Journal of Chemical Ecology, Vol. 14, No. 7, 1988

DEGRADATION OF JUGLONE BY SOIL BACTERIA

S.K. SCHMIDT

Department of Environmental, Population, and Organismic Biology
University of Colorado, Boulder, Colorado 80309

(Received July 27, 1987; accepted September 15, 1987)

Abstract—Bacteria that can degrade juglone (5-hydroxy-1,4-naphthoquinone) were isolated from soil beneath black walnut trees. Autecological studies with one of these bacteria (*Pseudomonas* J1), demonstrated that it could grow rapidly using juglone as its sole source of carbon and energy. Using nonlinear regression analysis and the Monod equation, it was determined that this bacterium had a high affinity for juglone (K_s = 0.95 μg/ml). *Pseudomonas* J1 can also utilize other aromatic compounds from plants as its sole source of carbon and energy. Compounds such as chlorogenic acid, ferulic acid, gallic acid, and 2-hydroxy-1,4-naphthoquinone (Lawson) were rapidly degraded by *Pseudomonas* J1. The rapid degradation of juglone and other suspected allelochemicals by soil bacteria make it unlikely that these compounds are important mediators of plant–plant interactions under natural conditions.

Key Words—Allelopathy, biodegradation, humic acids, *Juglans nigra*, juglone, nonlinear regression, *Pseudomonas putida* biovar A.

INTRODUCTION

Juglone is a toxic chemical produced by *Juglans nigra* L. and other members of the walnut family (Juglandaceae) (Thomson, 1971). It has long been known that juglone is toxic to certain plants, and this toxicity has been invoked to explain the antagonistic effect of black walnut trees on neighboring vegetation (Davis, 1928, Rietveld, 1983). Fisher (1978) presented evidence that the allelopathic potential of juglone was not expressed in well-drained soils, and he attributed this to the aerobic microbial mineralization of juglone in these soils. He went on to reason that, in wet soils, aerobic microorganisms capable of metabolizing juglone were not active, and therefore juglone was able to build up to concentrations high enough to inhibit the growth of red pines. However,

0098-0331/88/0700-1561$06.00/0

no evidence was presented by Fisher (1978) or subsequent workers (Rietveld, 1983; Rietveld et al., 1983) that juglone was metabolized by soil microorganisms. Other hypotheses for the disappearance of juglone from well-drained soils, such as increased leaching, incorporation into soil organic matter (Mathur, 1972; Saiz-Jimenez et al., 1975), or sorption onto the mineral fraction of the soil also have not been explored (Fisher, 1987).

Many studies have demonstrated the microbial breakdown of simple aromatic compounds in soil (Alexander, 1977; Reber, 1975), but very little work has been done on the metabolism of polycyclic quinones such as juglone. Polycyclic quinones are toxic, very reactive, and therefore more likely to be incorporated into the humic fraction of soil than to be completely metabolized by soil microorganisms (Saiz-Jimenez et al., 1975). The present study was undertaken to determine if soil microorganisms are able to breakdown juglone at concentrations likely to occur under field conditions.

METHODS AND MATERIALS

Bacterial Isolate. Soil samples were collected from the top 10 cm of soil beneath a stand of black walnut trees in Boulder County, Colorado. Soil was also collected 20 m from the walnut trees at a site in which no black walnut or other trees were growing. These soils were clay-loams of the Niwot series and are characterized as being somewhat poorly drained and mildly alkaline (Moreland and Moreland, 1975). Five soil samples from each site were used in enrichment studies to determine if juglone-utilizing microorganisms could be isolated. Juglone (98%, Aldrich Chemical Co., Milwaukee, Wisconsin) was added to 3-g soil samples in sterile 20-ml vials at a concentration of 2 μg/g of soil (2 ppm). After seven days, an additional 6 μg of juglone in 0.2 ml of distilled water was added to the soil. This process was repeated twice more, and 0.5 g of soil was then transfered to 100 ml of distilled water containing inorganic salts and 5 μg of juglone per milliliter. The culture was incubated for seven days at 23°C. Serial dilutions were then plated on a medium containing inorganic salts, 5 μg of juglone and 15 mg of Difco Bacto-Agar per milliliter. Isolated colonies were selected and streaked on juglone plates several times successively until pure cultures were obtained.

Identification of the bacteria obtained from each soil was done using standard bacteriological techniques (Palleroni, 1984). Flagella were stained using the technique of Heimbrook et al. (1986), and the capacity of the organisms to metabolize allelopathic compounds other than juglone was tested using the auxanographic technique as described by Parke and Ornston (1984). The inorganic salts solution used in all enrichments and experiments contained 750 μg KH_2PO_4, 480 μg Na_2HPO_4, 40 μg NH_4Cl, 10 μg $MgSO_4 \cdot 7H_2O$, 10 μg $CaCl_2 \cdot 2H_2O$, and 0.02 μg $FeCl_3 \cdot 6H_2O$ per milliliter of distilled–deionized water.

Three of the enrichments from the walnut soil yielded bacteria capable of metabolizing juglone. One of these bacteria was chosen at random for further studies and is henceforth designated as *Pseudomonas* J1. Stock cultures of *Pseudomonas* J1 were grown in the inorganic salts solution containing 5 μg of juglone per milliliter. The inoculum of *Pseudomonas* J1 used in the experiments described below was grown in inorganic salts solution containing 10 μg of juglone per milliliter. Cells were grown to the early stationary phase and were then diluted with inorganic salts solution to obtain the initial cell densities used in the experiments. Experiments were conducted in glass-stoppered 250-ml Erlenmeyer flasks containing 100 ml of the inorganic salts solution and the indicated concentrations of juglone. The flasks were incubated at 23°C without shaking. At regular intervals, the number of cells was determined by using the spread-plate technique. Triplicate 0.1-ml portions of 10-fold dilutions were plated on a medium containing 15 mg of Difco Bacto-agar and 3 mg of Trypticase soy broth with glucose (BBL, Cockeysville, Maryland) per milliliter of deionized water, and colony counts were made after 72 hr of incubation at 23°C. The data represents means of triplicate plate counts from individual flasks.

Juglone concentrations were determined with a spectrophotometer. To obtain a standard curve, juglone was dissolved in the inorganic salts solution at concentrations of 0.25, 0.5, 1.0, 2.5, 5.0, 7.5, 10, 20, and 40 μg/ml. These solutions were acidified with concentrated sulfuric acid to a pH of approximately 0.8 and the absorbance was read at 420 nm. During the experiments, 2-ml samples were removed from the cultures and immediately acidified with one drop of concentrated sulfuric acid to a pH of approximately 0.8 to stop all microbial activity in the sample. The absorbance values for each sample were then determined and the juglone concentration was calculated using the standard curve.

Data Analysis. Nonlinear regression analyses were performed on the curve of juglone disappearance. For this purpose, the MARQFIT computer program was used (Simkins and Alexander, 1984). This program fits nonlinear data by minimizing the square of the differences between the data and the model curve using the Marquardt method (Bard, 1974). Nonlinear regression is a statistical tool that can be used to determine values for ecologically important parameters describing the growth and metabolism of microorganisms (Schmidt et al., 1985b). This information can then be used to help estimate the persistence of organic chemicals in the environment.

RESULTS

Juglone-metabolizing cultures were isolated only from soils collected under black walnut trees. Bacteria that could tolerate juglone were obtained from the other soil samples, but these bacteria could not metabolize juglone even after

three months of incubation in the presence of juglone. In addition, juglone-utilizing cultures did not develop in flasks containing juglone but no soil, when these were left open to the laboratory air for three months. Therefore, it is reasonable to conclude that the bacteria obtained from soils beneath walnut trees were indigenous to those soils.

Pseudomonas J1 is a short (1–2 μm) gram-negative rod with one to four polar flagella, requires no growth factors, reacts positively to the oxidase and catalase tests, and can hydrolyze arginine under anaerobic conditions. In addition, *Pseudomonas* J1 produces a yellow fluorescent pigment when grown under iron limitation. These characteristics warranted placing it in rRNA group I of the genus *Pseudomonas* (Palleroni, 1984). Of the species in rRNA group I, *Pseudomonas* J1 matches almost exactly the characteristics of *P. putida* biovar A (Palleroni, 1984).

Table 1 summarizes the results of tests conducted with a number or organic compounds to determine what compounds could serve as sole carbon and energy sources for *Pseudomonas* J1. Many of the aromatic compounds have been reported to be allelochemicals (Rice, 1984). In addition to these suspected allelochemicals, other compounds such as sugars and amino acids were also tested for their ability to support the growth of *Pseudomonas* J1. Of note in these results was the inability of this organism to utilize most of the simple sugars that were tested. Only glucose and fructose were degraded, and even these sugars were not used as rapidly by *Pseudomonas* J1 as were the aromatic compounds tested.

Pseudomonas J1 was able to metabolize juglone and grow rapidly using this compound as its sole source of carbon and energy (Figure 1). In the inorganic salts solution in absence of juglone, no growth of *Pseudomonas* J1 was detected (Figure 1), and in the inorganic salts solution in the absence of *Pseudomonas* J1, no degradation of juglone was observed (data not shown). The yield of *Pseudomonas* J1 cells was also proportional to the concentration of juglone (1.4×10^7 and 3.1×10^7 cells/ml for 10 and 20 μg of juglone per milliliter, respectively), as would be expected for an organism using a substrate as its sole source of carbon and energy. The cell yields obtained are also close to those for other soil *Pseudomonas* spp. that completely mineralize simple aromatic compounds such as phenol (Schmidt et al., 1985b).

The data presented in Figure 2 show the disappearance of juglone when *Pseudomonas* J1 utilized juglone as its only source of carbon and energy. A similar curve of juglone disappearance was obtained with an initial juglone concentration of 20 μg/ml (data not shown). The data for the mineralization of 10 μg of juglone per milliliter was fit by the Monod equation using nonlinear regression analysis (Simkins and Alexander, 1984). From this analysis, the half saturation constant (K_s) for the growth of this organism on juglone was estimated to be 0.95 ± 0.71 μg/ml ($P = 0.05$). The values for the other parameters

TABLE 1. ORGANIC COMPOUNDS THAT CAN (+) OR CANNOT (−) ACT AS SOLE SOURCE OF CARBON AND ENERGY FOR GROWTH OF *Pseudomonas* J1

Aromatic compounds		Sugars	
Anthranilic acid	−	Lactose	−
Benzoic acid	+	D-Mannitol	−
Caffeic acid	+	D-Mannose	−
Catechol	+	L-Rhamnose	−
Chlorogenic acid	+	D-Ribose	−
trans-Cinnamic acid	−	Starch (soluble)	−
Ferulic acid	+	Sucrose	−
Gallic acid	+	Trehalose	−
p-Hydroxybenzoic acid	+	D-Xylose	−
m-Hydroxybenzoic acid	−	Amino acids	
2-Hydroxy-1,4-naphthoquinone	+	Aspartic acid	+
Naphthalene	−	Arginine	+
1-Naphthol	−	Betaine	+
Naphthoresocrinol	+	Glutamic acid	+
Phenol	−	Glycine	+
Protocatechuic acid	+	L-Lysine	+
Quinic acid	+	L-Ornithine	+
Salicin	−	L-Phenylalanine	+
Salicyclic acid	+	Serine	+
Tannic acid	+	D-Tryptophan	−
p-Toluic acid	−	L-Tryptophan	−
Vanillic acid	+	Other compounds	
Vanillin	+	Acetic acid	+
Sugars		Citric acid	+
L-Arabinose	−	Ethanol	+
D-Fructose	+	Fumaric acid	+
Glucose	+	Glycerol	+
i-Inositol	−	Succinic acid	+
m-Inositol	−	L-Tartaric acid	+
Lactose	−	Testosterone	−

of the Monod equation were $X_0 = 0.53 \pm 0.18$ μg/ml, $\mu_{max} = 0.47 \pm 0.09$/hr, and $S_0 = 9.76 \pm 1.3$ μg/ml. X_0 is the initial population density expressed in terms of juglone concentration, μ_{max} is the maximum specific growth rate for *Pseudomonas* J1 on juglone, and S_0 is the program's estimate for the actual initial concentration of juglone. Independent estimates of these parameters also were determined from the growth data shown in Figure 1 and simply by knowing the concentration of juglone and cells added at the beginning of the experiment. Thus, $X_0 = 0.63$ μg/ml, $\mu_{max} = 0.56$/hr, and $S_0 = 10.0$ μg/ml. These independent parameter estimates all fall within the 95% confidence intervals of the computer-generated estimates from the data presented in Figure 2.

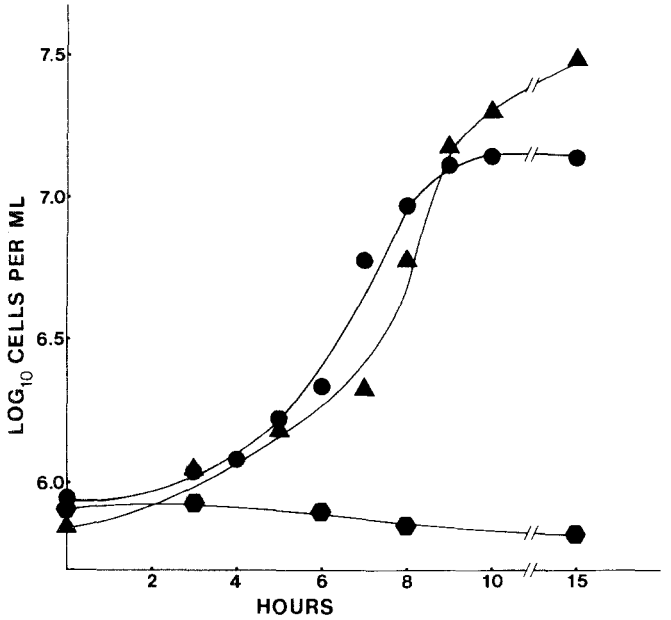

FIG. 1. Growth of *Pseudomonas* J1 on juglone at concentrations of 0 (⬣), 10 (●), and 20 (▲) μg/ml.

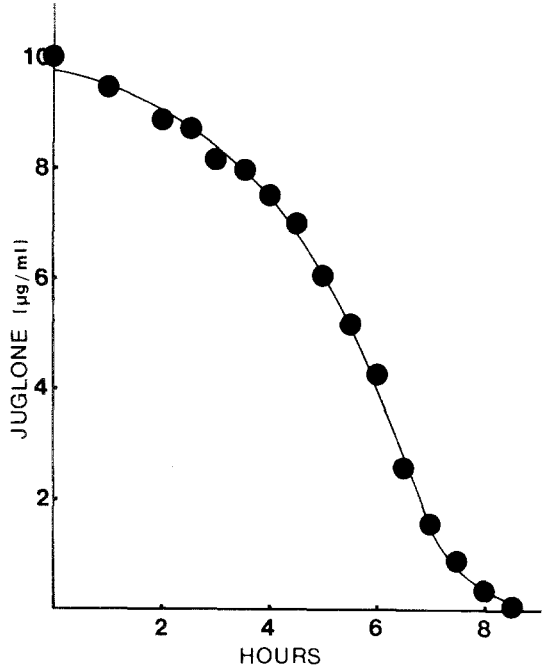

FIG. 2. Mineralization of 10 μg of juglone per milliliter by *Pseudomonas* J1. The curve is the best fit of the Monod equation to the experimental data (●). The curve was fit using nonlinear regression.

DISCUSSION

The presence of juglone-metabolizing bacteria in soils collected from under black walnut trees was confirmed in this study. This finding supports the hypothesis of Fisher (1978) that juglone is degraded and thus not allelopathically active in well-aerated soils. Juglone-mineralizing bacteria were not found in adjacent soils not exposed to black walnut litter or roots, indicating that juglone-utilizing bacteria are selected for in soils beneath black walnut trees. Juglone is structurally dissimilar to many aromatic compounds found in the soil solution (Alexander, 1977), and thus organisms adapted to metabolize simple compounds such as benzoate or phenol would not be expected to metabolize juglone. *Pseudomonas* J1, however, was able to grow on many simple aromatic compounds commonly reported to occur in soil. Compounds such as caffeic, chlorogenic, ferulic, and gallic acids have been reported to be allelochemicals (Rice, 1984), but most of these are readily degraded by a wide range of soil microorganisms (Alexander, 1977; Reber, 1975; Schmidt et al., 1987; Turner and Rice, 1975).

This is the first report of the microbial degradation of juglone. Previous work on the metabolism of polycyclic quinones has shown the fungus *Penicillum notatum* to be able to partially degrade lapachol (Rosazzo, 1982), an antitumor naphthoquinone from the wood of several species in the Bignoniaceae, Proteaceae, and Verbenaceae families (Thomson, 1971). *P. notatum* was able to carry out the partial degradation of lapachol using a monooxygenase enzyme under aerobic conditions (Rosazzo, 1982). Martin and Haider (1979), demonstrated that complexed anthroquinone mixtures were degraded by soil microorganisms, but they did not isolate the responsible organisms from soil.

Saiz-Jimenez et al. (1975) presented evidence for a different fate of polycyclic quinones in soil. In their work, the fungus *Eurotium echinulatum* catalyzed the oxidative linkage of anthroquinones and phenols to form dark-colored pigments similar to humic acids. Other workers have stressed the likelihood of an important role for polycyclic quinones in the formation of humic acids in soil (Mathur, 1972; Steelink and Tollin, 1967). Quinones also are very reactive with amino acids and proteins in the soil solution, forming complex molecules that are resistant to microbial degradation (Smith, 1982). Therefore, under aerobic conditions in soil, a multitude of fates is possible for polycylic quinones such as juglone. The fate of juglone in a particular soil may depend on several factors, such as aeration and the types of microorganisms present, but in any case it seems unlikely that juglone would persist in an unmodified form under aerobic conditions in soil in which black walnut trees are growing.

There is also reason to believe that juglone would not persist in an unmodified form under anaerobic conditions in soil. Recent work has demonstrated the bacterial breakdown of many aromatic compounds in anaerobic soil (Young, 1984). Soil bacteria can degrade aromatic compounds either by anaerobic (nitrate) respiration (Williams and Evans, 1975) or under methanogenic con-

ditions (Balba et al., 1979; Young, 1984). Even recalcitrant compounds such as halogenated benzoates are readily degraded by certain bacteria under anaerobic conditions (Suflita et al., 1982). In addition, 1,4-naphthoquinone and other naphthoquinones, which are structurally very similar to the K vitamins, have been shown to be utilized as growth factors by anaerobic bacteria such as *Succinivibrio dextrinosolvens* (Gomez-Alarcon et al., 1982). Thus, juglone may be subject to anaerobic attack, especially in soils that are repeatedly exposed to prolonged flooding. The persistence of juglone under wet moisture regimes in the lab (Fisher, 1978) may be due to the slower breakdown of juglone under anaerobic conditions, rather than a complete lack of degradation. It is also noteworthy that in his laboratory studies, Fisher (1978) used a soil that is not normally exposed to either anaerobic conditions or juglone. Thus, anaerobic bacteria adapted to metabolize juglone were probably absent from the soil used by Fisher (1978). Controlled studies are needed to determine if the die off of trees in certain soils is caused by juglone or if the effect is simply the result of anaerobiosis itself.

Autecological studies of microorganisms have been greatly enhanced by the use of nonlinear regression analysis and other computer-assisted techniques (Holder-Franklin and Tate, 1986; Simkins and Alexander, 1984). In the present study, the kinetic parameters obtained can be used to estimate the rate at which juglone will be degraded at different concentrations in the soil solution. Using the Monod equation and nonlinear regression techniques, an estimate for the half-saturation constant (K_s) for the utilization of juglone by *Pseudomonas* J1 was determined to be 0.95 μg/ml. K_s is inversely proportional to an organism's affinity for a given organic compound. A low K_s indicates a high affinity for the compound. At juglone concentrations greater than K_s, *Pseudomonas* J1 will be able to metabolize juglone at a rate approaching the maximum possible rate for this bacterium. The implications of this are that free juglone is unlikely to persist for long in the soil solution at concentrations around or above K_s. At concentrations below K_s, *Pseudomonas* J1 will metabolize juglone according to pseudo-first-order kinetics (Schmidt et al., 1985b). That is, juglone will be metabolized at a rate that is directly proportional to both the concentration of juglone and the size of the microbial population mineralizing juglone. The high affinity of *Pseudomonas* J1 for juglone is also evident from the rapid growth rate that this organism exhibited when growing on 10 μg of juglone per milliliter as its only source of carbon and energy.

At a juglone concentration approaching two orders of magnitude below K_s, *Pseudomonas* J1 may degrade juglone very slowly or not at all. Bacteria can exhibit threshold concentrations below which they are unable to mineralize organic compounds (Schmidt et al., 1985a). The threshold concentration for a *Pseudomonas* sp. that mineralizes *p*-nitrophenol (PNP) was approximately 100× lower than its K_s for growth on PNP (Schmidt et al., 1987). If *Pseudomonas*

J1 behaves similarly to this organism, it should be able to mineralize juglone at concentrations as low as 0.0095 μg/ml, a concentration below 10^{-7} molar. This is probably a conservative estimate of the lower limit for the degradation of juglone in soil. Scow et al. (1986) have demonstrated the rapid mineralization of many complex aromatic compounds at concentrations well below 1 ng/g of soil. In any case, *Pseudomonas* J1 is probably capable of mineralizing juglone at concentrations well below the lowest concentration that has elicited phytotoxicity, 10^{-6} molar (Rietveld, 1983), or significant toxicity to nitrogen fixing bacteria, 10^{-4} molar (Dawson and Seymour, 1983).

This study demonstrates the importance of considering the metabolic capabilities of soil microorganisms in studies of allelopathy (cf. Kaminsky, 1981). Soil microorganisms are carbon-limited most of the time (Lockwood and Filonow, 1981), and thus organisms such as *Pseudomonas* J1 are literally waiting to consume compounds such as juglone. The rapid degradation of potential allelochemicals by soil microorganisms should cause researchers to question claims of allelopathic activity if they are based solely on tests conducted in sterilized soil or laboratory media. Tests of the effects of a suspected allelochemical should be conducted using unsterilized soils from field sites where the alleged allelopathy is occurring. This simple procedure would give a more realistic assessment of the potential for a given chemical to cause allelopathic effects in nature.

Acknowledgments—This study was funded by the Council on Research and Creative Work, University of Colorado, Boulder. I thank T. Hess, L. Figueroa, and B. Martin for technical support, and K. Scow for helpful suggestions.

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