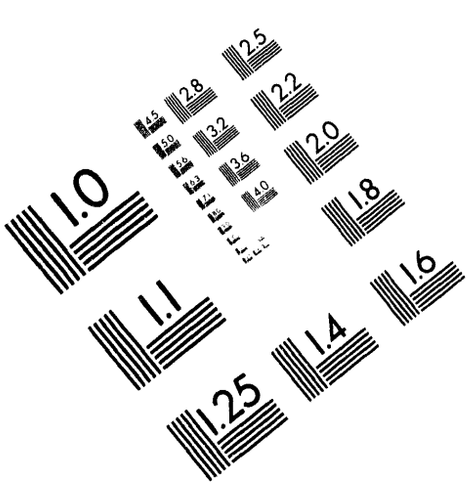

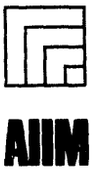

AIIM

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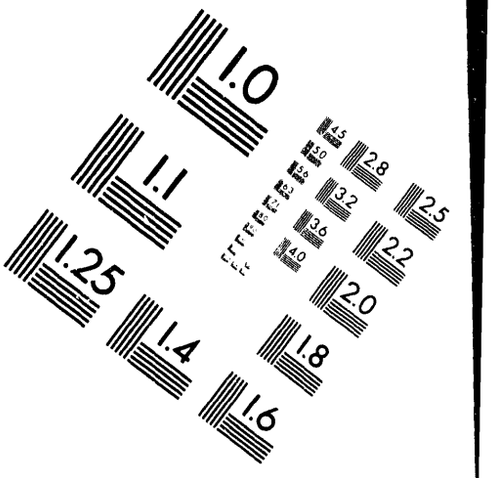

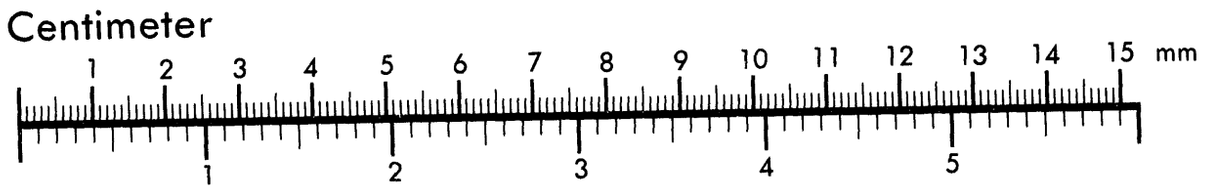

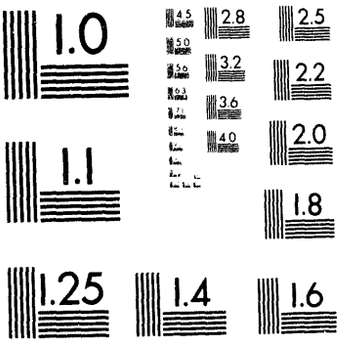

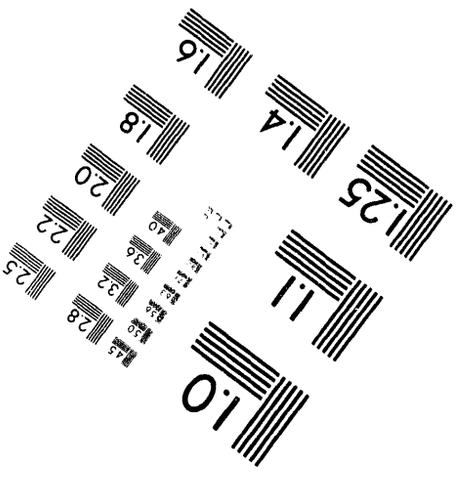

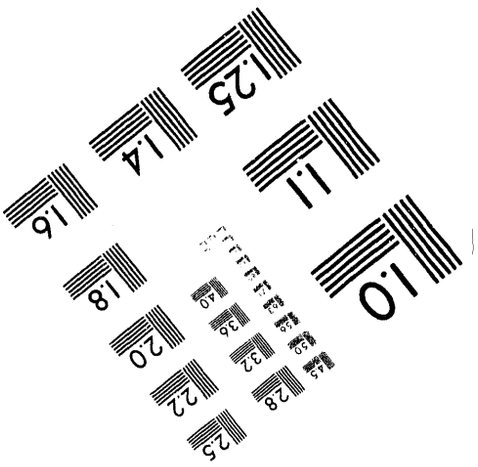

TOC

Taxonomic Characterization of the Cellulose-Degrading Bacterium NCIB 10462

C. Dees[1], D. Ringleberg[4], T.C. Scott[2] and T. Phelps[3]

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Key Words: cellulase, Sphingomonas, Pseudomonas fluorescens subsp. cellulosa

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

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ABSTRACT

The gram negative cellulase-producing bacterium NCIB 10462 has been previously named *Pseudomonas fluorescens* subsp. or var. *cellulosa*. Since there is renewed interest in cellulose-degrading bacteria for use in bioconversion of cellulose to chemical feed stocks and fuels, we re-examined the characteristics of this microorganism to determine its proper taxonomic characterization and to further define it's true metabolic potential. Metabolic and physical characterization of NCIB 10462 revealed that this was an alkalophilic, non-fermentative, gram negative, oxidase positive, motile, cellulose-degrading bacterium. The aerobic substrate utilization profile of this bacterium was found to have few characteristics consistent with a classification of *P. fluorescens* with a very low probability match with the genus Sphingomonas. Total lipid analysis did not reveal that any sphingolipid bases are produced by this bacterium. NCIB 10462 was found to grow best aerobically but also grows well in complex media under reducing conditions. NCIB 10462 grew slowly under full anaerobic conditions on complex media but growth on cellulosic media was found only under aerobic conditions. Total fatty acid analysis (MIDI) of NCIB 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 it's ability to degrade cellulose, we suggest it be called *Pseudomonas cellulosa*.

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INTRODUCTION

The importance of microorganisms that are capable of producing cellulases has increased with new demands for industrial processes to degrade or modify cellulosic biomass. The enzymes produced by these microorganisms are not only important in conversion of cellulosic waste to commercially useful feed stocks, but also to degrade cellulose and hemi-cellulose to materials 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 NCIB 10462 (1). This organism 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 (2,3,4,5). The bacterium isolated from soil in the early 1950's was placed in the National Council for Industrial Bacteriology (NCIB) repository but is now listed as a Pseudomonas species. Interest in the study of NCIB 10462 has recently increased (3,4,5,6) along with the recent upsurge of interest in biotechnological conversion of cellulose. Due to the new importance of this bacterium as a candidate for commercial biomass conversion, we re-examined the metabolic and physical properties of NCIB 10462 using growth, substrate utilization studies, total fatty acid analysis and examination of the total lipids.

MATERIALS AND METHODS

Source of Microorganism

The cellulase degrading bacterium was obtained from the National Council of Industrial Bacteriology repository. The microorganisms' catalog number NCIB 10462 is listed as *Pseudomonas fluorescens* subsp. *cellulosa*. Two separate cultures of this bacterium were obtained from the repository and examined in this study.

Culture Conditions.

NCIB 10462 was maintained on solid media consisting of M9 liquid medium (1) to which 15 grams per liter agarose had been added along with 0.1% carboxymethyl cellulose (CMC) as the sole carbon source. Cellulosic media consisted of a M9 salt solution as previously described (4). Agar was added to

15 grams per liter for solid media along with soluble cellulosic components (e.g. carboxymethyl cellulose, CMC) to 0.1% (w/v). Cellulosic liquid media 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 temperature for growth were performed in Trypticase soy broth or, for the MIDI analyses, on Trypticase soy agar plates with a 24 hour incubation period as previously described (7).

Substrate Utilization Assays

All bacteriologic assays and growth procedures were performed using standard bacteriology techniques, methods and medias. Non-fermentative analysis was performed using an API non-fermentor 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).

Lipid Analyses

Isolation of sphingoid bases was achieved with minor modifications to the assays developed previously (8, 9). Acetyl chloride was used to make the 2N 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 hours. 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. (1990) (10). The absence of any sphingoid bases was determined by analyses of the lipid extract on a GC/MS with positive electron impact ionization at 70 eV. The GC column was held at an initial temperature of 80 °C for 2 min. 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 0.10df.

RESULTS

Substrate Utilization Studies

Tables 1, 2 and 3 show that by substrate utilization studies and non-fermentative analysis that NCIB 10462 exhibits few characteristics that are typically associated with *P. fluorescens*. In contrast, 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. These studies show that this bacterium is not closely related to the genus Pseudomonas but instead has a very low probability match with the genus Sphingomonas.

Consistent with our other attempts to classify the organism using metabolic studies, Biolog analysis failed to identify the bacterium (Table 4). Biolog analysis at 4, 15 (data not shown) and 24 hours gave very low probability matches with *Sphingobacterium multivorum*, *Hemophilus ducreyi* and CDC Group DF-3 respectively.

Growth Studies

Figure 1 shows the growth of NCIB 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 (not shown). The organism grows poorly, if at all, at temperatures above 37 °C. No growth of the organism has been observed at 45 °C. Therefore, fatty acid analyses were performed at 27, 30, 35 and 37 °C in our efforts to taxonomically characterize this bacterium.

Since our initial examination revealed that the bacterium obtained from the repository was so different from *P. fluorescens*, we thought it necessary to re-examine the growth of the bacterium in different salt and pH conditions. NCIB 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 NCIB 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 from the starting pH of 7.2. Figure 3 shows that NCIB 10462 can tolerate up to 16 mM salt in the medium but

concentrations of salt above 16 mM results in reduced bacterial numbers. 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 approximately 16 mM.

Fatty Acid Analysis

Taxonomic characterization of NCIB 10462 using the total fatty acid profile (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 total ester-linked phospholipid fatty acids from bacteria at 37 °C are shown in Table 6. 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.

Since the non-fermentative 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

Due to renewed interest in the cellulose-degrading bacteria which can be used for biomass degradation and for the conversion of cellulosic materials to alternative fuels, it is important to fully understand the characteristics of bacteria and fungi that may be useful to these processes. One such bacterium is the cellulose-degrading bacterium NCIB 10462. This bacterium has been described as a *Pseudomonas fluorescens* subsp. *cellulosa* since its original isolation by Ueda in 1952 and its subsequent deposition in the NCIB repository.

Many features of NCIB 10462 make it an attractive candidate for use in fixed-bed bioreactor systems that can be used to degrade cellulase to commercially useful feedstocks or alternative fuels. The bacterium is small; has minimal nutritional requirements; not fastidious; and secretes a highly active cellulase. The growth temperature requirements of the bacterium also suggest that a fixed-bed bioreactor system using this bacterium 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 production under mildly alkalophilic conditions also make it attractive as a commercial producer of cellulase (Fig. 2). Down-stream processing costs of cellulase production would be reduced since at mild alkaline pH conditions adherence of the cellulase to filtration membranes will be reduced. This bacterium may also have some usefulness in removal of cellulose fibers from salt brine wastes produced during commercial paper-making processes. Due to the potential importance of NCIB 10462 in the biomass conversion of cellulose in a fixed-bed bioreactor, it is important to more closely determine the characteristics of this bacterium. Therefore, we examined the bacterium by a wide variety of standard bacteriological methods including assimilation studies and lipid analysis. NCIB 10462 was found to grow aerobically on a wide variety of solid and soluble cellulosic materials. NCIB 10462 grew in complex medium under reducing conditions and weakly on complex media under full anaerobic conditions. No anaerobic growth was observed in liquid cellulosic medium. Standard bacteriologic identification methods and substrate utilization studies suggested that the bacterium had few characteristics corresponding to the genus Pseudomonas and even less resemblance to a particular member of the genus *P. fluorescens.*

Since aerobic substrate utilization studies suggested that this bacterium might have a very low probability match to the genus Sphingomonas, we examined the lipids produced by this bacterium with a special interest in the production of sphingolipid. The lipid profile of this bacterium is unremarkable and is comprised of fatty acids indicative of a number eubacteria, specifically those utilizing anaerobic desaturation for fatty acid synthesis. Since no sphingolipid bases were detected, it cannot be properly classified in the genus Sphingomonas.

Total fatty acid patterns have been 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 *P. coronafaciens*, *P. syringae, P. facilis* and *P. pseudoflava.* We are performing 16 and 28s ribosomal RNA fingerprinting in an effort to more closely define 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 (NCIB 10462). However in view of its ability to degrade cellulose and characteristics identifying it as a pseudomonad, we propose that it is called *Pseudomonas cellulosa.*

SUMMARY

The cellulase-producing bacterium NCIB 10462 has many characteristics that make it ideal for use in a fixed-bed bioreactor system for the conversion of cellulose to chemical feedstocks or alternative fuel. The bacterium is: small; mildly alkalophilic; constituatively 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 it's 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.*

ACKNOWLEDGEMENTS

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Table 1. Metabolic and Physical Characteristics of NCIB 10462

Test	P. fluorescens	NCIB 10462
Growth on Blood Agar	+	+
Growth on McConkys Agar	+	-
Growth in Pseudomonas M10 Medium	+	-
Catalase	Strong Positive	Weak Positive
Growth in Thioglycollate	Aerobic	Anaerobic
Fluorescent	+	-
Oxidase	+	+
Tryptophanase	-	-
Argenine dihydrolase	+	-
Urease	-	-
Esculin	-	+
Gelatinase	+	-
PNGP	-	+
Glucose	+	+
Arabinose	+	+
Mannose	+	-
n-Acetyl-Glucosamine	+	+
Maltose	-	+
Gluconate	+	-
Caprate	+	-
Adipate	+	-
Malate	+	-
Phenylacetate	±	-

Table 2. Additional Characteristics of NCIB 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
Anaerobic Growth (37 °C.)	
Blood Agar	Positive
Trypticase Soy Broth	Positive
CMC Liquid Medium	Positive
Filter Paper Liquid Medium	Positive
Aerobic Growth on Cellulosic Media*	
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)

* Standing or shake cultures at 30 or 37 °C.

Table 4. Results of Biolog Carbon Substrate Utilization Analysis

	Incubation Time (Hours)	
	4	24
Substrates*		
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	-	+

* All other carbon substrates in the Biolog analysis were not utilized by NCIB 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-hydroxyphenylacetic acid, itaconic acid, α-keto butyric acid, α-ketoglutaric acid, α-keto valeric acid, D, L lactic acid, malonic acid, propionic acid, quinic acid, D-saccharic acid, sebacic acid, succinic acid, bromosuccinic acid, succinamic acid, glucuonamide, D-alanine, L-alanylglycine, L-asparagine, L-aspartic acid, L-glutamic, glycyl-L-aspartic acid, glycyl-L-glutamic acid, L-histidine, hydroxy-L-proline, L-leucine, L-ornithine, L-phenylalanine, L-proline, L-pyroglytamic acid, D-serine, L-serine, L-theonine, γ-butyric acid, urocanic acid, inosine, uridine, phenylethylamine, putrescine, 2-amino ethanol, 2,3, butanediol, glycerol, and D, L, glycerol phosphate.

Table 3. Additional Characteristics of NCIB 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-Ident*
Indoxyl-acetate hydrolysis	Positive
Argenine Utilization	Negative
Leucine aminopeptidase	Positive
Proline aminopeptidase	Positive
Pyroglutamic acid arylamidase	Negative
Tyrosine aminopeptidase	Negative
Argenine aminopeptidase	Positive
Alanine aminopeptidase	Positive
Histidine aminopeptidase	Positive (weak)
Phenylalanine aminopeptidase	Positive
Glycine aminopeptidase	Positive

*Colonies of NCIB 10462 are positive for β-galactosidase when grown on solid medium and tested for enzyme production by X-gal.

Table 5. MIDI similarity matches to NCIB 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		

Table 6. Ester-linked Phospholipid Fatty Acid Analysis

Phospholipid-linked Fatty Acid	Mole Percent
14:1w7c	0
14:0*	0.11
15:1w6c	0
15:0	0.09
16:1w7c**	5.89
16:1w7t**	6.72
16:0	15.81
17:0/17:1w8c	0.12
17:1w6c	0.05
cy17:0	0.06
17:0*	2.09
18:2w6	0.32
18:w9c	0.69
18:1w7c**	38.74
18:1w7t**	16.53
18:1w5c	0.26
18:0*	10.15
cy19:0*	2.39
Residue 1 2OH12:0	0
Total	100.00

*Structure verified by mass spectral analysis.
**Double bond position verified by mass spectral analysis.

Figure Legends

Figure 1 shows the effects of temperature on the growth of NCIB 10462 measured by the increase in absorbance at 600 nm or colony forming units.

Figure 2 shows the growth of NCIB 10462 under different pH conditions.

Figure 3 shows that NCIB 10462 can tolerate up to 16 mM NaCl added to the medium, but total bacterial growth is reduced at higher salt concentrations.

Figure 1.

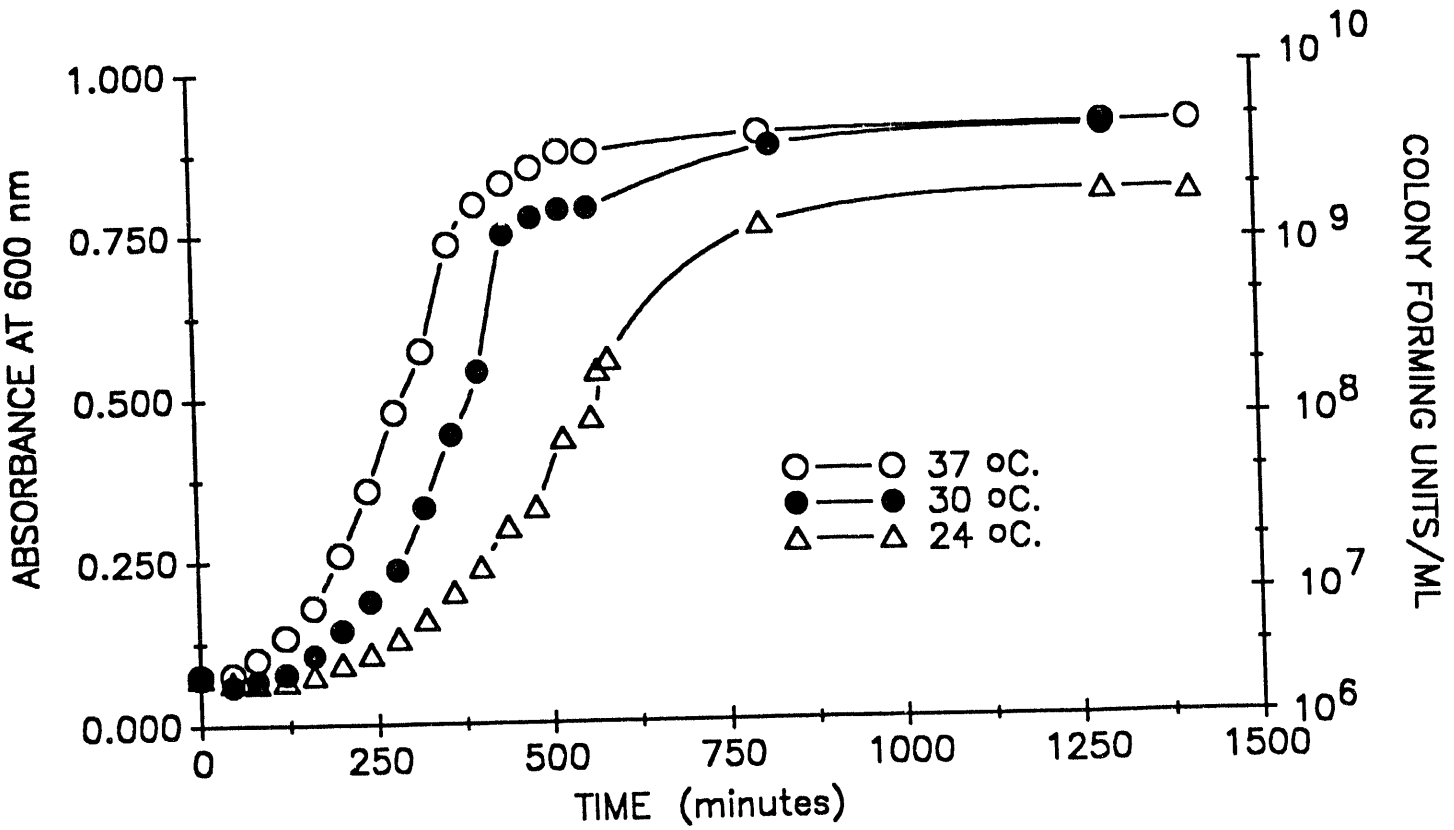

1.000
0.750
0.500
0.250
0.000
ABSORBANCE AT 600 nm
0
250
500
750
1000
1250
1500
TIME (minutes)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
COLONY FORMING UNITS/ML
37 oC.
30 oC.
24 oC.

FIGURE 2.

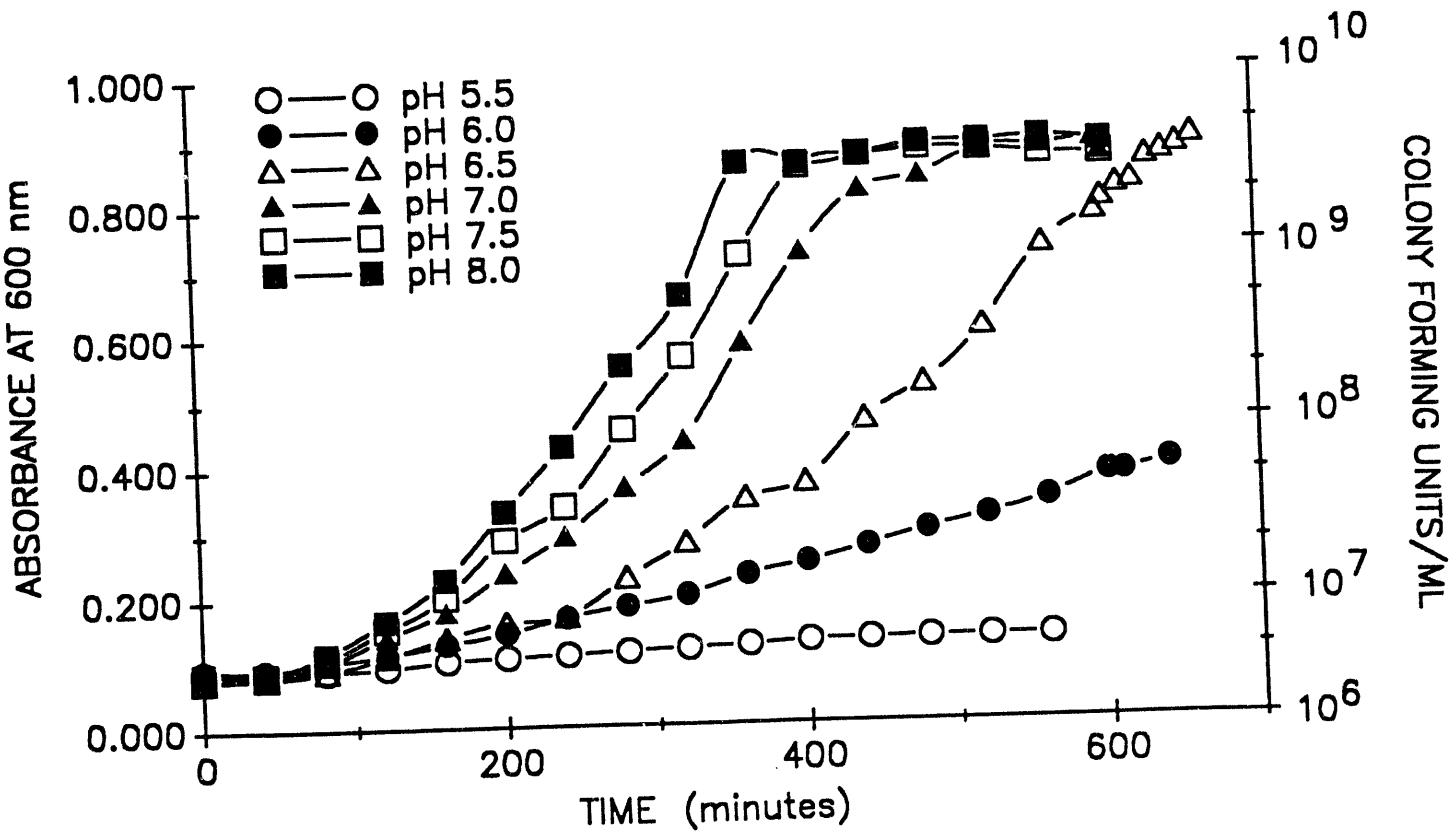
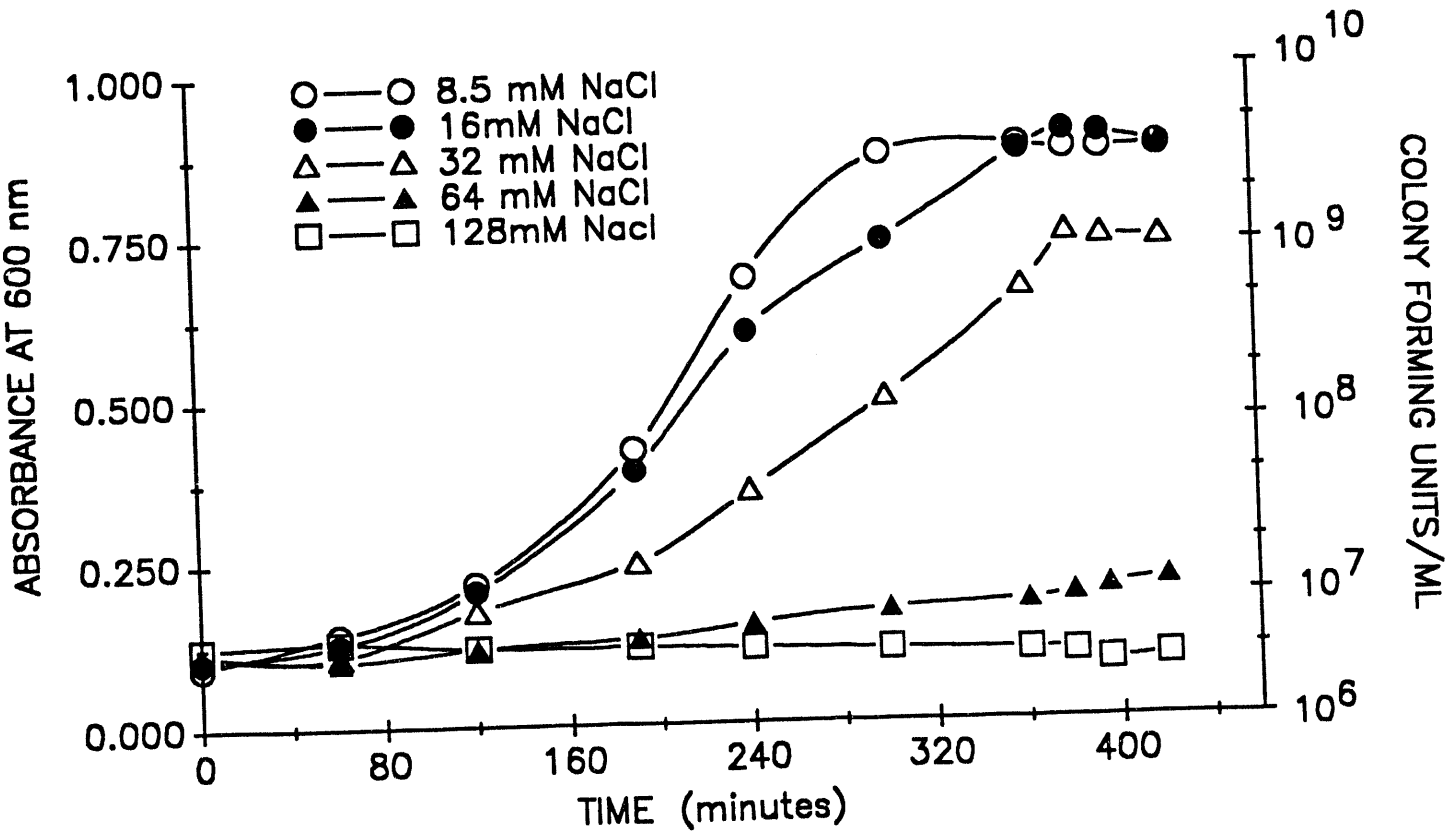

1.000
0.800
0.600
0.400
0.200
0.000
ABSORBANCE AT 600 nm
pH 5.5
pH 6.0
pH 6.5
pH 7.0
pH 7.5
pH 8.0
0
200
400
600
TIME (minutes)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
COLONY FORMING UNITS/ML

FIGURE 3.


8.5 mM NaCl
16mM NaCl
32 mM NaCl
64 mM NaCl
128mM Nacl
ABSORBANCE AT 600 nm
1.000
0.750
0.500
0.250
0.000
0
80
160
240
320
400
TIME (minutes)
10^{10}
10^{9}
10^{8}
10^{7}
10^{6}
COLONY FORMING UNITS/ML

DATE
FILMED
7/21/94
END