® GN and MT plates are useful tools for screening bacterial isolates and consortia for their ability to survive, metabolize, and potentially degrade selected organic chemicals.

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