J. Microbiol. Biotechnol. (2000), **10**(5), 643–650

JOURNAL OF
MICROBIOLOGY
AND
BIOTECHNOLOGY

Physiological and Phylogenetic Analysis of *Burkholderia* sp. HY1 Capable of Aniline Degradation

KAHNG, HYUNG-YEEL[1], JEROME J. KUKOR[1], AND KYE-HEON OH[2]*

[1]*Biotechnology Center for Agriculture and Environment, Rutgers University, New Brunswick, New Jersey 08901-8520, U.S.A.*
[2]*Department of Life Science, Soonchunhyang University, Chung-Nam 336-600, Korea*

Received: May 6, 2000
Accepted: August 17, 2000

Abstract A new aniline-utilizing microorganism, strain HY1 obtained from an orchard soil, was characterized by using the BIOLOG system, an analysis of the total cellular fatty acids, and a 16S rDNA sequence. Strain HY1 was identified as a *Burkholderia* species, and was designated *Burkholderia* sp. HY1. GC and HPLC analyses revealed that *Burkholderia* sp. HY1 was able to degrade aniline to produce catechol, which was subsequently converted to *cis,cis*-muconic acid through an *ortho*-ring fission pathway under aerobic conditions. Strain HY1 exhibited a drastic reduction in the rate of aniline degradation when glucose was added to the aniline media. However, the addition of peptone or nitrate to the aniline media dramatically accelerated the rate of aniline degradation. A fatty acid analysis showed that strain HY1 was able to produce lipids 16:0, 16:0 2OH, and 11 methyl 18:1 ω7c approximately 3.7-, 2.2-, and 6-fold more, respectively, when grown on aniline media than when grown on TSA. An analysis of the 16S rDNA sequence revealed that strain HY1 was very closely related to *Burkholderia graminis* with 95% similarity based on the alignment of a 1,435 bp fragment. A phylogenetic analysis of the 16S rDNA sequence based on a 1,420 bp multi-alignment showed that strain HY1 was placed among three major clonal types of β-Proteobacteria, including *Burkholderia graminis*, *Burkholderia phenazinium*, and *Burkholderia glathei*. The sequence GAT(C or G)G, which is highly conserved in several locations in the 16S rDNA gene among the major clonal type strains of β-Proteobacteria, was frequently replaced with GAT(C or G)A in the 16S rDNA sequence from strain HY1.

Key words: Aniline degradation, *Burkholderia*, phylogenetic analysis, fatty acids

Microbial degradation of aniline is of interest to environmental microbiologists because of the potentially serious environmental problems that may arise from aniline's ability to undergo a variety of chemical transformations. These transformations include the production of environmentally non-extractable humic acid-like compounds or persistent xenobiotics such as azobenzenes [11], azoxybenzene [13], nitrobenzene [9], and triazenes [16]. *Alcaligenes faecalis* [24], *Nocardia* sp. [3], *Moraxella* sp. [10, 26], *Rhodococcus erythropolis* [23], *Frateuria* sp. [2], *Pseudomonas putida* [15], *Pseudomonas acidovorans* [13], *Desulfobacterium anilini* [22], and *Achromobacter* sp. [10] have all been isolated based on their ability to utilize aniline as a sole source of carbon and nitrogen. However, only two aniline dioxygenase genes, originating from *Pseudomonas* species, have been cloned and characterized [6, 7]. Thus, detailed information about the phylogenetic diversity of aniline catabolism is very limited. Accordingly, in an effort to obtain more information about the diversity of aniline degradation, several aniline-degrading bacteria were isolated from soil using enrichment culture techniques. Strain HY1 was selected for its ability to rapidly degrade aniline. This report presents the physiological and phylogenetic characterization of strain HY1.

Materials and Methods

Aniline Enrichment Culture, Media, and Culture Conditions

Soil samples were taken from orchards in Cheju, Korea. Ten grams of each soil sample were incubated at 180 rpm in 100 ml of 0.9% sodium chloride solution at 30°C for 2 h. One milliliter of the supernatant was then transferred to liquid media containing 1 mM aniline for establishing an enrichment culture. The enrichment cultures were incubated at 30°C. Serial dilutions of each enrichment culture (10^{-1} to 10^{-3}) were transferred to a solid aniline medium containing 1 mM aniline. Thereafter, the fast-growing colonies of aniline-utilizing microorganisms were screened. Strain HY1 was isolated from one such aniline

*Corresponding author
Phone: 82-41-530-1353; Fax: 82-41-530-1353;
E-mail: kyeheon@sch.ac.kr

enrichment culture and used for the physiological and phylogenetic analyses in this study. Strain HY1 was routinely maintained on a TNA medium [18] containing 1 mM aniline owing to its tendency to lose its aniline-degrading ability during cultivation in complex media. For the aniline oxygenase assays and measurement of the aniline degradation rates, cells were grown in an aniline minimal medium (pH 6.8) that contained 1 g K_2HPO_4, 1 g KH_2PO_4, 1.39 g KCl, 1 g $MgSO_4$, 0.1 g $CaCl_2$, and 1 g $MgCl_2$ per liter.

Morphological, Physiological and Biochemical Tests for Aniline-Utilizing Microorganisms

Early log-phase cells from cultures grown at 30°C were used for the morphological, physiological, and biochemical tests. The morphological characteristics were tested by a Gram stain [8]. The cell motility was checked on motility media [4], by observing the number and morphology of the flagella using Mayfield and Inniss' method [14]. Other physiological and biochemical characteristics were tested according to protocols found in Methods for General and Molecular Bacteriology [8]. A GN2 MicroPlate™ (BIOLOG, Hayward, CA, U.S.A.) was used to characterize strain HY1 based on substrate utilization profiling.

Extraction and Analysis of Total Cellular Fatty Acids

Cells harvested following 24 h growth on a tryptic soy agar (TSA) were heated with NaOH-methanol to saponify the cellular lipids, and then the released fatty acids were methylated by heating with HCl-methanol. The fatty acid methyl esters (FAMEs) were solvent-extracted and analyzed by GC-FID (gas chromatography with flame ionization detection) and GC-MS (gas chromatography-mass spectrometry). The FAMEs were identified by comparing their retention times and mass spectra with those of the authentic standards.

Determination of Aniline Degradation Rate and Release of NH_4^+ Ion

Cells were grown in a minimal medium containing 1 mM aniline and incubated at 30°C with shaking for 48 h. Every 4 h, 1 ml of the culture was withdrawn and used to determine the cell density (A_{600}) as well as the aniline concentration determinations. To measure the amount of aniline remaining in the medium, the sample was centrifuged to remove the cells and the supernatant was analyzed at 280 nm by UV spectrophotometry. One milliliter of the culture was also withdrawn and vigorously mixed with 1 ml of cold methanol, and then centrifuged at 4°C for 10 min. The resulting supernatants were carefully transferred to autosampler vials and analyzed by high performance liquid chromatography (HPLC) and gas chromatography (GC). Reverse-phase HPLC was performed with a PhaseSep H4726 column (4.6×250 mm) filled with Spherisorb ODS2 (particle diameter, 5 μm) proceded by a Whatman CSKI guard column (6.5×065 mm) coupled to a Shimadzu SCL-6B solvent delivery system and a CR501 chromatopac computing integrator. A methanol-water (90:10) solvent was used at a flow rate of 1 ml/min. The analytes were detected by monitoring at A_{280} and the concentration was calculated by a comparison with a standard curve. A GC analysis coupled with flame ionization detection was performed on a Varian Star 3500 (Chromatography Systems, Walnut, CA, U.S.A.). The GC was installed with a Rtx-5 silica column crossbonded with 5% diphenyl and 95% dimethylpolysiloxane (30 m×0.53 mm inner diameter; Restek Corporation, Bellefonte, PA, U.S.A.). The oven temperature was programmed at 40°C for 6 min, followed by a linear increase of 10°C per min to 300°C, and thereafter the temperature was held for 15 min. The injector and detector temperatures were maintained at 300°C. Nitrogen was used as a carrier gas. The amount of NH_4^+ ion released from aniline was determined at 630 nm using an indophenol blue reaction [1, 5]. The catechol 1,2- and 2,3-dioxygenase activities were assayed by the procedure of Aoki [2] and Nozaki [17], as well as by spraying an aqueous 0.3% catechol solution onto cells grown on aniline minimal medium plates as described previously [19].

Measurement of Oxygen Consumption Rates

Cells were grown in 500 ml of an aniline minimal medium for 48 h with shaking, collected by centrifugation, and then resuspended in a 50 ml of 50 mM sodium phosphate buffer (pH 7.0). Simultaneous induction experiments were carried out with a Clark-type oxygen electrode (Rank Brothers, Cambridge, U.K.). The electrode was kept at 30°C. The well of the oxygen electrode was filled with 2.8 ml of an aerated 50 mM sodium phosphate buffer (pH 7.0). One-hundred microliter of the resuspended cells adjusted to a final protein concentration of 1.0 or 5.0 mg/ml was used for the assay. The rate of oxygen consumption was first measured in the absence of the substrate for 5 min, and subsequently monitored for 30 min in the presence of 100 μl of aniline (final concentration, 1.0 mM) or 100 μl of catechol (final concentration, 3.0 mM). The oxygen consumption attributable to aniline or catechol utilization was then calculated by subtracting the amount of oxygen consumption in the absence of the substrate from the amount of oxygen consumption in the presence of substrate. This value was indicated as QO_2. The oxygen consumption experiments were performed three times under identical conditions and the QO_2 values were then determined as the mean of these three values.

Effects of Glucose and Peptone on Aniline Oxygenase Activity

To examine the effect of a secondary carbon source on aniline degradation by strain HY1, 0.05%, 0.1%, and 0.2% glucose-containing aniline minimal media were separately prepared. One-hundred microliter of cells previously grown in a TNB medium containing 1 mM of aniline were added to each medium, which was then incubated at 30°C for

3 days. Every 4 h, a 1 ml sample was taken, mixed with 1 ml methanol, and used for GC analysis. To investigate the influence of a secondary nitrogen source, 0.1%, 0.2%, and 1.0% peptone or nitrate were added separately to the aniline media, and subsequently tested in a similar manner to that described above for secondary carbon sources. These experiments were performed three times under the same conditions and the rate of aniline degradation was then determined as the mean of these three values.

Effect of Other Environmental Factors on Aniline Utilization Activity

To evaluate the effects of environmental factors on aniline oxygenase activity, strain HY1 was examined at different pHs, temperatures, and substrate concentrations. Six aniline minimal media were separately prepared at pHs of 4, 5, 6, 7, 8, and 9. These media were individually inoculated with strain HY1 and then incubated at 30°C for two days. To test the effect of temperature, cells were separately grown at 24°C, 30°C, and 37°C, for two days. A 1 ml sample of each culture was taken, mixed with 1 ml ice-cold methanol, and then centrifuged for 10 min. The resultant supernatant was immediately assayed by both GC and reverse-phase HPLC, and the data obtained was analyzed to determine the rate of aniline degradation and the identity of any metabolic intermediates. These experiments were conducted in triplicate. The aniline oxygenase activity was determined as the mean of the triplicate determinations.

PCR Amplification of 16S rDNA and Sequence Analysis

Total genomic DNA of strain HY1 was purified using a Nucleospin Tissue Kit (Clonetech, Palo Alto, CA, U.S.A.). For PCR amplification, two universal eubacterial primers, 27f (5'-AGAGTTTGATCCTGGCTCAG) and 1522r (5'-AAGGAGGTGATCCA(AG)CCGCA) were used as the sense and antisense primers, respectively. The PCR reactions were run for 1 min at 95°C, cycled 25 times (1 min at 95°C, 1 min at 55°C, 1 min 72°C), and then extended for 10 min at 72°C. The PCR products were inserted into a pGEM-T vector and transformed into *E. coli* JM109 (Promega Co., Madison, WI, U.S.A.). Two-hundred nanogram of the double stranded DNA was used as a template for sequencing together with both 27f and 1522r primers. The nucleotide sequencing was carried out using an ABI 373A automated sequencer. The sequence analysis was performed using the Lasergene software (DNA STAR, Inc., Madison, U.S.A.), along with GCG and BLAST searches of the Genbank database.

Nucleotide Sequence Accession Number

The nucleotide sequences of the 16S rDNA from *Burkholderia* sp. strain HY1 have been deposited in the Genetic Sequence Data Bank (GenBank) under accession number AF210314.

Results and Discussion

Physiological and Biochemical Characterization of Strain HY1

Strain HY1 was selected from several aniline-degrading bacteria for its ability to rapidly degrade aniline under aerobic conditions. Strain HY1 was Gram-negative, rod-shaped, catalase-positive, oxidase-positive, motile, tryptophanase-negative, urease-positive, and positive for citrate, phenylacetate, mannitol, sucrose, fructose, trehalose, L-valine utilization, and was not able to degrade aniline under anaerobic conditions in the presence of nitrate as an electron acceptor. Based on these results, strain HY1 appeared to be similar to the *Burkholderia* species with a 92% confidence based on a comparison with organisms in the API database. The physiological and biochemical characteristics of strain HY1 obtained from the BIOLOG system [25] are listed in Table 1. An analysis of carbohydrate utilization profiles based on the GN2 MicroPlate™ and analysis of the total cellular fatty acids by GC-FID and GC-MS also placed HY1 as a *Burkholderia* species with a greater than 90% confidence. These results were consistent with the data obtained from the analysis of the 16S rDNA sequence from HY1.

Rate of Aniline Degradation and Release of NH_4^+

The GC and HPLC analyses of the aniline cultures revealed that 1 mM aniline was completely degraded to catechol and then to *cis,cis*-muconic acid through an *ortho*-ring fission pathway under aerobic conditions within 36 h by strain HY1 (data not shown). Significant aniline degradation was detected within 6 h after inoculation of the cultures, and then there was a rapid degradation between 30 and 36 h post-inoculation. The concentration of NH_4^+ ions released from aniline was measured every 4 h during the incubation. The amount of NH_4^+ liberated from aniline during the 48 h of incubation increased to a maximum concentration of 170 μM (Fig. 1). However, the catechol produced from the aniline degradation did not increase proportionally to the amount of NH_4^+. This inconsistency most likely resulted from the continuous metabolism of catechol during the aniline degradation. A small catechol peak was only detected in the GC or HPLC analysis at the 24-h time point (data not shown). The catechol 1,2- and catechol 2,3-dioxygenase activity were assayed by spraying a 0.3 M catechol solution onto the cells grown on aniline media. No yellow color developed, thereby suggesting that no meta-cleavage product was produced from catechol. Catechol 1,2-dioxygenase activity of strain HY1 was 0.438 and 0.331, respectively, when catechol or 3-methylcatechol was used as a substrate for the enzyme assay by the procedure of Aoki *et al.* [2]. Moreover, catechol 2,3-dioxygenase activity as evaluated by the Nozaki method [17] was not observed in strain HY1. Therefore, it was concluded that strain HY1 probably utilizes catechol *via* the *ortho*-cleavage pathway to *cis,cis*-muconate.

Table 1. Physiological and biochemical characterization of strain HY1 using the BIOLOG analysis system.[a]

Water	-	β-Hydroxybutyric acid	+
α-Cyclodextrin	-	p-Hydroxyphenylacetic acid	+
Dextrin	ND[b]	γ-Hydroxybutyric acid	-
Glycogen	+	α-Ketobutyric acid	-
Tween-40	+	α-Ketoglutaric acid	ND
Tween-80	+	α-Ketovaleric acid	-
N-Acetyl-D-galactosamine	+	D,L-Lactic acid	+
N-Acetyl-D-glucosamine	+	Malonic acid	-
Adonitol	+	Propionic acid	+
L-Arabinose	+	Quinic acid	+
D-Arabitol	+	D-Saccharic acid	+
Cellobiose	-	Sebacic acid	+
i-Erythritol	-	Succinic acid	+
D-Fructose	+	Bromo-succinic acid	+
L-Fructose	+	Succinamic acid	+
D-Galactose	+	α-D-Glucose	+
m-Inositol	+	α-D-Lactose	-
Lactulose	-	D-Maltose	-
Mannitol	-	D-Mannose	+
D-Mellibiose	-	β-Methyl-D-glucoside	-
D-Psicose	-	D-Raffinose	-
L-Rhamnose	+	D-Sorbitol	+
Sucrose	-	D-Trehalose	-
Turanose	-	Xylitol	+
Methylpyruvate	+	Mono-methylsuccinate	+
Acetic acid	+	*cis*-Aconitic acid	-
Citric acid	+	Formic acid	+
D-Galactonic acid lactone	+	D-Galacturonic acid	+
D-Gluconic acid	+	D-Glucosaminic acid	+
D-Glucuronic acid	+	Glucuronamide	-
α-Hydroxybutyric acid	+	Itaconic acid	-
Alanineamide	+	D-Alanine	+
L-Alanine	+	L-Alanyl-glycine	+
L-Asparaginase	+	L-Aspartic acid	+
L-Glutamic acid	+	Glycyl-L-aspartic acid	-
Glycyl-L-glutamic acid	+	L-Histidine	+
Hydroxy L-proline	+	L-Leucine	+
L-Ornithine	+	L-Phenylalanine	+
L-Proline	+	L-Pyroglutamic acid	+
D-Serine	+	L-Serine	+
L-Threonine	+	D,L-Carnithine	+
γ-Aminobutyric acid	+	Urocanic acid	-
Inosine	+	Uridine	+
Thymidine	-	Phenylethylamine	-
Putrescine	-	2-Aminoethanol	+
2,3-Butanediol	-	Glycerol	+
D,L-α-Glycerol phosphate	-	Glucose-1-phosphate	-
dGlucose-6-phosphate	-	Gentiobiose	-

[a]A single HY1 colony grown on a TSA plate was streaked on BUG agar media containing 5% sheep blood and incubated overnight at 30°C. Cells were suspended in normal saline and inoculated into the GN2 MicroPlate™. After incubation for 20 h, the resulting pattern was read with Biolog's automated Micro-Station™ instrument. [b]ND indicates Not Determined.

Effect of Glucose and Nitrate (and/or Peptone) as a Second Carbon and Nitrogen Source on Aniline Degradation

The addition of glucose as a secondary carbon source to aniline minimal media resulted in a drastic reduction in the aniline degradation rate for strain HY1. The rate of aniline degradation decreased as the amount of glucose increased. In the absence of additional glucose, HY1 completely degraded 1 mM of aniline in 36 h, yet in the presence of

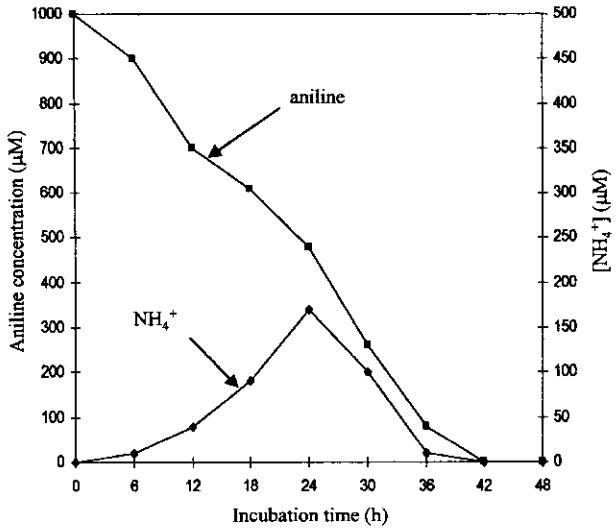

Fig. 1. Aniline degradation under aerobic condition by strain HY1.
Analytes were detected as described in Materials and Methods.

0.2% additional glucose HY1 took 60 h to degrade 1 mM of aniline (Fig. 2). Even greater reductions in the rate of aniline degradation resulted when the aniline media contained 0.5% or more glucose (data not shown). It is not surprising that a reduction in the rate of aniline degradation was observed in aniline media containing 0.5% or more glucose, since strain HY1 demonstrated a type of diauxic response as seen in many other bacteria capable of degrading aromatic compounds. From these results, it would appear that HY1 utilizes glucose first as a nutrient source and then catabolizes aniline. Schukat *et al.* [23] reported that *Rhodococcus* sp. AN117 was able to cometabolize 2- and 3-chloroaniline in the presence of glucose, and that addition of supplemental glucose did not have an inhibitory effect on the rate of aniline degradation. These finding suggest the possibility that aniline catabolism in strain HY1 may be regulated by the mechanisms different from those found in other aniline degrading bacteria.

Aniline can be used as both a carbon and a nitrogen source by strain HY1. To investigate various aspects of nitrogen metabolism in strain HY1, the effect of secondary nitrogen sources on the rates of aniline degradation were investigated. Interestingly, the addition of peptone as a second nitrogen source in aniline minimal media resulted in an acceleration of the aniline degradation rate. The addition of 0.2% peptone resulted in an approximately 1.6-fold higher rate of aniline degradation at the 18-h and 24-h time points for HY1. The rate of aniline degradation was enhanced more by the addition of 0.2% peptone than the addition of 1% peptone (data not shown), thereby suggesting that the rate of aniline degradation is not proportional to the amount of peptone added. The acceleration of the aniline degradation rate in the presence of peptone was unexpected. Peptone is a hydrolyzed protein in which nitrogen is abundant. It is possible that the nitrogen source in peptone had an effect on the rate of aniline degradation by HY1. Since peptone is a mixture of many different substances, it is unclear whether the nitrogen source, or some other component in peptone, was responsible for the elevated rates of aniline degradation. Therefore, the aniline degradation by strain HY1 was tested in the presence of 0.5% nitrate and it was found that nitrate also efficiently accelerated the rate of aniline degradation (Fig. 2). The mechanism through which these secondary nitrogen sources enhanced the aniline degradation by strain HY1 remains to be further investigated.

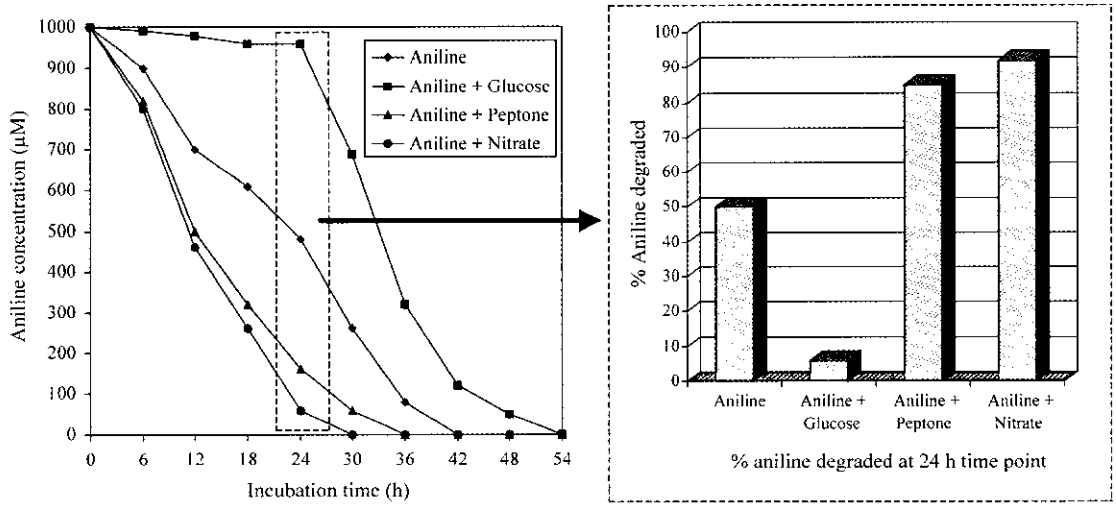

Fig. 2. Effect of secondary carbon and nitrogen sources on aniline degradation.
Rates of aniline degradation over the 54-h time course of the experiment are presented in the panel to the left. In the right-hand panel, the relative percentages of the initial aniline concentration (1 mM) are compared for the 24-h time point.

Oxygen Consumption Rates and Effect of Environmental Factors on Aniline Oxygenase Activity

The rates of oxygen consumption by bacterial cell suspensions grown on aniline were monitored in the presence of aniline and catechol; catechol being the first intermediate produced in the aniline degradative pathway. The rates of oxygen consumption (QO_2) for HY1 were 124.3 nM and 58.2 nM per min when provided with 1 mM aniline and 3 mM catechol, respectively. HY1 was shown to consume much more oxygen in the presence of aniline than catechol (Table 2). In addition, washed resting cells of HY1 that had been previously grown on aniline took up oxygen without a lag when catechol was given as the substrate. These results are consistent with catechol being an intermediate in aniline degradation by strain HY1, and they agree to the observations made on other bacterial isolates capable of degrading aniline under aerobic conditions [10, 13, 23, 24, 27].

Effect of pH

HY1 was separately grown for 24 h on aniline minimal media initially adjusted to pHs of 4, 5, 7, and 9. The cells were collected, washed three times with a 40 mM phosphate buffer (pH 7.0), and then transferred to separate containers to produce an A_{600} of 1.0 in a 10 ml total volume of the buffer. Before the addition of the cells, each of the buffers contained 1 mM aniline and was adjusted to pHs of 4, 5, 7, or 9, respectively. One milliliter samples were taken at 4-h and 24-h time points, and analyzed by GC and HPLC. The aniline oxygenase activity was determined based on the rate of decrease in the aniline concentration. From these experiments, it was determined that there were no significant differences in the rate of aniline degradation among the different pHs (Table 2). Thus, it would appear that the aniline oxygenase in strain HY1 is capable of functioning over a broad pH range.

Effect of Temperature

HY1 was tested on aniline media at 24°C, 30°C, and 37°C. Aside from varying the temperatures, all the enzyme assay procedures were performed at pH 7 and in the same manner as the pH experiments described above. While HY1 exhibited a very weak aniline oxygenase activity at 37°C, and a moderate aniline oxygenase activity at 24°C, the strongest aniline oxygenase activity occurred at 30°C (Table 2). This result indicates that, in the presence of aniline, aniline oxygenase and the related enzymes of the aniline degradative pathway are most active at 30°C or just below 30°C.

Shifts of Total Cellular Fatty Acids of HY1 When Grown on Aniline

The GC-FID and GC-MS analyses showed that the total cellular fatty acids of HY1 were composed of 13 C-even and 7 C-odd fatty acids (fatty acids that comprised less than 0.2% of the total were not considered in this calculation). The predominant lipid 18:1 ω7C made up 38.5% of the total cellular fatty acids when grown on TSA, yet decreased to 10.5% when grown on aniline. Lipid 16:0 made up 7% of the total cellular fatty acids on TSA, yet increased to 27% on aniline, thereby indicating that the total cellular fatty acid composition of HY1 is greatly affected by aniline as a growth substrate (Fig. 3). This is the first report of the effect of aniline on shifts in cellular fatty acid composition for bacteria that can degrade aniline. Shifts in cellular fatty acid composition have been reported previously for other bacterial systems, largely in response to solvent stress or to the presence of other stress agents in the environment. For example, Pinkart *et al.* [20] reported that solvent-tolerant and solvent-sensitive *Pseudomonas putida* strains were able to produce *trans*-unsaturated fatty acids following exposure to *o*-xylene. In addition, Ryu and Jang [21] reported that oxygen and unsaturated fatty acids might make an effect on the ethanol tolerance of yeast strains. Ko *et al.* [12] reported the shifts of fatty acid composition in *Pseudomonas* sp. DJ-12 exposed to benzoate and 4-chlorobenzoate. However, the preceding results differ significantly from our findings in that we have observed an increase in only a few *trans*-unsaturated fatty acids, and we observed an increase in many saturated fatty acids in strain HY1 in response to aniline. These

Table 2. Effect of environmental factors on aniline oxygenase activity and rate of oxygen consumption by strain HY1.

Environmental factors		Aniline oxygenase activity (μmol/h/mg protein)[a]	Oxygen consumption (nmol/min/mg dry weight)		Characteristics[b]
			1 mM Aniline	3 mM Catechol	
pH	4	152.0	124.3	ND	AO[+]/C12O[+]
	7	193.0		58.2	
	9	148.0		ND	
Temperature	24°C	45.0		ND	
	30°C	207.0		Same as pH 7 result	
	37°C	[c]ND		ND	

[a]Values measured following initial incubation period (4 h) represent results for mean concentrations of substrate removal obtained from three independent experiments.

[b]AO and C12O indicates aniline oxygenase and catechol 1,2-dioxygenase, respectively.

[c]ND, not determined (activity levels lower than 30.0 were not calculated).

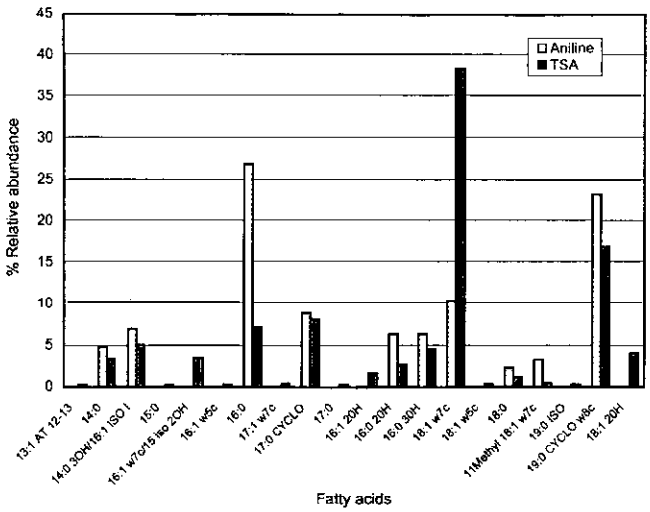

Fig. 3. Fatty acid profiles of strain HY1 analyzed by GC-FID and GC-MS when grown on TSA (■) or aniline (□).
Twenty-four-hour grown cells on TSA or aniline were used for extraction of total cellular fatty acids, and the lipids were identified based on the retention of authentic references.

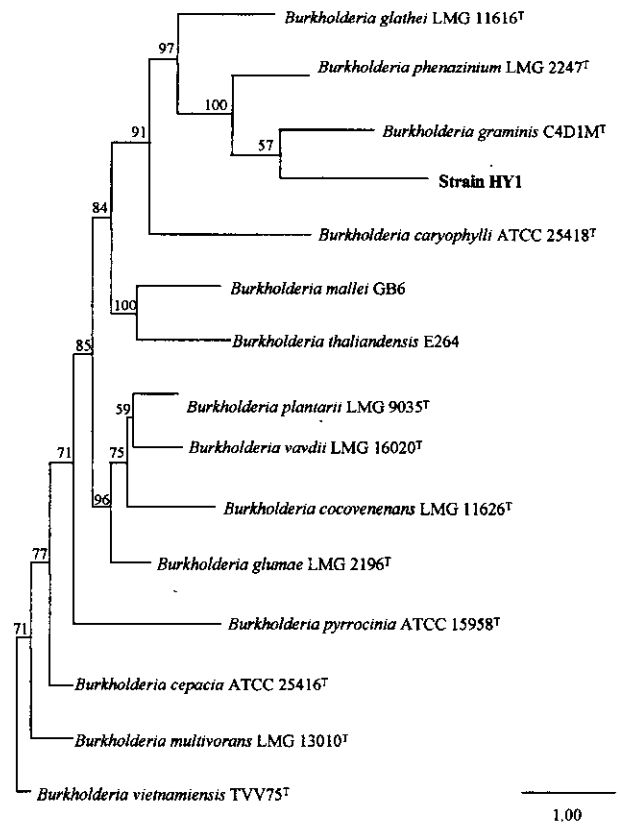

Fig. 4. Phylogenetic tree of strain HY1 and representative organisms of the β-subdivision of the *Proteobacteria*.
To build the tree 1,420 bases were used. Bootstrap values at nodes of the dendrogram indicate the percentage of occurrence of the branching order in 500 bootstrapped trees (only values of 50 or above are shown). The bar scale represents 1 nucleotide substitution per 100 nucleotides.

results suggest the possibility that an increase in saturated fatty acids, such as 14:0, 16:0, and 18:0, might be linked with an extension in membrane permeability allowing for uptake of aniline into the cell. It should be noted that in our system, aniline is used as a carbon and nitrogen source, therefore changes in membrane fatty acid composition in strain HY1 must reflect not only adaptation to the presence of toxic compound in the cell's environment, but also adaptation allowing for the utilization of this substrate as a nutrient and energy source. The specific cellular response mechanisms by which membrane fatty acid shifts allow strain HY1 to adapt to a new environment warrants further intensive study.

Phylogenetic Analysis of Strain HY1

The 16S rDNA of strain HY1 was amplified and sequenced to evaluate its phylogenetic placement of strain HY1. Based on a comparative sequence analysis of the 16S rDNA, HY1 falls within β-Proteobacteria. HY1 is most closely related to three major clonal types of the β-Proteobacteria group, including *Burkholderia graminis*, *Burkholderia phenazinium*, and *Burkholderia glathei* (Fig. 4). There is scant information about these *Burkholderia* species, and nothing is known about the biodegradation of pollutants by these microorganisms. A multi-alignment analysis of the

B. caryophylli ATCC 25418T	224 GACG 227	249 GACG 252	271 GAGA 275	348 GAAG 351	425 GATG 428
B. cepacia ATCC 25416T	220 GACG 223	245 GACG 248	267 GAGA 270	344 GAAG 347	421 GATG 424
B. cocovenans LMG 11626T	226 GACG 229	251 GACG 254	273 GAGA 276	350 GAAG 353	427 GATG 430
B. glatheia LMG 14190T	213 GACG 217	239 GACG 242	261 GAGA 264	338 GAAG 341	415 GATG 418
B. glumae LMG 2197T	223 GACG 226	248 GACG 251	270 GAGA 273	347 GAAG 350	424 GATG 427
B. graminis C4D1MT	227 GACG 230	252 GACG 255	274 GAGA 277	351 GAAG 354	428 GATG 431
B. mallei GB6	247 GACG 250	272 GACG 275	294 GAGA 297	371 GAAG 374	448 GATG 451
B. multivorans LMG 13010T	255 GACG 258	280 GACG 283	302 GAGA 305	379 GAAG 382	456 GATG 459
B. phenazinium LMG 2247T	213 GACG 216	240 GACG 243	260 GAGA 263	337 GAAG 340	412 GATG 415
B. plantarii LMG 9035T	224 GACG 227	249 GACG 252	271 GAGA 274	348 GAAG 351	423 GATG 426
B. pyrrocina ATCC 15958T	253 GACG 256	278 GACG 281	300 GAGA 303	377 GAAG 380	454 GATG 457
B. thaliandensisa E264	247 GACG 250	272 GACG 275	294 GAGA 297	371 GAAG 374	448 GATG 451
B. vandii LMG 16020T	164 GACG 167	189 GACG 192	211 GAGA 214	297 GAAG 300	374 GATG 377
B. vietnamiensis TVV75T	217 GACG 220	244 GACG 247	264 GAGA 267	350 GAAG 353	427 GATG 430
B. avium ATCC 35086T	265 GACG 268	290 GACG 293	312 GAGA 315	398 GATG 401	466 AATG 469
HY1	265 GACA 268	290 GACA 293	312 GAAA 315	398 GAAA 401	466 GATA 469
	*	*	*	*	*

Fig. 5. Multi-alignment of conserved regions within the 16S rDNA genes of *Burkholderia* species related to strain HY1.
The (G--->A) substitutions that occur in strain HY1 are indicated by asterisks.

16S rDNA sequences revealed that a GAT(C, or G)G sequence, which is highly conserved in the major clonal type strains, was frequently substituted to GAT(C, or G)A in several locations of the 16S rDNA sequence from HY1 (Fig. 5). These results suggest the possibility that strain HY1 may have unique characteristics that distinguish it from the majority of *Burkholderia* species.

Acknowledgments

This work was partially supportod by grant P-42-ES-04911 from the National Institute of Environmental Health Sciences Superfund Basic Research Program to JJK.

REFERENCES

1. Allen, S. E., H. M. Grimshaw, J. A. Parkinson, and C. Quarmby. 1974. Inorganic constituents: Nitrogen, pp. 184-206. *In* S. E. Allen (ed.), *Chemical Analysis of Ecological Materials*. Blackwell Scientific Publications, London, U.S.
2. Aoki, K., K. Ohtsuka, R. Shinke, and H. Nishira. 1984. Rapid biodegradation of aniline by *Frateuria* species ANA-18 and its aniline metabolism. *Agric. Biol. Chem.* **48:** 865-872.
3. Bachofer, R. and F. Lingens. 1975. Conversion of aniline *into pyrocatechol by a Nocardia sp.: Incorporation of* oxygen-18. *FEBS Lett.* **50:** 288-290.
4. Edward, D. R. and D. W. Bruner. 1942. Serological identification of *Salmonella* culture. *Circ. Ky. Agric. Exp. Sta.* **110:** 54.
5. Franson, M. A. 1976. *Standard Methods for the Examination of Water and Wastewater*, 14th ed. American Public Health Association, Washington DC, U.S.A.
6. Fujii, T., T. Masahiro, and M. Yoshimichi. 1997. Plasmid-encoded genes specifying aniline oxidation from *Acinetobacter* sp. strain YAA. *Microbiology* **143:** 93-99.
7. Fukumori, F. and C. P. Saint. 1997. Nucleotide sequences and regulational analysis of genes involved in conversion of aniline to catechol in *Pseudomonas putida* UCC22 (pTDN1). *J. Bacteriol.* **179:** 399-408.
8. Gerhart, P., R. G. E. Murray, R. N. Costillow, E. W. Nester, W. A. Wood, N. R. Kreig, and G. B. Phillips. 1981. *Methods for General and Molecular Bacteriology*. American Society for Microbiology. U.S.A.
9. Itoh, N., M. Naoki, and K. Toyoko. 1993. Oxidation of aniline to nitrobenzene by nonheme bromoperoxidase. *Biochem. Molec. Biol. Intl.* **29:** 785-791.
10. Kahng, H.-Y., S.-I. Kim, M.-J. Woo, Y.-K. Park, and Y.-N. Lee. 1992. Isolation and characterization of aniline-degrading bacteria. *Kor. J. Microbiol.* [presently *J. Microbiol.*] **30:** 199-206.
11. Kearney, P. C., J. R. Plimmer, and F. B. Guardia. 1969. Mixed chlorobenzene formation in soil. *J. Agric. Food Chem.* **17:** 1418-1419.
12. Ko, Y.-J., S.-H. Park, Y.-K. Park, and C.-K. Kim. 1999. Responses of *Pseudomonas* sp. DJ-12 to pollutant stresses of benzoate and 4-chlorobenzoate. *J. Microbiol. Biotechnol.* **9:** 422-428.
13. Loidle, M., C. Hinteregger, G. Ditzemuller, A. Ferschl, and F. Streichsbier. 1990. Degradation of aniline and monochlorinated anilines by soil-born *Pseudomonas acidovorans* strains. *Arch. Microbiol.* **155:** 56-61.
14. Mayfield, C. I. and W. E. Inniss. 1977. A rapid method for staining bacterial flagella. *Can. J. Microbiol.* **23:** 1311-1313.
15. McClure, N. C. and W. A. Venables. 1987. PTDN1, a catabolic plasmid involved in aromatic amine catabolism in *Pseudomonas putida* mt-2. *J. Gen. Microbiol.* **133:** 2073-2077.
16. Minard, R. D., S. Russel, and J. M. Bollag. 1997. Chemical transformation of 4-chloroaniline to a triazene in a bacterial culture medium. *J. Agric. Food Chem.* **25:** 841-844.
17. Nozaki, M. 1970. Metapyrocatechase (*Pseudomonas*). *Methods Enzymol.* **17A:** 522-525.
18. Olsen, R. H. and J. Hansen. 1976. Evolution and utility of a *Pseudomonas aeruginosa* drug resistance factor. *J. Bacteriol.* **125:** 837-844.
19. Park, Y.-I., J.-S. So, and S.-C. Koh. 1999. Induction by carvone of the polychlorinated biphenyl (PCB)-degradative pathway in *Alcaligenes eutrophus* H850 and its molecular monitoring. *J. Microbiol. Biotechnol.* **9:** 804-810.
20. Pinkart, H. C., J. W. Wolfram, R. Rogers, and D. C. White. 1996. Cell envelope changes in solvent-sensitive *Pseudomonas putida* strains following exposure to *o*-xylene. *Appl. Environ. Microbiol.* **62:** 1129-1132.
21. Ryu, H.-Y. and H.-W. Jang. 1991. Effect of oxygen and unsaturated fatty acids on the ethanol tolerance of yeast strains. *J. Microbiol. Biotechnol.* **1:** 6-11.
22. Schnell, S. and B. Schink. 1991. Anaerobic aniline degradation *via* reductive deamination of 4-aminobenzoyl-CoA in *Desulfobacterium anilini*. *Arch. Microbiol.* **155:** 183-190.
23. Schukat, B., D. Janke, D. Krebs, and W. Fritsche. 1983. Metabolism of aniline by *Rhodococcus erythropolis* AN-13. *Agric. Biol. Chem.* **47:** 1611-1616.
24. Surovtseva, E. G. and A. I. Volnova. 1972. Aniline as the sole source of carbon, nitrogen, and energy for *Alcaligenes faecalis*. *Microbiologiya* **50:** 49-53.
25. Yoon, S.-H., J.-K. Song, S.-J. Go, and J.-C. Ryu. 1998. Production of biopolymer flocculant by *Bacillus subtilis* TB11. *J. Microbiol. Biotechnol.* **8:** 606-612.
26. Zeyer, J. and P. C. Kearney. 1982. Microbial degradation of *para*-chloroaniline as sole source of carbon and nitrogen. *Pestic. Biochem. Physiol.* **17:** 215-223.
27. Zeyer, J., A. Wasserfallen, and K. N. Timmis. 1985. Microbial mineralization of ring-substituted anilines through an *ortho* cleavage pathway. *Appl. Environ. Microbiol.* **50:** 447-453.