0273-2289/95/5152—0263$06.40

Characterization of the Cellulose-Degrading Bacterium NCIMB 10462†

C. DEES,*[,1] D. RINGELBERG,[4]
T. C. SCOTT,[2] AND T. J. PHELPS[3]

[1]Health Sciences Research Division, [2]Bioprocessing Research and Development Center and [3]Environmental Sciences Division, Oak Ridge National Laboratory,†† Oak Ridge, TN 37831; and [4]Center for Environmental Biotechnology/ The University of Tennessee, Knoxville, TN

ABSTRACT

The gram-negative cellulase-producing bacterium NCIMB 10462 has been previously named *Pseudomonas fluorescens* subsp. or var. *cellulosa*. Because of renewed interest in cellulose-degrading bacteria for use in the bioconversion of cellulose to chemical feed stocks and fuels, we re-examined the characteristics of this microorganism to determine its true metabolic potential. Metabolic and physical characterization of NCIMB 10462 revealed that this is an alkalophilic, nonfermentative, gram-negative, oxidase-positive, motile, cellulose-degrading bacterium. The aerobic substrate utilization profile of this bacterium has few characteristics consistent with a classification of *P. fluorescens* and a very low probability match with the genus *Sphingomonas*. However, total lipid analysis did not reveal that any sphingolipid bases are produced by this bacterium. NCIMB 10462 grows best aerobically, but also grows well in complex media under reducing conditions. NCIMB 10462 grows slowly under anaerobic conditions on complex media, but growth on cellulosic media occurred only under aerobic conditions. Total fatty acid analysis (MIDI) of NCIMB

*Author to whom all correspondence and reprint requests should be addressed.

††Managed by Martin Marietta Energy Systems, Inc., under contract no. DE-AC05-84OR21400 with the US Department of Energy.

10462 failed to group this bacterium with a known pseudomonas species. However, fatty acid analysis of the bacteria when grown at temperatures below 37°C suggest that the organism is a pseudomonad. Since a predominant characteristic of this bacterium is its ability to degrade cellulose, we suggest that it be called *Pseudomonas cellulosa*.

Index Entries: Cellulase; *Pseudomonas fluorescens* subsp. *cellulosa*.

INTRODUCTION

Microorganisms that are capable of producing cellulases have become more important as the demand has increased for industrial processes to degrade or modify cellulosic biomass. The enzymes produced by cellulase-producing microorganisms are potentially useful to convert cellulosic waste to commercially useful feed stocks that can be used in the production of alternative fuels. A wide variety of anaerobic and aerobic bacterial cellulase producers have been described, including the bacterium NCIMB 10462 (*1*). This bacterium was originally named *Pseudomonas fluorescens* var. *cellulosa*. The bacterium has been called *Pseudomonas fluorescens* subsp. *cellulosa* in the many recent studies performed using this microorganism (*2–5*). The bacterium, isolated from soil in the early 1950s, was placed in the National Council for Industrial and Marine Bacteriology (NCIMB, Aberdeen, Scotland) repository where is now listed as a *Pseudomonas* species. Interest in the study of NCIMB 10462 has recently increased (*3–6*), along with the recent upsurge of interest in biotechnological conversion of cellulose to fermentable carbohydrates. Because of the importance of this bacterium as a candidate for commercial biomass conversion, we re-examined the metabolic and physical properties of NCIMB 10462 using growth, substrate-utilization studies, total fatty acid analysis, and total lipid examination.

MATERIALS AND METHODS

Source of Microorganism

The cellulase-degrading bacterium was obtained from the NCIMB repository. The microorganism's catalog number NCIMB 10462 is listed as *Pseudomonas* species. Two separate cultures of this bacterium were obtained from the repository and examined in this study.

Culture Conditions

Cellulosic media consisted of a M9 salt solution as previously described (*4*). Agar was added to 15 g/L for solid media along with soluble cellulosic components (e.g., carboxymethyl cellulose, CMC) to 0.1% (w/v). Cellu-

losic liquid medium consisted of the M9 salts solution to which strips of Whatman No. 1 filter or newspaper had been added. Alternatively, cellulose-containing medium was made by adding Avicell powder (0.1% w/v) to M9 salt solution. All studies to determine optimal pH and the effects of temperature on growth were performed in Trypticase Soy broth (TSB) with vigorous shaking. MIDI analyses were formed on bacteria grown on Trypticase soy agar (TSA) plates with a 24-h incubation period as previously described (*7*).

Substrate Utilization Assays

All bacteriologic assays and growth procedures were performed using standard bacteriology techniques, methods, and medias. Nonfermentative analysis was performed using an API nonfermentor identification system (BioMerieux-Vitek, Hazelwood, MO). Utilization of carbon sources was also performed using a Biolog GN system (Biolog Inc., Hayward, CA). MIDI analysis was performed using standard protocols developed by the company (MIDI Inc., Newark, DE). Motility studies were performed using TSA and on M10 medium (Difco Inc., Detroit, MI).

Lipid Analyses

Isolation of sphingoid bases was achieved with minor modifications to the assays previously developed (*8,9*). Acetyl chloride was used to make the 2*N* methanolic HCl hydrolysis solution, which was then added to a lyophilized pellet (10 mg) of the isolate and incubated at 80°C for a period of 18 h. The lipids were then isolated, recovered, and saponified in a strong alkaline solution. The lipids were again isolated and recovered, and the trimethylsilyl esters formed using *N,O*-bis(trimethylsilyl)-trifluoroacetamide (BSTFA, Sigma Chemical Inc., St. Louis, MO) as described in Wollenweber et al. (*10*). The absence of any sphingoid bases was determined by analyses of the lipid extract on a gas chromatograph/mass spectrometer (GC/MS) with positive electron-impact ionization of 70 eV. The GC column was held at an initial temperature of 80°C for 2 min, and then raised at a rate of 5°C/min to a final temperature of 280°C, where it was held for 5 min. The injector was maintained at a temperature of 280°C, and the detector at 300°C. The GC/MS was equipped with an RT_x-1 60 m capillary column with an internal diameter of 0.25 mm and a film thickness of 0.10 df.

RESULTS

Substrate-Utilization Studies

Tables 1, 2, amd 3 show that substrate-utilization studies and nonfermentative analysis of NCIMB 10462 revealed few characteristics that are typically associated with *P. fluorescens*. For example, NCIMB 10462 did

Table 1
Metabolic and Physical Characteristics of NCIMB 10462

Test	*P. fluorescens*	NCIMB 10462
Growth on blood agar	+	+
Growth on MacConkeys Agar	+	–
Growth in M10 medium	+	–
Catalase	Strong positive	Weak positive
Growth in thioglycollate	Aerobic	Anaerobic
Fluorescent	+	–
Oxidase	+	+
Tryptophanase	–	–
Arginine dihydrolase	+	–
Urease	–	–
Esculin	–	+
Gelatinase	+	–
PNGP	–	+
Glucose	+	+
Arabinose	+	+
Mannose	+	–
n-Acetyl-Glucosamine	+	+
Maltose	–	+
Gluconate	+	–
Caprate	+	–
Adipate	+	–
Malate	+	–
Phenylacetate	±	–

not exhibit growth on MacConkeys Agar or Pseudomonas M10 medium. NCIMB 10462 produced a weakly positive catalase reaction, but did grow in anaerobic thioglycollate medium. In large cultures (500 L) of NCIMB 10462, a bright green pigment was found in culture supernatants. No evidence for the production of a fluorescent pigment was found (Table 2). In contrast to *P. fluorescens*, as shown in Table 2, this bacterium can utilize a wide variety of solid and soluble cellulosic media as a sole carbon source, which is consistent with previous observations. The organism is oxidase- and catalase-positive, motile, and aerobic, which are the few characteristics that suggest it might be related to the pseudomonads. The bacterium only weakly clots a lymulus lysate test, which is interesting, since it appears to be a small gram-negative rod by gram stain tests. These studies show that this bacterium is not closely related to *P. fluorescens*, but instead has a very low probability match with the genus *Sphingomonas* and *Pseudomonas* species.

Consistent with our other attempts to classify the organism using metabolic studies, Biolog analysis failed to identify the bacterium (Table 4).

Table 2
Additional Characteristics of NCIMB 10462

Gram morphology	Small gram-negative rod, approx 0.5 μm
Motility	Motile at 25 and 37°C
Starch hydrolysis (amylase)	Positive for starch hydrolysis
β-Galactosidase	Positive
β-Glucosidase	Positive
Optimal growth temperature (complex media)	25–30°C
Optimal pH (complex media)	pH 7.2–8.0
Pigment production	Green pigment in 500-L culture, which was not observed in smaller cultures
Anaerobic Growth (37°C)	
Blood agar	Positive (slow growth)
Trypticase soy broth	Positive (slow growth)
CMC liquid medium	Negative
Filter paper liquid medium	Negative
Aerobic growth on cellulosic media[a]	
CMC	Positive (solid and liquid)
Avicel	Positive (solid and liquid)
Filter paper	Positive (solid media)
Cellobiose	Positive (solid media)
Newspaper	Positive (liquid media)
Cellulose acetate	Positive (liquid media)
Nitrocellulose	Negative (liquid media)
Polyacrylamide	Negative (liquid media)

[a]Standing or shake cultures at 30 or 37°C.

Table 3
Additional Characteristics of NCIMB 10462

Indole production (tryptophane metabolism)	Negative
N-acetyl glucosaminidase	Positive
α-Glucosidase	Positive
α-Arabinosidase	Positive
β-Glucosidase	Positive
α-Fucosidase	Negative
Phosphatase	Negative
α-Galactosidase	Positive
β-Galactosidase	Negative on An-Indent[a]
Indoxyl-acetate hydrolysis	Positive
Arginine utilization	Negative
Leucine aminopeptidase	Positive
Proline aminopeptidase	Positive
Pyroglutamic acid arylamidase	Negative
Tryosine aminopeptidase	Negative
Arginine aminopeptidase	Positive
Alanine aminopeptidase	Positive
Histidine aminopeptidase	Positive (weak)
Phenylalanine aminopeptidase	Positive
Glycine aminopeptidase	Positive

[a]Colonies of NCIMB 10462 are positive for β-galactosidase when grown on solid medium and tested for enzyme production by X-gal.

Table 4
Results of Biolog Carbon Substrate Utilization Analysis

	Incubation time, h	
	4	24
Substrates[a]		
α-Cyclodextrin	+	+
Dextrin	+	+
Glycogen	+	+
A-acetyl-glucosamine	+	+
Cellobiose	+	+
D-Galactose	+	+
Gentiobiose	+	+
α-D-glucose	+	+
α-Lactose	+	+
Maltose	+	+
D-mannose	+	+
D-melibiose	+	+
D-trehalose	+	+
Furanose	+	−
Glucose-1-phosphate	−	+
Glucose-6-phosphate	−	+
Alanimide	−	+
Thymidine	−	+

[a] All other carbon substrates in the Biolog analysis were not utilized by NCIMB 10462, including: tween-40, tween 80, *N*-acetyl-galactosamine, adonitol, arabitol, erythritol, fructose, fructose, inositol, mannitol, mannose, 6-methyl glucoside, raffinose, rhamnose sorbitol, sucrose, xylitol, methyl pyruvate, mono-methyl succinate, acetic acid, cis-aconic acid, citric acid, formic acid, D-galactouronic acid, D-gluconic acid, D-glucosaminic acid, D-glucuronic acid, α-hydroxybutyric acid, 6-hydroxybutyric acid, γ-hydroxybutyric acid, *p*-hydroxypehnylacetic acid, itaconic acid, α-keto butyric acid, α-ketoglutaric acid, α-keto valeric acid, D, L-lactic acid, malonic acid, proprionic acid, quinic acid, D-saccaric acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, gluconamide, D-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic, glycl-L-aspartic acid, glycyl-L-glucamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglytamic acid, D-serine, L-serine, D-theonine, γ-butyric acid, urocanic acid, inosine, uridine, phenylethylamine, putrescine, 2-amino ethanol, 2,3, butanediol, glycerol, and D, L-glycerol phosphate.

Biolog analysis at 4, 15 (data not shown), and 24 h gave very low probability matches with *Sphingobacterium multivorum, Hemophilus decreyi,* and CDC Group DF-3, respectively.

Growth Studies

Figure 1 shows the growth of NCIMB 10462 at various temperatures. The organism grows optimally at 25–30°C, although good growth is obtained at 37°C. The bacterium dies rapidly after reaching stationary phase when grown at 37°C (data not shown). The organism grows poorly, if at

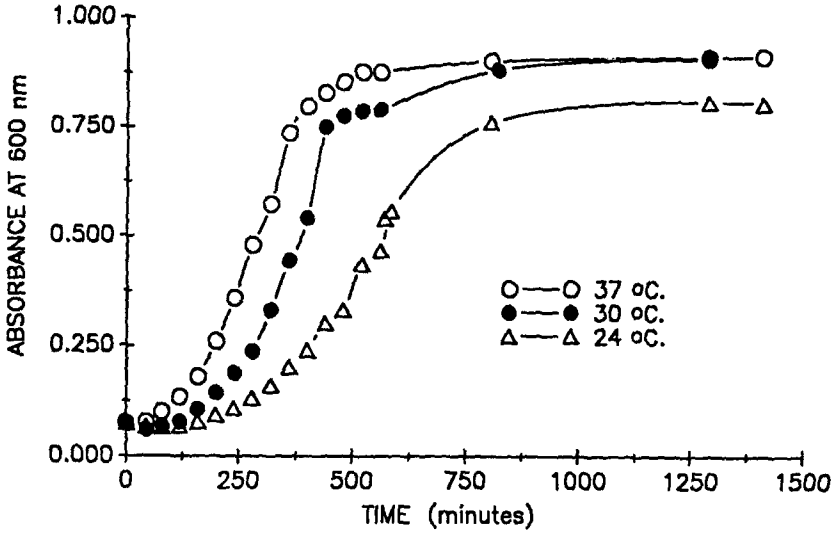

Fig. 1. The effects of temperature on the growth of NCIMB 10462 measured by the increase in absorbance at 600 nm. Bacterium were grown in TSB at pH 7.4.

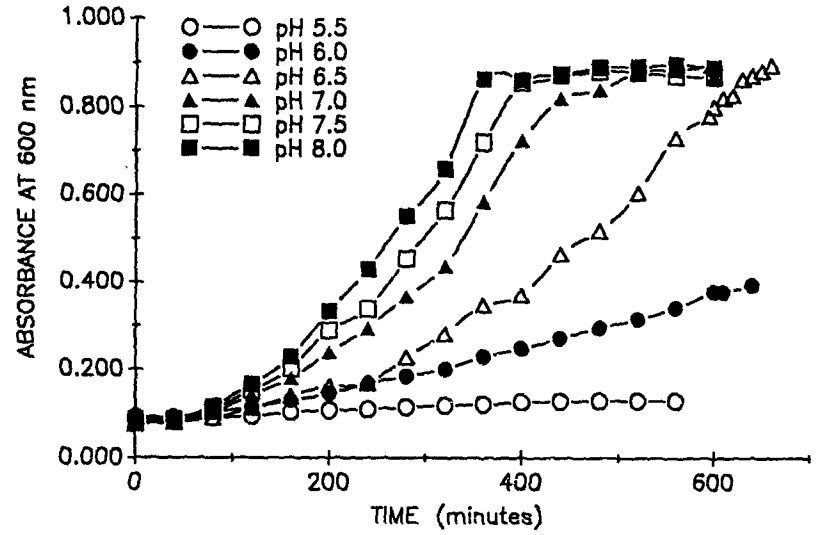

Fig. 2. The growth of NCIMB 10462 in TSB under different pH conditions.

all, at temperatures above 37°C. Therefore, fatty acid analyses were performed at 27, 30, 35, and 37°C to characterize this bacterium taxonomically.

Since our initial examintion revealed that the bacterium obtained from the repository was very different from *P. fluorescens*, we thought it necessary to re-examine the growth of the bacterium in different salt and pH conditions. NCIMB 10462 was found to grow well at pH 7.4–8.0 with growth decreasing after the pH is adjusted below pH 7.0 (Fig. 2). In complex media, NCIMB 10462 reduced the pH of the medium below pH 6.8, whereas in liquid cellulosic medium, the pH does not fall more than 0.3 U

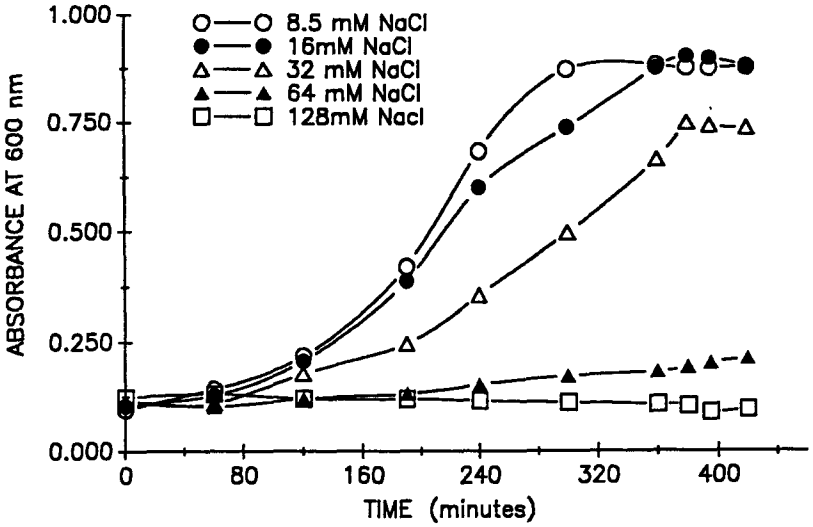

Fig. 3. NCIMB 10462 can tolerate up to 16 m*M* NaCl added to the medium (TSB), but total bacterial growth is reduced at higher salt concentrations.

from the starting pH of 7.2. Figure 3 shows that NCIMB 10462 can tolerate up to 16 m*M* salt in the medium, but concentrations of sodium chloride above 16 m*M* result in reduced bacterial numbers. Direct degradation of cellulosic waste by this organism in salt brines, like those produced in the paper-making process, would require dilution of salt concentration to approx 16 m*M*.

Fatty Acid Analysis

Taxonomic characterization of NCIMB 10462 using MIDI identified the isolate as a pseudomonad when grown at 27 or 30°C with different species being identified at each temperature (Table 5). The analysis failed to produce a match when the isolate was grown at either 35 or 37°C, except for an identification of *Pasteurella* in one of the 35°C replicates. The different identifications can be attributed to membrane lipid changes in response to the different growth temperatures. As the temperature increased, the ratio of palmitoleic (16:1w7c) to vaccenic (18:1w7c) acid also increased.

The total ester-linked phospholipid fatty acids from bacteria at 37°C are shown in Table 6. The profile is comprised, predominantly, of monounsaturated fatty acids (>70%). Of these monounsaturates, the two isomers of vaccenic acid were the most abundant, followed by both isomeric configurations of palmitoleic acid. These acids are formed via the anaerobic desaturase fatty acid biosynthetic pathway, a pathway utilized by a number of gram-negative bacteria, including the *Pseudomonads* (*12,13*). The deletion of trans-fatty acids at the observed levels was unusual, since the synthesis of trans-fatty acids (in *Pseudomonads*) has been associated with the presence of xenobiotic (*14*) or environmental stress (*15*).

Table 5
MIDI Similarity Matches to NCIMB 10462 Grown at Different Temperatures

Growth temp.	Sample #	MIDI identification	Index	
27 °C	27.1	*P. syringae*	0.119	
		P. coronafaciens		0.081
		P. facilis	0.093	
	27.2	*P. syringae*	0.137	
		P. coronafaciens		0.070
		P. facilis	0.087	
30°C	30.3	*P. coronafaciens*		0.034
		P. syringae	0.026	
		P. facilis	0.031	
		P. pseudoflava	0.027	
	30.4	*P. coronafaciens*		0.039
		P. syringae	0.032	
		P. facilis	0.031	
		P. pseudoflava	0.026	
35°C	35.5	*Pasteurella multocida*	0.014	
		P. pseudoflava	0.011	
		Neisseria meningitidis	0.011	
	35.6	No match		
37°C	37.7	No match		
	37.8	No match		

Since the nonfermentatiave analysis suggested that this isolate might be a distantly related member of the genus *Sphingomonas,* we also analyzed for the presence of sphingoid bases and detected none.

DISCUSSION

Many features of NCIMB 10462 make it an attractive candidate for use in fixed-bed bioreactor systems that can be used to degrade cellulose to commercially useful feed stocks or alternative fuels: the bacterium is small, has minimal nutritional requirements, is not fastidious, and continuously secretes cellulase (Dees, C. and Scott, T. C. unpublished data). The growth temperature requirements of the bacterium suggest that a fixed-bed bioreactor system could be run at reduced temperatures (below 30°C) to minimize contamination with other organisms (Fig. 1). The preference of the bacterium for growth and cellulase (Fig. 2). Downstream processing costs of cellulase production would be reduced, since at mild alkaline pH conditions, adherence of the cellulase to filtration membranes

Table 6
Ester-Linked Phospholipid Fatty Acid
Analysis of NCIMB 10462[a]Table Title

Phospholipid-linked fatty acid	Mole Percent
14:1w7c	0
14:08[a]	0.11
15:1w6c	0
15:0	0.09
16:1w7c[c]	5.89
16:1w7t[c]	6.72
16:0	15.81
α17:0/17:1w8c	0.12
17:1w6c	0.05
cy17:0	0.06
17:0[b]	2.09
18:2w6	0.32
18:w9c	0.69
18:1w7c[c]	38.74
18:1w7t[c]	16.53
18:1w5c	0.26
18:0[b]	10.15
cy19:0[b]	2.39
Total	100.00

[a]NCIMB grown at 27°C on TSA.
[b]Structure verified by mass spectral analysis.
[c]Double-bond position verified by mass spectral analysis.

will be reduced. NCIMB 10462 may be useful in the removal of cellulose fibers from salt brine wastes produced during commercial paper-making processes. Because of the potential importance of this bacterium in the conversion of cellulose in a fixed-bed bioreactor, it is important to determine its characteristics and of metabolic capabilities more closely. Therefore, we examined the bacterium by a wide variety of standard bacteriological methods, including assimilation studies and lipid analysis. NCIMB 10462 was found to grow aerobically on a variety of solid and soluble cellulosic materials (Table 2). It also grew in complex medium under reducing conditions and weakly on complex media under anaerobic conditions. No anaerobic growth was observed in the liquid cellulosic medium. Standard bacteriologic identification methods and substrate utilization studies suggested that the bacterium had few characteristics corresponding to *P. fluorescens.*

Total fatty acid patterns are used to identify both anaerobic and aerobic bacteria (*11*). Total fatty acid analysis grouped this bacterium into

the *Pseudomonas* genus (at low similarity indices, <0.150 with 1.0 being a perfect match), but was not consistent with the species identification, indicating *Pseudomonas coronafaciens, Pseudomonas syringae, Pseudomonas facilis,* and *Pseudomonas pseudoflava.* We are performing 16- and 28-s ribosomal RNA fingerprinting in an effort to define more closely the taxonomic position of this bacterium. Preliminary results suggest that this bacterium is a pseudomonad. Until this bacterium's phylogenetic profile is fully established, it could be described by its repository number (NCIMB 10462). However, in view of its ability to degrade cellulose and characteristics identifying it as a pseudomonad, we propose that it be called *Pseudomonas cellulosa.*

SUMMARY

The cellulase-producing bacterium NCIMB 10462 has many characteristics that make it ideal for use in a fixed-bed bioreactor system for the conversion of cellulose to chemical feed stocks or alternative fuel: the bacterium is small, grows under mildly alkalophilic conditions, constitutively produces a highly active cellulase, is not fastidious in its nutritional requirements, and grows well at temperatures below 30°C. The bacterium may also be useful in the bioreduction of cellulosic wastes from commercial paper-making processes. Since its first description in 1952, this bacterium has been designated *Pseudomonas fluorescens* var. or subsp. *cellulosa.* We re-examined the characteristics of this bacterium and found few traits that would characterize it as member of the fluorescent pseudomonads. Substrate-utilization studies, total fatty acid analysis, and preliminary characterization of the ribosomal RNA group this bacterium with the genus *Pseudomonas,* but fail to identify it with a particular species. Since the predominant characteristic of this bacterium is its ability to degrade cellulose, we propose that it be called *Pseudomonas cellulosa.*

ACKNOWLEDGMENTS

We appreciate the use of equipment and materials generously provided by Dr. D. C. White at the University of Tennessee Center for Environmental Biotechnology. We acknowledge Dr. A. Sonnesson's expertise in examining the isolate for the presence of sphingolipid bases. We are also grateful for the assistance with the Biolog analysis provided by Dr. Anthony Palumbo of the Environmental Sciences Division for the Oak Ridge National Laboratory. This research was sponsored by Laboratory Directed Research and Development Fund, United States Department of Energy.

REFERENCES

1. Ueda, K. S. Ishikawa, Itami, T., and Asai, T. (1952), *J. Agr. Chem. Soc. Jpn.* **26,** 35.
2. Yame, K., Suzuki, H., Hirotani, M., Ozawa, H., and Nisizawa, K. (1970), *J. Biochem.* **67,** 9–35.
3. Hall, J. and Gilbert, H. J. (1988), *Mol. Gen. Genet.* **213,** 112–117.
4. Hazlewood, G. P., Laurie, J. I., Ferria, L. M. A., and Gilbert, H. J. (1992), *J. Appl. Bacteriol.* **72,** 244–251.
5. Hong, Y., Pasternak, J. J., and Glick, B. R. (1990), *Curr. Microbiol.* **20,** 339–342.
6. Wolff, B. R., Lewis, D., Pasternak, J. J., and Glick, B. R. (1990), *J. Ind. Microbiol.* **5,** 59–64.
7. Osterhout, G. J., Schull, V. H., and Dick, J. D. (1991), *J. Clin. Microbiol.* **29,** 1822–1830.
8. Ikuya, Y., Ikuko, T., and Eiko, Y. (1982), *FEMS Microbiol.* **15,** 303–307.
9. Akihiro, Y., Ikuya, Y., Masamiki, M., and Eiko, Y. (1978), *J. Biochem.* **83,** 1213–1216.
10. Sonnesson, A., Moll, H., Jantzen, E., and Zähringer, U. (1993), *FEMS Microbiol.* **106,** 315–320.
11. Mills, C. K., Kefauver, M. B., and Gherna, R. L. (1989), Abstracts of the 89th Annual Meeting of the American Society for Microbiology, p. 283.
12. Hamwood, J. I. and Russell, N. J. (1984), in *Lipids in Plant and Microbes.* George Allen, p. 78.
13. Rutledge, C. and Wilkinson, S. G. eds. (1988), *Microbial Lipids,* vol. 1, Academic, pp. 333–339.
14. Heipieper, H. J., Piefenbach, K., and Keveloh, H. (1992), *Appl. Environ. Microbiol.* **88,** 1847–1852.
15. Guekat, J. B., Ringelberg, D. B., and White, D. C. (1987), *Can. J. Microbiol.* **88,** 749–754.