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HYDROLYSIS OF A FUNGICIDE, BUPRIMATE BY INDIGENOUS *ACHROMOBACTER* SPECIES

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ABSTRACT

A soil bacterium (CMG500) capable of degrading a fungicide buprimate (Nimrod), was isolated from vegetable fields and identified as *Achromobacter* species. It harbors a catabolic plasmid pNS3. The isolate was resistant to streptomycin, penicillin, and ceferozone. The structure of the degraded product was elucidated using mass spectra, U. V. and N. M. R. It was found that the fungicide bupirimate (5-Butyl – 2-ethyl amino 6-methyl-4pyrimidyl dimethyl sulfamate was converted into 5- Butyl-2 ethyl amino 6-methyl –4 –pyrimidinol by *Achromobacter* species. Hydrolysis of buprimate fungicide resulted in the removal of dimethyl sulfamate group. The degrading genes for buprimate were present on a conjugative plasmid pNS3.

Key-words: Achromobacter, buprimate, fngicidal hydrolysis,

INTRODUCTION

Pesticides being a promise for higher yield of food and fiber are an input in agriculture all over the world. These pesticides being synthetic persist in the environment and are reaching to levels in the environment where the life span of human being is being shortened by both immediate and long-term effects of these chemicals, which include carcinogenesis, mutagenesis and system damage (Deaven, 1985).

In Pakistan substantial quantities of agricultural chemicals are used annually to enhance the yield. Rivers and streams are contaminated with these toxicants (Baloch, 1985) and thus they are a constant threat to public health. Residues of organochlorine, methyl mercury fungicide, DDT have been reported to persist in the food grains in many parts of the world (Khan, 1995). For the removal and detoxification of such toxic chemicals from the environment, studies have been conducted around the world to isolate soil bacteria capable of biodegradation of these agrochemicals using catabolic route. These include, *Achromobacter, Flavobacterium, Arthrobacter, Pseudomonas,* and *Alcaligenes* etc. Biodegradation of 1, 2, 3 and 1, 2, 4 trichlorobenzene (fungicides) has been demonstrated by liquid enrichment culture. Moos *et al.* (Moose *et al.*, 1983) have demonstrated the biodegradation of chlorinated fungicides like pentachlorophenol (Radehaus and Schmidt, 1992). The *Achromobacter* sp. WM111 is reported to rapidly degrade *N*-methylcarbamate insecticide by producing a hydrolyzing enzyme (Karens *et al.,* 1986) and its gene has been cloned in gram-negative bacteria (Tomasek and Karen, 1998).

In most cases microbial metabolism of pesticides involve an initial ester hydrolysis followed by complete mineralization /incorporation of one or more of the hydrolysis products and to be used as energy source (Tam *et al.*, 1987; Racke *et al.*, 1988). The genes responsible for degradation of these toxic fungicides and pesticides are reported to be plasmid borne (Deaven, 1985; Mulbry *et al.,* 1986; Ahmed and Afzal, 1992; Laemmli *et al.,* 2000) in soil bacteria.

Buprimate is a fungicide, widely used in barley fields as spray in U.K. Present study was carried out to isolate and study a fungicide buprimate (dimethylsulphamoyl derivative of pyrimidine) degrading organism, elucidate structure of the degraded product, and study the genes involved in the degradation.

MATERIALS AND METHODS

Isolation and identification

Bacterial isolate CMG500 was isolated from soil sample collected from vegetable fields of village Kandri (District Sukkar, Pakistan). For isolation soil suspension was inoculated in the nutrient broth supplemented with varying concentrations (from $10gL^{-1}$ to $60gL^{-1)}$ of Buprimate fungicide (Merck). Bacterial isolate CMG500 was picked from the agar plate (inoculated with the broth grown culture) supplemented with the highest concentration ($59gL^{-}1$) of buprimate. Organism was purified and identified by API test kit and maintained at $4^{o}C$. Buprimate is a white waxy solid. The working concentrations were prepared from a stock solution of 100g buprimate per litter hexane.

Antibiotic resistance

Resistance to various antibiotics was determined by standard procedure (Ahmed and Afzal, 1992). Ten different antibiotics (Ampicillin, Kanamycin, Chloramphenicol, Streptomycin, Erythromycin, Tetracycline, Neomycin, Penicillin, Ceferozone and Rifampicin) of sigma grade. Stock solution were prepared according to (Ahmed and Afzal, 1992) and stored at 4^{o}C.

Degradation of Buprimate fungicide

Bacterial isolate CMG500 was aerobically grown in triplicate in one L of nutrient broth containing $59gL^{-1}$ buprimate fungicide, incubated at 37^{o}C in an orbital shaker. Two sets of controls were used one without CMG500 but containing $59gL^{-1}$ of buprimate in nutrient broth and the other containing CMG500 without buprimate, and maintained at the same conditions, for the evaluation of autolytic growth or transformation of buprimate with the culture medium. 5ml sample was eluted at 2days interval up to 22days of incubation. The sample was used for the extraction of the metabolite.

Extraction of metabolite (s)

Five-ml samples were extracted twice with 10 ml. of ethyl acetate by vigorous shaking in a separating funnel. The ethyl acetate layer and the aqueous layer (bottom layer) were separated. The ethyl acetate fractions were pooled and the resulting solution was used for thin layer chromatography. Same procedure was practiced for controls.

Thin layer chromatography and purification of metabolite(s)

Ethyl acetate extracts of buprimate containing culture and controls (with and without buprimate were loaded on silica gel plates (Dc cards SIF, 5x10 cm, Riedel de Haen), the plates were allowed to run in the solvent mixture of petroleum ether: acetone in a ratio of 7:3 (vol/vol) and visualized under UV illuminator at 254 and 366 nm. Each band was scratched separately, and extracted with a mixture of chloroform: methanol (9:1 vol/vol). After 20-30 minutes, the contents were filtered, concentrated in Eyala rotary evaporator and checked for purity of the metabolites by TLC in the same solvent system. The purified product was re-extracted with ethyl acetate and analyzed spectroscopically for identification and structure elucidation.

Analysis of metabolite(s)

Approximately 20mg of purified metabolite product and the fungicide buprimate were then analyzed through;

1. UV spectra:

The UV spectra were recorded on Pye Unicam 5P-200G and Shimadzu UV240 ultraviolet spectrophotometer.

2. Mass spectra:

The EI (electron impact) mass spectra were recorded on Finiigan (Varian) MAT-112 and MAT-312 mass spectrometers connected to MAT 188 data system with POP 11/34 DEC computer system.

3. NMR spectra:

The one dimensional and two dimensional NMR spectra were recorded at 300 MHZ on Bruler AC-300, AM-300 nuclear magnetic resonance spectrometers.

Plasmid isolation

Plasmid DNA of the isolate CMG500 was isolated by Birnboim and Doly method (Birnboim and Doly, 1979). Plasmid DNA bands were observed on UV Transilluminator. They were then analyzed by the electrophoresis documentation analysis system 120. Curing of plasmid DNAThe isolate CMG500 was cured of plasmid DNA according to Miller, (1972). For conjugation studies E. *coli* strain J-53, which was, buprimate, Am, Sm, Km sensitive and Rif resistant, used as recipient strain. Whereas CMG 500 was used as donor. Conjugation was done as described by Rotimi and Dureden, (1980).

RESULTS

Identification

The bacterial isolate was identified as *Achromobacter* species on the basis of API test kit and coded as (CMG500).

Resistance to antibiotics

The bacterial isolate CMG500 showed resistance to high concentrations of streptomycin, penicillin and ceferozone only and showed sensitivity to most of the tested antibiotics (Fig. **1**).

Degradation of fungicide buprimate

TLC analysis showed the presence of one major and two very monir spots after 22days of incubation, which had different Rf values as compared to that of buprimate (fungicide) in the control.

Structure elucidation of degraded product.

In order to elucidate the structure of the degraded product spectroscopic analysis was carried out. EI mass spectra of fungicide (control) and the degraded product showed that buprimate was degraded and resulted in a metabolite of m/z 209 where as the mass of fungicide was observed at m/z416 (Fig. **2**) Comparison of mass analysis, nuclear magnetic resonance analysis and ultra violet spectrum of the fungicide and the metabolite with the standard mass spectra in The NIST Mass Spectral Search Programme for the NIST/EPA/NIH Mass Spectral Library. (Ver.1.6d 06//24/98 © 1977,USA.) revealed that hydrolysis of the fungicide has occurred in presence of *Achromobacter* sp., which has resulted in the removal of dimethylsulfamate group of the buprimate (Fig. **3**). This was particularly evident from the presence ofm/z 209 of the metabolite titled as Ethrimol (in the NIST Mass Spectral Library) (Fig. 4), which is also used as fungicide for seed dressing.

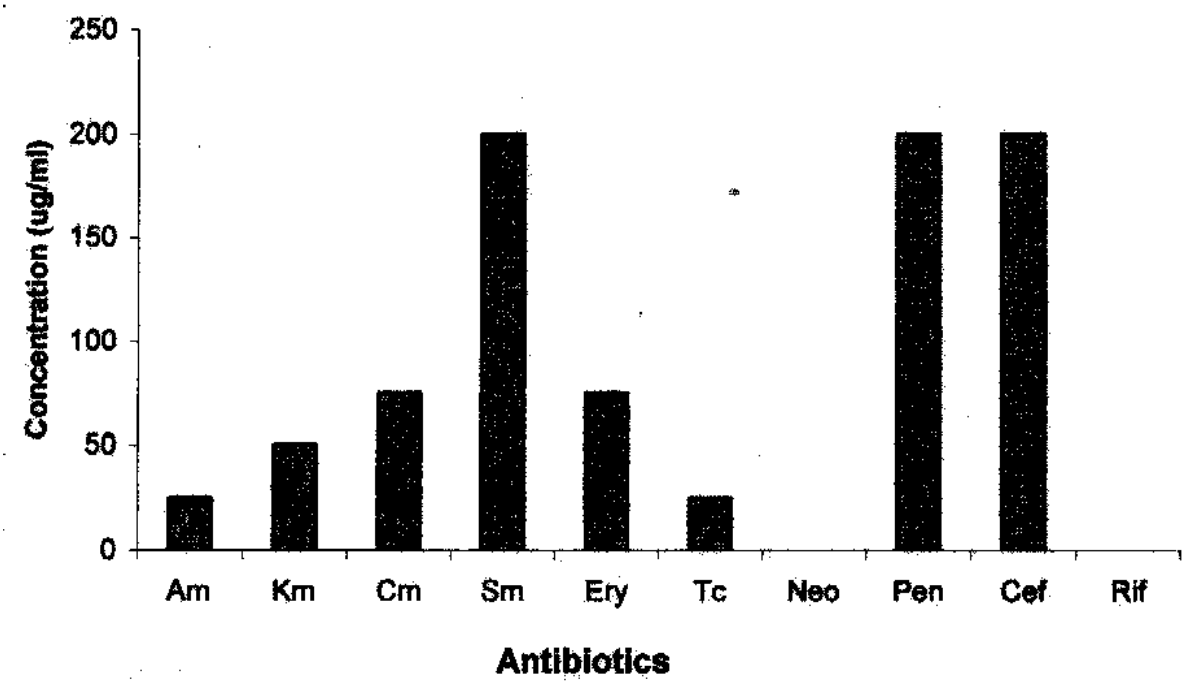

Fig.1. Antibiotic resistance in CMG500 (*Achromobacter* sp). Am. Cef, Cm, Km, Ery, Neo, Pen, Sm, Tc.

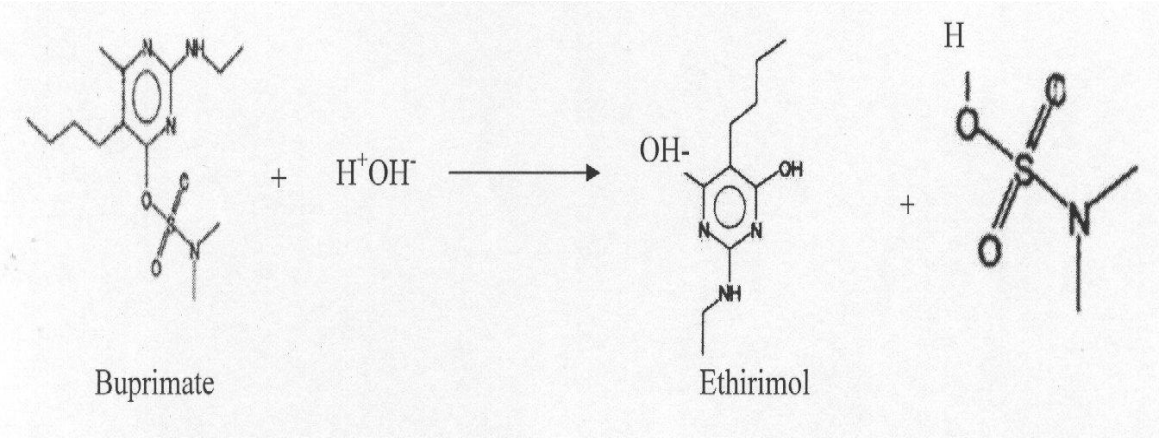

Fig. 2. Mass spectrum of burimate, A: buprimate from control samples. B; NIST mass spectrum of buprimate .

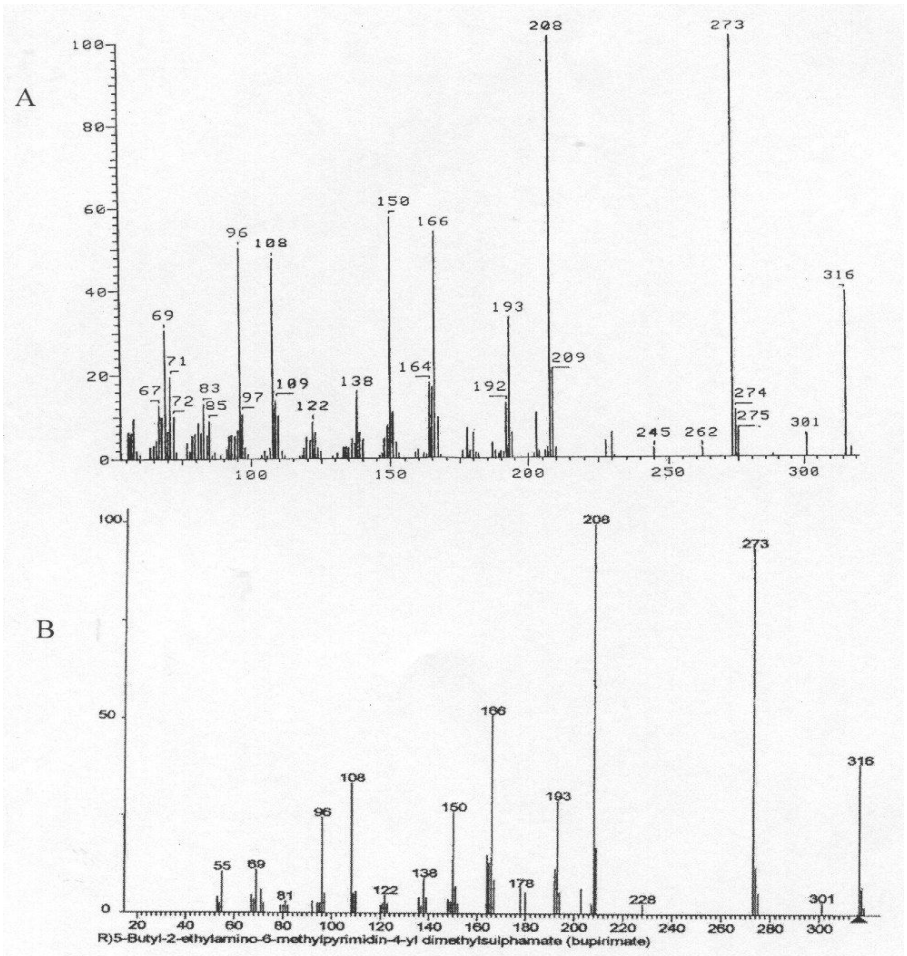

Fig. 3. Mass spectrum of ethirimol, A; mass spectrum of ethirimol from test samples, B; NIST reference mass spectrum of ethirimol.

Curing of plasmid DNA

Bacterial isolate CMG500 harbors a catabolic plasmid pNS3 which was cured by acridine orange at a concentration of $0.001gL^{-1}$. Curing of plasmid DNA resulted in the loss of buprimate degradation potentials of CMG500. This suggested that catabolic gene(s) are present on plasmid pNS3. Whereas genetic determinants for antibiotic resistance reside on chromosome as none of the antibiotic resistance marker was lost with plasmid curing.

Conjugation

Conjugation between bacterial strain CMG500 (buprimate degrading, Sm+, Pen+, Cef+, Rif-, Neo-) used as donor and E.*coli* J-53 (Am-, Sm-, Km-, Rif+) used as recipient strain has resulted in the Am-, Sm-, Km-, Rif+ and Bup+ i.e. buprimate degrading transconjugants.

DISCUSSION

An *Achromobacter* species isolated from the fungicide-contaminated soil of a vegetable field in Sind province of Pakistan is capable of degrading buprimate fungicide in nutrient-enriched environment. Curing and conjugation experiments have revealed that the genetic determinants for the buprimate degradation are present on conjugative plasmid pNS3. These findings correspond to the results of other workers (Ahmed and Afzal, 1992; Laemmli *et al.,* 2000; Mae *et al.,* 1993; Parekh *et al*., 1995; Hayatsu *et al.,* 1999) who have shown that degrading genes are plasmid borne. It has been reported that conjugal transfer of catabolic pathways has facilitated adaptation of microbial communities to the utilization of toxic chemicals in nature (Van der Meer, 1992) therefore, repeated application of pesticides have evolved microorganisms which have demonstrated, to be involved in the utilization of pesticides as

carbon/energy or nutrient sources and produce intermediate metabolites (Karens *et al*., 1986; Tam *et al.,* 1987; Racke and Coats, 1987; Muller *et al.,* 1989).

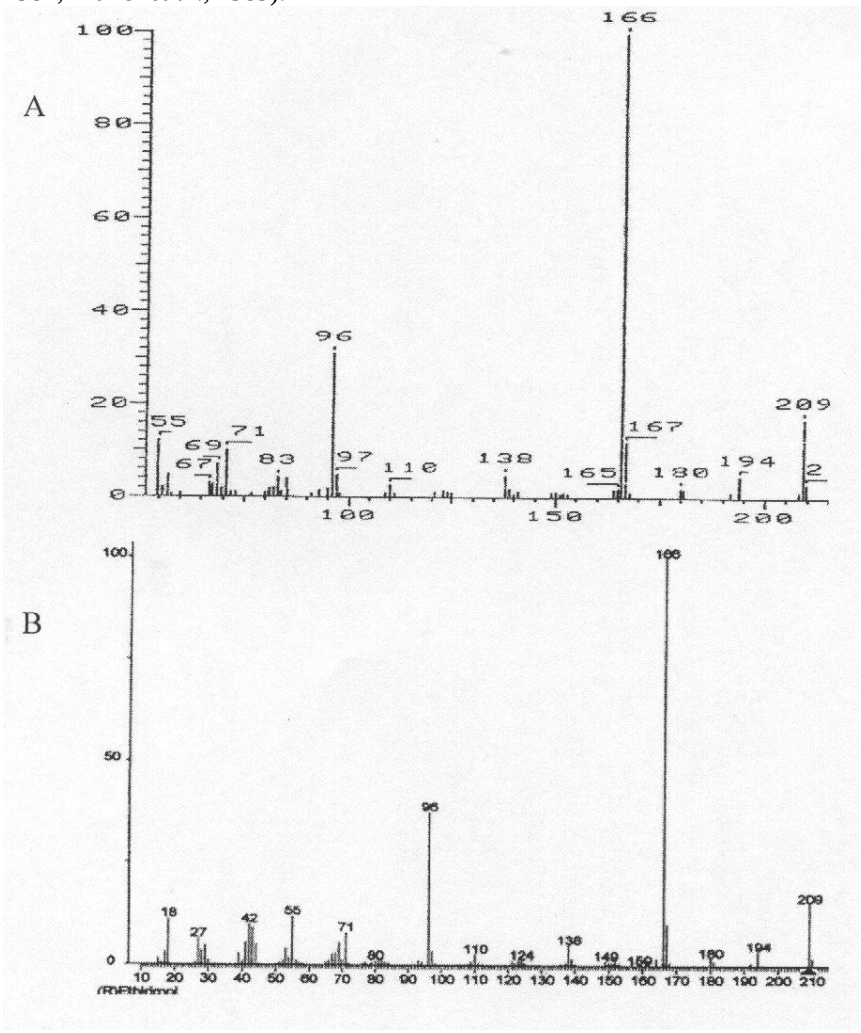

Fig. 4. Hydrolysis of buprimate into ethirimol.

Buprimate 5-butyl-2-(ethylamino)-6-methyl-4-pyrimidnyl dimethylsulfamate was hydrolyzed and degraded to the metabolite 5-butyl-2-ethylamino)-6-methyl-4-pyrmidinol by the indigenous soil isolate CMG500 (*Achromobacter* sp.) in this study. However no transformation of buprimate was found in the control samples without bacterial culture. Removal of dimethylsulphamoyl ester from buprimate has transformed it into highly action specific fungicide i.e. Ethirimol, which is active only against *Erysiphe graminis* a powdery mildew of barley. It has half-life less than a week in barely plants whereas buprimate has a wider spectrum of action against apple mildew and mildews of a number of soft fruits and horticulture crops. In most cases microbial metabolism of pesticides involves an initial ester hydrolysis followed by complete mineralization/incorporation of one or more of the hydrolysis products (Khan, 1995; Laemmli *et al.,* 200). Hydrolysis of carbofuran by *Achromobacter* sp. completely destroys its pesticide properties (Myra *et al*., 1987). The *Achromobacter* sp. WM111 is reported to rapidly degrade N-methylcarbamate insecticide by producing a hydrolyzing enzyme (Karens *et al.,* 1986). In general, pesticide-hydrolyzing enzymes seem to be able to act on a range of compounds with similar chemical linkages rather than on specific compounds. Examples include degradation of diuron, isoprotturon, chlorotoluron, linuron and monolinuronoccur herbicides through hydrolysis of the urea carbonyl group rather than by the usual route of successive demethylation by *Arthrobacter globiformis* strain D47 (Cullington and Walker, 1999). In the present study the hydrolysis of fungicide resulted in the formation of Ethirimol, which has a half life less than a week in plants when applied through roots. Break down of parent pesticide to another pesticide had been also reported in the case of diuron herbicide to 3,4-dichloroaniline (Cullington and Walker, 1999). Therefore, the term degradation in this study only refers to partial break down of parent buprimate molecule. It may also be an

enzymatic hydrolysis as there was no transformation of buprimate in the control samples (without CMG500). Like many other studies the genetic determinants were found on plasmid pNS3, which was cured and transferred to *E. coli* J-53 during conjugation. Since Pakistan is an agricultural country, where pesticides are used in hundreds of tons every year and their residues persist for long periods, therefore, such types of studies are important. This isolate if applied shall result in the production of ethirimol, which does not persist for long.

CONCLUSION

Partial degradation of fungicide buprimate by indigenous soil *Achromobacter* sp. into another but action specific fungicide indicates that microorganisms play direct or indirect role in the bioremediation of soils, either by complete mineralization of parent compound or by producing less persistent and action specific intermediate(s) in the course of degradation, such as transformation of buprimate into ethirimol.

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