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Biodegradation of diphenyl ethers by a copper-resistant mutant of *Erwinia* sp.

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SUMMARY

A bacterium tentatively identified as an *Erwinia* sp. was isolated from sewage by enrichment on methanol and lignin. Several mutants developed from this strain were studied for their ability to degrade aromatic ethers. Different concentrations of the chemicals were incubated with the organisms and the degradation was estimated by high-performance liquid chromatography (HPLC). Among these mutants, one isolate, *Erwinia* sp. strain CU3614, showed resistance to copper ions (> 20 mM $CuSO_4$) and the ability to degrade 4-hydroxydiphenyl ether (4-HDPE), 4-chlorodiphenyl ether (4-CDPE), 4-nitrodiphenyl ether (4-NDPE) and 2,7-dichlorodibenzo-*p*-dioxin (2,7-DCDD) in the presence of copper ions. Increased concentrations of copper in the medium resulted in higher degradation of 4-HDPE. Further studies with copper-sensitive mutants obtained from *Erwinia* sp. CU3614 by Tn5 transposon-induced mutagenesis showed a corresponding decrease in the ability to degrade 4-HDPE. These results suggest the presence of a copper-associated activity in the biotransformation of aromatic ethers.

INTRODUCTION

Next to cellulose, lignin is the second most abundant organic polymer on earth, comprising about 25% of the woody tissues in plants. It is a complex and heterogeneous polymer of phenyl propane units bound covalently, mainly through three different linkages: aryl-alkylether, aryl-arylether and diphenyl [2]. Degradation of lignin by microorganisms is hindered by the unique polyphenolic structure of lignin. Similar recalcitrant molecules also include lignin-derived compounds or other xenobiotic aromatic compounds such as diphenyl ethers or dibenzo-*p*-dioxins.

Highly toxic chlorinated dibenzo-*p*-dioxins have been recognized as possible contaminants in the manufacture of certain herbicides and pesticides. The degradation of xenobiotic aromatic compounds, especially chlorinated dibenzo-*p*-dioxins, is inefficient [8,18]. A number of soil microorganisms were screened for their ability to degrade ^{14}C-labeled 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). Only a few of them were capable of degrading this compound after prolonged incubation [12]. Co-oxidation of dibenzo-*p*-dioxin to *cis*-1,2-dihydrobenzodioxin and 1,2-dihydroxy-dibenzodioxin by a *Pseudomonas* sp. was reported by Gibson et al. [9]. They also demonstrated that a mutant strain of a *Beijerinckia* sp. oxidized monochlorinated dibenzo-*p*-dioxin to *cis*-dihydrodiols [10]. Recently, a ligninolytic basidiomycete *Phaenerochate chrysosporium* has been reported to degrade TCDD, DDT and lindane [4].

We have been interested in the characterization of aromatic ether cleavage systems of ligninolytic bacteria. Since diphenylethers and dibenzo-*p*-dioxins are aromatic ethers, it was of interest to investigate the potential degradation of such compounds. A ligninolytic bacterium which was isolated by a novel enrichment technique from sewage and identified as *Erwinia* sp. degraded such lignin-derived aromatic ethers as vanillin and vanillic acid [15]. We used several techniques to improve lignin degradation [15]. Recently, we isolated several copper-resistant mutants. All grew on LB agar with $CuSO_4$ at concentrations over 20 mM. In this report, we present the rationale for the isolation of copper resistant mutants and the results of the experiments on biodegradation of 4-NDPE, 4-HDPE, 4-CDPE, and 2,7-DCDD in the presence of copper by one of these isolates, *Erwinia* sp. strain CU3614.

MATERIALS AND METHODS

Organisms. Erwinia sp. was isolated from sewage obtained from a plant in Louisiana, using the method of Srinivasan and Cary [15]. Briefly, sewage sludge was introduced into

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the reactor vessel of a continuous culture apparatus. The medium with 0.5% methanol as sole carbon source was pumped continuously at a dilution rate of 0.1 h^{-1}. The pH was maintained at 9.0 by automatic addition of 1 M NaOH with the aid of a pH controller. After 10 days, when the chemostat culture was in a steady state, the carbon source was altered to 0.2% Kraft lignin (Crown Zellerbach, Bogalusa, LA) dissolved in NaOH. The reactor was maintained at the steady-state dilution rate for one week. Several isolates were obtained by selection on agar medium with Kraft lignin as the only carbon source. One of the isolates was tentatively identified as a saprophytic *Erwinia* based on the characteristics of fermentation of carbohydrates and other biochemical tests. Mutants used in this study were derived from this isolate. Other bacterial strains are listed in Table 1. *Escherichia coli* strain S17-1 with plasmid pSUP2021 [14] was used in transposon Tn5-induced mutagenesis.

Chemicals and media. All solvents were of analytical grade. Chlorinated diphenylethers and dibenzo-*p*-dioxins were custom synthesized by AccuStandard, New Haven, CT. 4-Hydroxydiphenyl ether and 4-nitrodiphenyl ether were purchased from Aldrich Chemical Co., Milwaukee, Wisconsin.

The organisms were maintained on NY agar medium which contained nutrient broth 0.8% (w/v) and yeast extract 0.2% (w/v) with the addition of 20 μg/ml of $CuSO_4 \cdot 5H_2O$ for copper-resistant mutants. SM medium contained sodium succinate (0.5%, w/v) and basal salts; it was adjusted to pH 8.0 before autoclaving. SM medium was used in biotransformation studies of diphenyl ethers and dibenzo-*p*-dioxins. SM medium with the addition of 10 μg/ml kanamycin was used in selection and maintenance of Tn5-induced copper-sensitive mutants. LB medium [11] was used in studies of copper resistance.

Mutation and selection. Organism was inoculated in 10 ml of NY medium and incubated overnight in an incubator shaker at a 32°C. One hundred μg/ml of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) was added to the culture and shaking was continued for 60 min. NTG-treated cells were harvested, washed with 0.1 M pH 6.8 potassium phosphate buffer. The suspension of cells in buffer was diluted and spread on agar gradient plates containing SM medium with 100 – 500 μg/ml of $CuSO_4$ or 10^{-2} M 2,4-dinitrophenol (DNP). Copper- or DNP-resistant strains were isolated from these plates.

Transposon Tn5-induced mutagenesis. The transposon mutagenesis system developed by Simon et al. [14] was used to isolate Tn5-induced mutants. Plasmid pSUP2021 was transferred from *E. coli* S17-1 into *Erwinia* sp. strain CU3614 by the filter mating technique described by Hom et al. [5]. The mating mixture was spread on SM agar plates containing 10 μg/ml kanamycin. Kanamycin-resistant colonies on SM agar plates were replicated onto SM agar plates containing 10 μg/ml kanamycin and 25 μg/ml $CuSO_4.5H_2O$, and copper-sensitive mutants were isolated.

Growth measurement. In order to measure bacterial growth, 5 ml overnight culture of copper-mutants grown on SM medium was inoculated into a Bellco 300-ml sidearm flask containing 50 ml of the same medium plus 0.2 mM $CuSO_4$, 0.3 mM 4-HDPE or 10 μg/ml kanamycin. Flasks were shaken at 250 rpm and at 32°C. Turbidity of the culture was determined using a Klett-Summerson photoelectric colorimeter with a green filter.

Biotransformation of diphenyl ethers and dibenzo-p-dioxins. Studies on biotransformation were carried out using stationary phase cultures. Cells were grown in SM medium containing 0.2 mM $CuSO_4$ for 24 h before the addition of diphenyl ethers or dioxins. 4-HDPE was added directly to cultures using a stock solution of 0.1 N NaOH. Chlorodiphenyl ethers were dissolved in benzene, and 4-NDPE was dissolved in ethanol for addition to cultures. Dioxins were introduced into cultures using a sand matrix or a filter paper coated with these chemicals. Killed controls were autoclaved or received 5 mM $HgCl_2$. Sterile controls were also used. The culture was then incubated for 24 to 48 h. Forty μl concentrated perchloric acid was added per ml of culture to terminate the reaction. Acidified broths were extracted three times with two volumes of ethyl acetate. The organic extracts were combined and concentrated under a stream of nitrogen, and the dry residue was taken up in a small volume of methanol for HPLC analysis of diphenyl ethers. Otherwise, dichloromethane was used for extraction of dibenzo-*p*-dioxins and as solvent to dissolve the dry residue.

High-performance liquid chromatography analysis. The HPLC system consisted of a model 660 solvent programmer, a model 6000A solvent delivery system, a M45 pump, a data module, a μ-Bondapak C18 column (all from Waters), and an ISCO V4 absorbance detector operated at 280 nm. The mobile phase used was methanol-water (85 : 15) at a flow rate of 1.0 ml/min. However, a gradient from 70% to 100% methanol was used for dibenzo-*p*-dioxins.

Thin layer chromatography (TLC). Samples were also analyzed on TLC plates (Silica gel 60 F-254, Alltech Assoc., Inc.) using a solvent system of chloroform, hexane, acetone, and acetic acid (50 : 50 : 10 : 1), v/v. Compounds were detected under UV light (254 nm).

Table 1

Copper resistance of strains used.

Strains		MIC of $CuSO_4$ (mM)	Source
Erwinia sp.		11	This study on LB agar
	CU36	>20	This study
	CU3614	>20	This study
E. coli	NM522	10	This study
	JM83	14	This study
	HB101	9	This study
	RJ 92–95	12–20	Tetaz et al. [16] on N agar
	K-12	6	Tetaz et al. [16]
Pseudomonas syringae		2–0.4	Bender et al. [3] on MGY agar
Xanthomonas campestris		7.2	Adaskaveg et al. [1] on LB agar

RESULTS AND DISCUSSION

Development of copper-resistant strains

In the ligninase system of *Phaenerochaete chrysosporium*, the enzyme generates a radical cation by Fe-porphyrin-mediated transfer of a single electron from the aromatic ring, in the presence of H_2O_2, during the biodegradation of lignin model compounds [13]. In a number of bacterial systems a copper-binding protein is involved in single-electron transfer as one of the components of the respiratory chain [6,7]. Therefore, attempts were made to isolate copper-resistant mutants by chemical mutagenesis as a means of obtaining strains with enhanced degradative characteristics. A further selection of a spontaneous mutant, CU3614, resistant to 2,4-dinitrophenol was made from a copper-resistant strain, CU36, on the assumption that such a mutant might possess a respiratory chain with low sensitivity to phenolic compounds generated by arylether cleavage.

Table 1 presents the minimum inhibitory concentrations (MIC) of $CuSO_4$ required to inhibit colony formation by the isolate. Several species of bacteria from diverse sources are insensitive to high concentrations of $CuSO_4$ [1,3,17]. MIC values for copper of several organisms are also presented in the table for comparison. Copper-resistance is associated with conjugated plasmids in *Escherichia coli* RJ 92-95, *Pseudomonas syringae* and *Xanthomonas campestri* [3,16,17]. However, it has not been determined whether a plasmid is involved in copper-resistance in *Erwinia* strains used in the present study.

Effect of copper on the biodegradation of 4-hydroxydiphenyl ether

The copper-associated function of copper-resistant strains isolated from *E. coli*, *X. campestris*, and *P. syringae* was not reported [1,3,17]. In the present study, we demonstrate that copper resistant mutants in *Erwinia* sp. can degrade aromatic ethers such as 4-HDPE in the presence of copper. Degradation of 4-HDPE by two mutants, CU36 and CU3614, was enhanced by increasing the concentration of $CuSO_4$ in the medium (Table 2). Strain CU3614 did not show increased activity in degrading 4-HDPE if $CuSO_4$ was not added. In the presence of $CuSO_4$, especially at higher concentrations such as 0.4 mM, strain CU3614 degraded about 90% of 0.25 mM 4-HDPE. Most of this degradation came from its copper-associated activity. This was confirmed by comparing HPLC profiles of the extracts from cultures incubated with or without the addition of 0.4 mM $CuSO_4$. As shown in the HPLC profiles in Fig. 1, cultures incubated with 0.4 mM $CuSO_4$ showed almost complete depletion of 4-HDPE and the appearance of two new peaks. Cultures without the addition of $CuSO_4$ demonstrated little depletion of 4-HDPE and did not produce metabolic intermediates. The minimal 4-HDPE degradation detected

Table 2

Biodegradation of 4-HDPE by *Erwinina* sp. strains.
Cultures were incubated with 0.25 mM 4-HDPE and different concentrations of $CuSO_4$. Degradation was then analyzed as described in the text

$CuSO_4$ (mM)	% Degradation by:		
	Erwinia sp.	Mutant CU36	Mutant CU3614
0.00	25.7	25.7	19.3
0.05	14.7	30.6	23.4
0.10	22.3	33.2	28.0
0.20	31.0	41.3	36.9
0.40	15.5	47.8	90.7

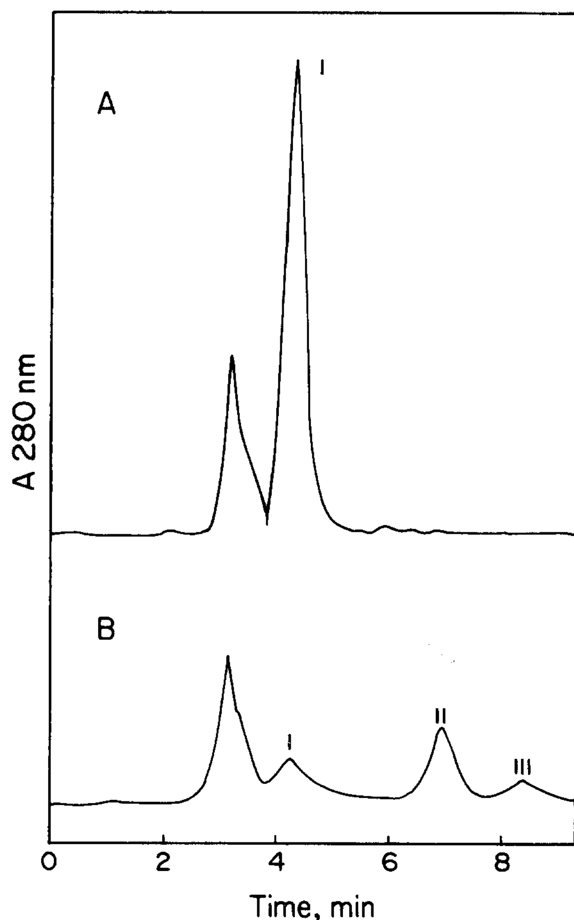

Fig. 1. HPLC profiles of the extracts from 24-h cultures of *Erwinia* sp. CU3614. (A) Culture incubated with 0.25 mM 4-HDPE shows only 4-HDPE peak (I). (B) Culture incubated with 0.25 mM 4-HDPE and supplemented with 0.4 mM $CuSO_4$ shows a small 4-HDPE peak (I) and two additional peaks (II and III).

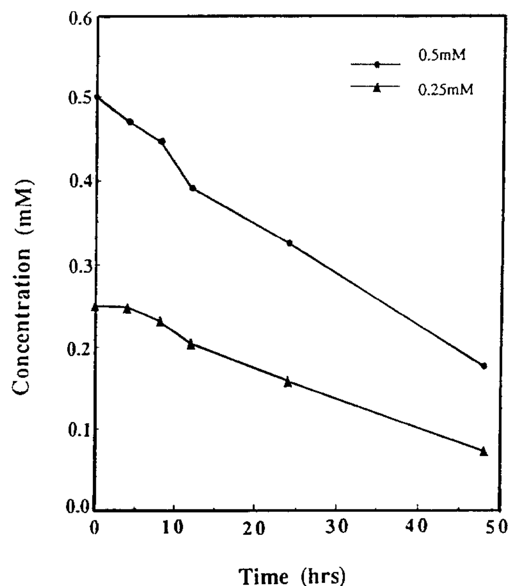

Fig. 2. Time course of 4-DHPE degradation. Stationary phase cultures of *Erwinia* sp. CU3614 were incubated with 0.5 mM 4-HDPE (-●-) or 0.25 mM 4-HDPE (-▲-) as described in the text. Samples from extracts of cultures were analyzed using HPLC. Symbols represent averages of three values.

from this culture without the addition of copper may be due to a copper-independent activity (Table 2). No 4-HDPE depletion was found in sterile or killed controls which were treated in the same way as experimental samples.

Kinetic studies on 4-hydroxydiphenyl ether degradation

The time-course of 4-HDPE degradation by strain CU3614 was observed using cultures incubated with 0.2 mM $CuSO_4$, and 0.25 mM or 0.5 mM 4-HDPE. Transformation of 4-HDPE occurred immediately in cultures containing 0.5 mM 4-HDPE (Fig. 2). The rate increased between 8 and 10 h of incubation, then it shifted to a slower rate through the remainder of the incubation period. It was likely that multiple activities were involved in the biotransformation of 4-HDPE. Two products appeared from the degradation of 4-HDPE by these activities (Fig. 1). Biodegradation of 4-HDPE by strain CU3614 was slower in cultures incubated with 0.25 mM 4-HDPE than with 0.5 mM 4-HDPE, nevertheless, the reaction in both concentrations showed similar kinetics (Fig. 2). Cultures at low concentrations of 4-HDPE might need a longer period to initiate the degradation of this compound. Optimum conditions for the biodegradation of 4-HDPE were observed at pH 8.0 to 9.0 and at 33 to 37 °C (data not shown).

Biodegradation of other diphenyl ethers and dibenzo-p-dioxins

Besides 4-HDPE, mutant CU3614 also degraded 4-CDPE, 4-NDPE, and 2,7-DCDD (Table 3). Copper-associated activities might also be involved in the degradation of other aromatic ethers. However, the efficiency differs depending on the structure of these chemicals. In the presence of $CuSO_4$, most of 4-HDPE was degraded by CU3614 within 24 h. This mutant also degraded 4-CDPE but less efficiently. The degradation of 4-CDPE was probably hindered by the chlorine group which substitutes the hydroxy group in 4-HDPE. 2,7-DCDD whose chemical structure is even more divergent from 4-HDPE was degraded at the lowest rate. The lower degradation of 2,7-DCDD or 4-NDPE may also be due to poor solubility of these compounds in water. The degradation product of 4-NDPE was yellow and tentatively identified as

Table 3

Biodegradation of diphenyl ethers and dibenzo-*p*-dioxins by *Erwinia* sp. CU3614[a]

Chemical	Concentration (ppm)	Degradation %
4-Hydroxydiphenyl ether	100	84.2
2-Chlorodiphenyl ether	100	–[b]
4-Chlorodiphenyl ether	100	51.4
4-Nitrodiphenyl ether	86.1	29.3
2-Chlorodibenzo-*p*-dioxin	5	–
2,7-Dichlorodibenzo	5[c]	12.4
-*p*-dioxin	2[d]	27.6

[a] Cultures were incubated for 24 h.
[b] –: No degradation was observed.
[c] 2,7-DCDD was absorbed on to a sand matrix.
[d] 2,7-DCDD was absorbed on to filter paper.

p-nitrophenol by its absorption maximum at 400 nM (Fig. 3), HPLC, and TLC analyses. The HPLC analysis of the degradation product of 4-NDPE showed that the product and a pure sample of *p*-nitrophenol had the same retention time of 3.58 min. The product and *p*-nitrophenol each had an Rf of 0.63 in TLC with a solvent system of

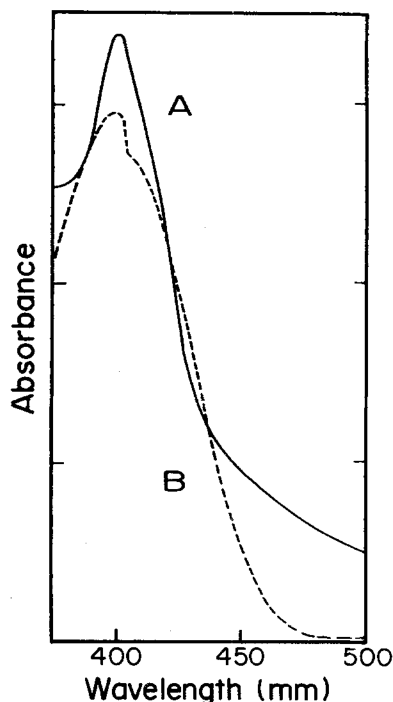

Fig. 3. Absorption spectra of the 4-NDPE degradation product and *p*-nitrophenol. (A, ———) 4-NDPE degradation product. (B, -------) *p*-nitrophenol.

chloroform, hexane, acetone, and acetic acid (50:50:10:1), v/v. Mutant CU3614 did not degrade 2-chlorodiphenyl ether or 2-chlorodibenzo-*p*-dioxin in these experiments. Their structures may interfere with the specific site of attack.

Studies of copper-sensitive mutants

Copper-sensitive mutants were isolated from *Erwinia* sp. CU3614 with transposon Tn5-induced mutagenesis. Among these copper-sensitive mutants, III-14-1 and III-26-31, which were also sensitive to 4-HDPE, were used in this study. Mutant III-14-1 had an MIC value of 5 mM for $CuSO_4$ in LB medium, and mutant III-26-31 had MIC value of 9 mM. Fig. 4 presents the effect of copper or 4-HDPE on the growth of these copper-sensitive mutants. $CuSO_4$ (0.2 mM) or 0.3 mM 4-HDPE did not interfere with growth of the copper-resistant mutant, *Erwinia* sp. CU3614, however, they inhibited growth of both copper-sensitive mutants (Fig. 4B, C). The growth of strain III-14-1 was completely inhibited by copper or 4-HDPE. The same concentrations of $CuSO_4$ and 4-HDPE were less inhibitory to the growth of III-26-31. Strain CU3614 could not grow in a medium containing kanamycin, but both III-14-1 and III-26-31 which are Tn5-induced mutants (Km^r) grew (Fig. 4D). Overall, these Tn5-induced copper-sensitive mutants exhibited slower growth, even in the absence of inhibitors (Fig. 4A). Degradation of 4-HDPE was also inefficient in these copper-sensitive mutants (data not shown). The sensitivity to copper perhaps interfered with their ability for 4-HDPE degradation which required increased copper concentration as described for strain CU3614 (Table 2).

These results suggest that copper-associated activities may be involved in degradation of 4-HDPE and other aromatic ethers. Although the protein(s) involved in the transformation of 4-HDPE has not been identified yet, several blue proteins were found in the crude cell extract of *Erwinia* CU3614 growing on a high concentration of copper (data not shown). Further studies on these copper-sensitive mutants pointed to the relationship between copper-resistance and 4-HDPE degradation. Moreover, the same system may not be involved in both copper-resistance and 4-HDPE degradation. In this study, the biotransformation of chemicals was only demonstrated using intact cells. Preliminary experiments using crude cell extracts or broken cells showed transformation only in the presence of an electron acceptor, 2,6-dichlorophenol-indophenol. It is possible that a rusticyanin-like copper protein may be involved in the reaction, as described by Ingledew [7] for single electron transfer to the components of the respiratory chain of *Thiobacillus ferrooxidans*.

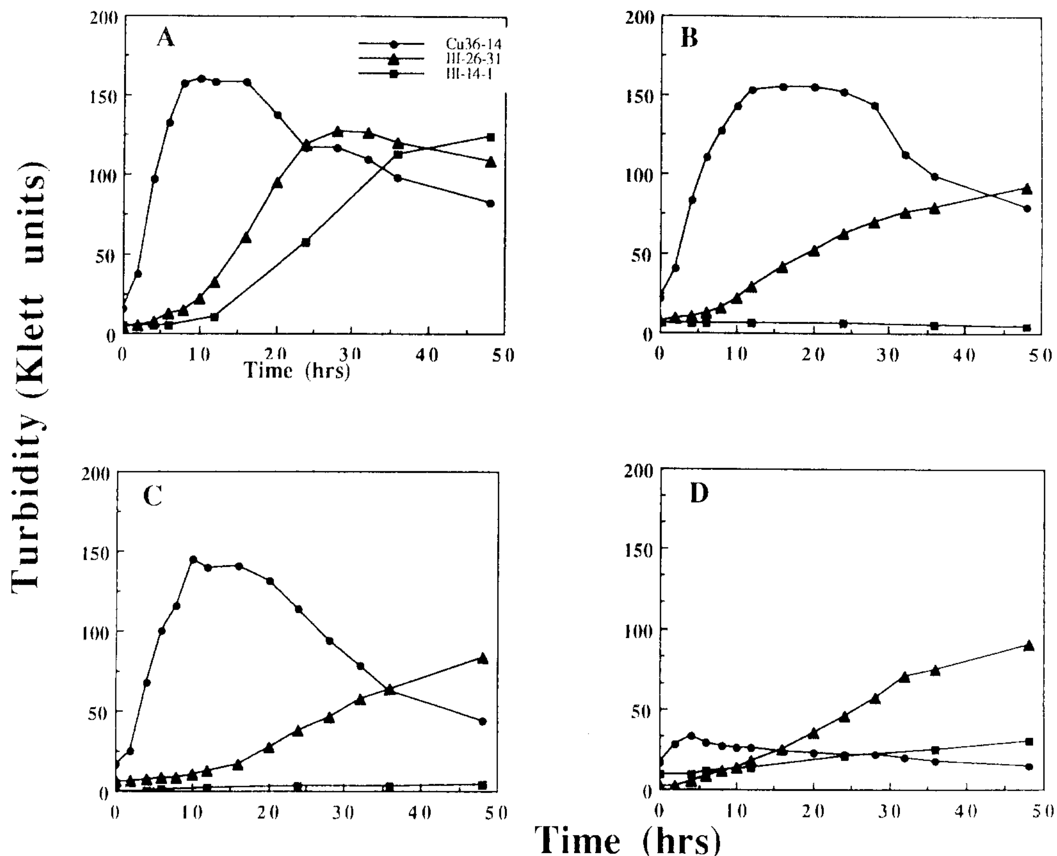

Fig. 4. Growth curves of *Erwinia* sp. CU3614 and its copper-sensitive mutants III-14-1 and III-26-31. Cultures were grown in 50 ml SM (A), SM containing 0.2 mM $CuSO_4$ (B), SM containing 0.3 mM 4-HDPE (C), and SM containing 10 μg/ml kanamycin (D). Turbidity in cultures of CU3614 (-●-), III-26-31 (-▲-) and III-14-1 (-■-) was monitored at 660 nm with a Klett-Summerson photoelectric colorimeter.

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