ISOLATION OF AN ACETOCHLOR DETOXIFYING BACTERIUM AND CLONING OF AN ASSOCIATED GENE

by

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PREFACE

I hereby declare that unless specifically indicated this thesis is the result of my own investigation.

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ABSTRACT

A *Pseudomonas* strain, A108, which was capable of detoxifying the herbicide acetochlor (2-chloro-N-ethoxymethyl-6'-ethylacet-o-toluide) was isolated from soils. The microbe was isolated using a combination of batch culture enrichment techniques, phenotypic agar plate based assays and a qualitative bioassay for detecting acetochlor detoxification. With the aid of a bioassay developed specifically for the quantification of acetochlor concentrations, it was determined that over a 21 day period A108 was capable of detoxifying 20 % of the acetochlor present in a medium containing no other organic carbon and 53 % of the herbicide in a medium containing glucose and yeast extract at concentrations of 0.02 $g.l^{-1}$ and 0.005 $g.l^{-1}$ respectively. A fragment of A108 DNA was cloned in *Escherichia coli* which produced recombinant cells with both elevated acetochlor resistance and the ability to detoxify 15 % of the acetochlor present in a minimal nutrient medium (containing 0.02 $g.l^{-1}$ glucose and 0.005 $g.l^{-1}$ yeast extract) over a 21 day period. Partial sequencing of the cloned A108 DNA revealed that it encoded an amino acid sequence with significant homology with the dihydrolipoyltransacetylase component of the pyruvate dehydrogenase complexes of *Azotobacter vinlandii*, *E. coli* and *Alcaligenes eutrophus*. Theories are proposed as to the possible biochemical mechanisms whereby expression of the dihydrolipoyltransacetylase gene of A108 in recombinant *E. coli* cells may function in the detoxification of acetochlor.

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1 LITERATURE REVIEW

1.1 Introduction

The development of recombinant DNA technology is believed to be one of the most significant advances made in the history of cell biology. In the years immediately following the first successfully accomplished genetic engineering projects many researchers involved in crop improvement envisaged the production of crop varieties specifically and conveniently tailored with any of a virtually unrestricted supply of genes. It was soon realised, however, that ambitious objectives, such as the creation of crops capable of nitrogen fixation, could not be achieved until both improved recombinant DNA techniques were developed, and the depth of basic molecular genetic knowledge had increased. More modest ventures such as those involving the direct transfer of single dominant genes encoding herbicide resistance into crops, have contributed to a greater understanding of plant molecular genetics. This improved knowledge has in turn permitted the refinement of techniques for plant genetic manipulation.

Large scale release of xenobiotic[1] pesticides into the environment has occurred since the end of the second world war (Bartha, 1990). While many of these chemicals had prior to this time never been encountered by living organisms, most have been shown to undergo some form of metabolic transformation in at least a few members of every biological kingdom. Although in evolutionary terms 50 years is an extremely short period, it appears that the genetic systems of a variety of soil bacteria and fungi, either in isolation or as members of microbial communities, have developed the capacity to utilise many of these xenobiotic molecules as nutrient sources. The potential for bacterial genes which mediate the degradation of xenobiotic herbicides in the soil to be used in the engineering of herbicide resistance in crops has been realised in a number of instances (Buchanan-Wollaston *et al.*, 1992; Maier-Greiner *et al.*, 1991a; Stalker *et al.*, 1988; Streber and Willmitzer, 1989).

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[1]Defined by Bartha (1990) as being "synthesised organic substances that are in their composition and/or structure foreign to the world of organic products created by biosynthetic processes."

Because of the ongoing detection of herbicide residues in water supplies (Cartwrite *et al.*, 1991; Pimentel *et al.*, 1991) there is genuine concern that the production of herbicide tolerant crops will contribute to environmental contamination by permitting the excessive use of herbicides (Goldberg *et al.*, 1990; Hindmarsh, 1991). It has, however, been suggested that herbicide resistance derived from the incorporation into crop species of degradative genes which contribute to natural processes of herbicide mineralisation, could significantly decrease the environmental impact of increased herbicide application rates (Lyon, 1991). Also, the isolation of genes encoding herbicide degradation for purposes of crop improvement simultaneously makes these genes available for use in the production of microbial strains employable in the clean-up of herbicide contaminated soil and water.

In order to provide a means of genetically engineering resistance in crops to the xenobiotic herbicide, acetochlor, the aim of this study has been the isolation of an acetochlor degrading soil bacterium and the cloning and partial sequencing of a gene encoding this herbicide's detoxification.

1.2 Herbicide Usage in Agriculture

Every year competition between crops and weeds for water, light and nutrients is responsible for seriously reducing the world's agricultural output (Mazur and Falco, 1989). In the USA alone annual crop losses due to weed infestations account for a twelve percent reduction in total agricultural productivity. Despite spending in excess of $2.8 billion on weed control this decreased productivity costs American farmers an estimated $49 billion per year (Pimentel *et al.*, 1991). Because of the ability to selectively kill almost any weed species without damaging crop species, it is not surprising that herbicide treatment is the method of weed control most often employed in modern agriculture. For example, in the USA 85 % of all pesticides applied to major crops are herbicides (LeBaron, 1990). The use of herbicides for controlling the proliferation of weeds has become such an integral part of modern agricultural practice that an estimated 35% reduction in worldwide food production would occur if herbicide use was totally abandoned (Combellack, 1989). The employment of herbicides in weed control thus serves to significantly improve, at a reasonable cost, the quality and quantity of the world's agricultural output (Cork and Kreuger, 1991; Mazur and Falco, 1989).

There are, however, various commercial and ecological problems associated with the use of herbicides. One problem which decreases the commercial viability of many herbicides is that their use is restricted by the availability of tolerant crop species. Numerous instances exist in which herbicides that provide a broad spectrum of weed control cannot be used effectively because of their toxicity to crops (Lyon, 1991). While many herbicides do exist which selectively kill certain weeds without effecting the growth of certain crops, it is only very rarely that a herbicide is available which is selective enough to be used during the cultivation of a crop for the control of weed species closely related to the crop. A lack of tolerance among a few crop species to a particular selective herbicide may restrict its use when, for example, plants resistant to two different groups of herbicides are either grown in adjacent fields or in rotation with one another. Under these conditions herbicide drift between fields and herbicide carryover as a soil residue into the next growing season can seriously damage non-target crops. Farmers may, therefore, be compelled to use one of only a few, possibly unsatisfactory, herbicides (Lyon, 1991).

A second difficulty encountered with herbicide usage is that there is a tendency among weed species to develop a degree of resistance following repeated exposure to herbicides such as paraquat and the triazines (LeBaron and Gressel, 1982; Lehoczki *et al.*, 1992). This problem is complicated by the fact that the established approach for developing new herbicides to deal with these pests, is becoming increasingly ineffective. While in the 1950's the procedure of synthesising a chemical and screening it for herbicidal activity yielded one new herbicide for every two thousand compounds tested, by the 1980's the efficiency of this laborious process had decreased ten fold so that the examination of twenty thousand new chemicals was required to discover one useful herbicide (Lyon, 1991; Mazur and Falco, 1989).

Lastly, the most serious consequence of widespread herbicide usage is probably the ongoing contamination of water supplies. Monitoring programmes in South Africa (Hasset *et al.*, 1987) and other parts of the world where herbicides are widely used in agriculture, have revealed extensive herbicide contamination of both surface and ground-waters (Cartwrite *et al.*, 1991; Pimentel *et al.*, 1991). The possible health hazards posed by such widespread pollution will almost certainly lead to increased monitoring of agricultural chemicals in surface and ground waters and result in stricter regulations regarding both the

use of the herbicides detected and the maximum concentrations of these chemicals permissable in drinking water (Chesters *et al.*, 1989). The result of increased governmental control over herbicide use will be to provide substantial commercial incentives for the development of strategies permitting the effective use of more environmentally acceptable herbicides (Gasser and Fraley, 1989).

1.3 Genetic Manipulation of Crops and the Enhancement of Herbicide Effectiveness

Genetic manipulation of crop species by means of selective breeding has been carried out since the dawn of civilisation. More recently, however, developments in recombinant DNA technology have made it possible to directly alter the genetic characteristics of crop varieties. Both breeding and recombinant DNA techniques have been used to improve the tolerance of crops to a variety of herbicides. Despite the fact that most of the successes in this area have been achieved using traditional breeding practices, there are a number of reasons why, in the foreseeable future, conventional plant breeding may be largely surpassed in importance by more direct genomic manipulations. Firstly, the biochemical mechanisms responsible for herbicide selectivity are often both well understood and governed by the inheritance of single dominant genes which may be conveniently transferred using current recombinant DNA techniques (Mazur and Falco, 1989). Secondly, the direct transfer of herbicide resistance genes can potentially yield herbicide resistant crops far more rapidly than conventional breeding programs. Lastly, recombinant DNA technology permits the transfer of genes between completely unrelated species thus enormously broadening the range of genes available for use in improving the resistance of crops to herbicides.

Although ethical objections have been raised against the development of herbicide tolerant plants (Fox, 1990; Goldburg *et al.*, 1990; Hindmarsh, 1991), there are many ways in which herbicide resistant crop varieties may be constructively employed to help overcome many of the problems encountered with herbicide usage.

The most obvious requirement for increased herbicide resistance among crop varieties is to provide safeguards against crops being damaged by herbicides (Gasser and Fraley, 1989). Improved herbicide tolerance in crops which permits greater flexibility of herbicide

dosages may be especially important during times of worsening environmental stress when certain usually resistant crop varieties become increasingly sensitive to herbicides (Mazur and Falco, 1989).

The development of herbicide resistant crops may also provide solutions to the problem of herbicide tolerance that sometimes occurs among weeds following their long term exposure to certain herbicides. One simple solution would be to produce improved resistance in a crop which would then permit farmers to increase application rates in order to regain effective weed control (Mazur and Falco, 1989). A second solution would be to simultaneously transfer two genes providing resistance to two unrelated herbicides into a crop which could then be treated with both compounds. While this would decrease the probability of tolerance occurring among weeds, a sensible choice of the two herbicides could allow both to be applied at lower rates than if either was being applied alone (Mazur and Falco, 1989). A third solution would be to produce a crop variety that is resistant to a widely used herbicide against which tolerance in weeds is known not to occur. In fact, the cost effectiveness of providing a new market for an already established herbicide could compare very well with the price of either increased application rates or the development of a new herbicide (Comai and Stalker, 1984; Lyon, 1991).

The production of a number of crop species which are resistant to the same well established herbicides could allow farmers greater flexibility when selecting crop species for either rotations or double plantings (Mazur and Falco, 1989). Such broad crop tolerance could, by significantly improving herbicide selectivity, be employed to facilitate the use of herbicides that have wide spectrums of weed control, have short lifetimes in the soil, are effective at low application rates, do not leach from soils, are inexpensive and have no ill effects on non-target organisms in the environment (Burnside, 1992; Gasser and Fraley, 1989; Mazur and Falco, 1989).

1.4 Types of Herbicide Resistance

The sensitivity of an organism to any biocide is determined by the efficiency with which that biocide is able to interfere with cellular processes which are essential for the organism's survival. Like most other biocidal chemicals, herbicides function at the

molecular level by interacting with and disrupting integral components of cellular metabolism (Hatzios, 1987).

A number of mechanisms have been observed which may render organisms insensitive to the effects of herbicides. These include physiological barriers to herbicide uptake and translocation, alterations in the structure or production of herbicide targeted molecules and the expression of herbicide detoxifying genes. Because the toxicity of most herbicides is dependent upon their ability to bind with a specific receptor situated on either an enzyme or a structural protein, the basis of all these modes of resistance is the prevention of herbicide-receptor attachment. A fourth, more complex mechanism of herbicide resistance which has been observed involves the induction of metabolic pathways which actively reverse herbicide-induced cellular effects.

The failure of transport systems to bring herbicide molecules into contact with their target sites may afford plants a high degree of herbicide tolerance (Hatzios, 1987). Section a in Table 1.1 provides examples of modifications in herbicide uptake, translocation or cellular compartmentalisation providing plants with herbicide resistance.

A degree of herbicide tolerance is also displayed by plants and microbes containing herbicide targeted proteins which are either overexpressed (Donn *et al.*,1984; Rogers *et al.*, 1983; Shah *et al.*, 1986a) or lack the appropriate herbicide binding sites (Comai *et al.*, 1983). While these forms of resistance occur naturally, they have also been artificially induced in cultured plant cells and microorganisms of interest in herbicide resistance studies (Mullineaux, 1992). Examples of plants and microorganisms displaying a high degree of herbicide tolerance by virtue of alterations in the structure or production of herbicide targeted proteins are presented in section b of Table 1.1.

TABLE 1.1. Examples of the mechanisms responsible for the provision of resistance a variety of herbicides in different plant and microbial species

	Resistance Mechanism	Herbicide	Resistant Organism	Reference
a.	Altered Translocation, Uptake or Compartmentalisation	Atrazine	*Cucumis sativus*	Werner and Putnam, 1980
		Glufosinate	*Hordium* sp.	Mersey *et al.*, 1990
		Glyphosate	*Ligustum japonicum*	Neal *et al.*, 1986
		Paraquat	*Conyza linefola*	Feurst *et al.*, 1985
b.	Insensitive Target	Atrazine	*Amaranthus hybrius* *Solanum nigrum*	Hirschberg and McIntosh, 1983 Goloubinoff *et al.*, 1984
		Chlorsulfuron	*Salmonella typhimurium* *Arabidopsis thaliana* *Kochia scoparia*	LaRossa and Schloss, 1984 Haughn and Somerville, 1986 Saari *et al.*, 1990
		Glyphosate	*Salmonella typhimurium* *Lotus corniculatus*	Comai *et al.*, 1983 Boerboem *et al.*, 1990
	Overproduced Target	Glufosinate	*Medicago sativa*	Donn *et al.*, 1984
		Glyphosate	*Daucus carota* *Petunia hybrida*	Amrhein *et al.*, 1983 Shah *et al.*, 1986a
c.	Detoxification	Acetochlor	*Zea mays*	Breux, 1987
		Atrazine	*Zea mays*	Guddewar and Dauterman, 1979
		Bromoxynil	*Viola arvensis*	Saunders and Pallett, 1985
		Chlorsulfuron	*Solanum nigrum*	Hutchenson *et al.*, 1984
		2,4-D	*Lotus corniculatis*	Davis, 1984
d.	Corrective Metabolism	Paraquat	*Conyza canadensis*	Lehoczki *et al.*, 1992
		Dichlofop methyl	*Lolium rigidum*	Holtum *et al.*, 1990

Another mechanism resulting in herbicide tolerance is the occurrence in most organisms of metabolic pathways specifically aimed at the detoxification of harmful molecules. These pathways, by transforming the structure of herbicide molecules, may effectively prevent herbicides interfering with cellular activities. In plants the basis for the selective toxicity of most currently used herbicides is dependent upon differential rates of detoxification in

tolerant and sensitive species (Hatzios, 1987). Crop species resistant to particular herbicides by virtue of efficient detoxification pathways are shown in section c of Table 1.1.

Finally, tolerance to certain herbicides occurs in some plants due to corrective metabolism which is able to repair or reverse herbicide-induced cellular damage (Holtum *et al.*, 1990; Lehoczki *et al.*, 1992). Because of its relative rarity and the potential complexity of the diverse mechanisms involved, this mode of resistance has not been extensively documented and is still poorly understood. Two examples which have been studied are listed in section d of Table 1.1.

1.5 The Introduction of Herbicide Resistance into Plants Using Recombinant DNA Technology

The only characteristics which may be conveniently transferred using current recombinant DNA techniques are those which are determined by one or, at most, a few dominant genes. Because of both the monogenic inheritance of certain herbicide resistance genes and the relative ease with which transformants containing such genes may be isolated, the engineering of herbicide resistance was one of the first areas in which recombinant DNA techniques were successfully applied (Mazur and Falco, 1989). Being dependent on the use of dominant genes, attempts to engineer herbicide resistance in crops have focused almost exclusively on the transfer of genes coding for either altered catalytic enzymes targeted by herbicides or detoxification enzymes (Comai and Stalker, 1984).

The first successful incorporation of a herbicide resistance gene into a crop variety using recombinant DNA technology was achieved by Comai and coworkers who, in 1985, transferred a glyphosate resistance gene from *Salmonella typhimurium* into tobacco (*Nicotiana tabacum*). The primary target of the herbicide glyphosate is the enzyme 5-enolpyruvyl-shikimate-3-phosphate synthase (EPSPS) which is involved in aromatic amino acid synthesis. A mutant form of *S. typhimurium*'s *aroA* gene, encoding an overexpressed and glyphosate insensitive EPSPS was used to supply transgenic tobacco plants with increased glyphosate resistance.

The feasibility of a plant obtaining substantial herbicide resistance through the acquisition of a foreign gene encoding a herbicide detoxifying enzyme was first demonstrated by DeBlock *et al.* (1987). By incorporating into tobacco, potato and tomato a *Streptomyces hygroscopicus* gene encoding an acetyl transferase which specifically detoxified the herbicide glufosinate, DeBlock and coworkers were able to supply these plants with a high degree of glufosinate resistance. Examples of other successful genetic engineering projects that have aimed at providing crops with herbicide resistance are listed in Table 1.2.

Recently, genes from both tomato and pea, encoding Cu,Zn superoxide dismutases functional in oxidative stress defence, have been transferred into potato and tobacco respectively. These genes have been observed to provide the transgenic plants in which they were expressed with slightly increased tolerance to the superoxide generating herbicide, paraquat (Perl *et al.*, 1993; Gupta *et al.*, 1993). Research in this area has, however, not been concerned with the production of paraquat resistant crops but has rather aimed at the investigation of how different levels of superoxide dismutase expression protect cells from oxidative stresses in general. Because transferred genes encoding corrective metabolism are unlikely to provide a practical means of engineering useful levels of herbicide resistance in crops, they will not be considered as potential herbicide resistance genes in this review.

1.5.1 Considerations when Selecting a Mechanism for Engineering Herbicide Resistance

A number of factors should be considered before deciding whether either an altered herbicide target gene or a herbicide detoxifying gene should be isolated for use in a crop improvement project. If use is to be made of an altered target gene it is necessary to determine whether enough information is available on the herbicide's mode of action to facilitate isolation of the gene. The reason for this is that, unlike the detoxifying enzyme approach, the altered target approach to engineered herbicide resistance cannot be used until the biochemical site of action of the herbicide has been identified and characterised (Lyon, 1991; Oxtoby and Hughes, 1989). Apart from this information being essential for isolating the altered target gene required, it also indicates, firstly, whether or not the herbicide has more than one target and, secondly, whether the herbicide targets a catalytic or a non-catalytic protein. An attempt to engineer resistance to a herbicide which has

TABLE 1.2. Genes encoding herbicide resistance, their sources and the crops into which they have been successfully transferred

Mechanism of Engineered Resistance	Herbicide	Source of gene	Gene Transferred	Recipient Plant	Reference
Altered Target	Glyphosate	*Salmonella typhimurium*	Mutant *aroA* (coding for EPSPS)	Tobacco Tomato Poplar	Comai *et al.*, 1985 Fillatti *et al.*, 1987 Fillatti *et al.*, 1988
			Mutant *aroA*-transit sequence fusion[a]	Tobacco	Larson-Kelly *et al.*, 1988
		Escherichia coli	Mutant *aroA*-transit sequence fusion[b]	Tobacco	Della-Cioppa *et al.*, 1987
		Petunia hybrida	Gene coding for EPSPS[c,d]	Petunia	Shah *et al.*, 1986a
	Sulphonylurea herbicides	*Nicotiana tabacum*	SuRB-Hra(coding for mutant acetolactate synthase)[d]	Tobacco Tomato	Mazur *et al.*, 1987a
		Arabidopsis thaliana	*csr1* (coding for a mutant acetolactate synthase)[d]	Tobacco Flax Poplar Sugar beet Chicory	Haughn *et al.*, 1988 McHughen, 1989 Brasileiro *et al.*, 1992 D'Halluin *et al.*, 1992 Vermeulen *et al.*, 1992
		Brassica napus	*ahas3r* (coding for a mutant acetolactate synthase)[d]	Tobacco	Brandle *et al.*, 1994
	Imidozolinone herbicides	*Arabidopsis thaliana*	*imr1* (coding for a mutant acetolactate synthase)[d]	Tobacco	Hattori *et al.*, 1992
	Atrazine	*Amaranthus hybrius*[e]	*psbA* (coding for Q_B protein)-transit sequence fusion[a]	Tobacco	Cheung *et al.*, 1988
		Solanum nigrum[e]	*psbA*	Soyabean[f]	Liu *et al.*,1990
	Glufosinate	*Medicago sativa*	Gene coding for Glutamine synthetase[c]	Tobacco	Eckes *et al.*, 1989
	Asulam	*Escherichia coli*[g]	*sul1* (coding for dihydropteroate synthase)-transit sequence fusion[a]	Tobacco	Guerineau *et al.*, 1990
	Norflurazon	*Erwinia uredovora*	*crtI* (coding for phytoene desaturase)[ac]	Tobacco	Misawa *et al.*, 1993
Detoxification	Glufosinate	*Streptomyces hygroscopicus*	*bar* (coding for a phosphinothricin acetyl transferase)[c]	Tobacco Potato Tomato Alfalfa Maize Cauliflower Rice Poplar Wheat Tall fescu Sugar cane Oats Pea	DeBlock *et al.*, 1987 D'Halluin *et al.*, 1990 Gordon-Kamm *et al.*, 1990 Mukhopadhyay *et al.*, 1991 Cao *et al.*, 1992 DeVillard, 1992 Vasil *et al.*, 1992 Wang *et al.*, 1992 Chowdhury and Vasil, 1992 Somers *et al.*, 1992 Russell *et al.*, 1992
		Streptomyces viridochromogenes	*pat* (coding for Phosphinothricin acetyl transferase)[c]	Tobacco Alfalfa Carrot	Wohlleben *et al.*, 1988 Donn *et al.*, 1990 Droge *et al.*, 1992

TABLE 1.2. (Cont.)

Mechanism of Engineered Resistance	Herbicide	Source of gene	Gene Transferred	Recipient Plant	Reference
Detoxification	Bromoxynil	*Klebsiella ozaenae*[a]	*bxn* (coding for a bromoxynil specific nitrilase)[b]	Tobacco	Stalker *et al.*, 1988
	2,4-D	*Alcaligenes eutrophus*[a]	*tfdA* (coding for a 2,4D monooxygenase)[c]	Tobacco Cotton	Streber and Willmitzer, 1989 Lyon *et al.*, 1989 Bayley *et al.*, 1992
	Dalapon	*Pseudomonas putida*	*dehI* (coding for a dehalogenase)	Tobacco	Buchanan-Wollaston *et al.*, 1992
	Cyanamide	*Myrothecium verrucaria*	*cah* (coding for Cyanamide hydratase)[c]	Tobacco	Maier-Greiner *et al.*, 1991a

[a] Chloroplast transit sequence from a ribulosebisphosphate carboxylase (RuBisCo) small subunit gene.
[b] Chloroplast transit sequence from the EPSPS gene of petunia.
[c] Chimeric gene construct containing the Cauliflower Mosaic Virus 35s promoter.
[d] Gene transferred together with its native transit sequence.
[e] Gene isolated from the chloroplast genome.
[f] Gene transferred directly into chloroplast genome.
[g] Gene isolated from a naturally occurring plasmid.
[h] Chimeric gene construct containing the light inducible promoter and transit sequence of the RuBisCo small subunit gene from tobacco.

more than one target would, at present, be impractical using genes encoding altered herbicide targets because it would require the isolation and transfer of more than one gene. Similarly, attempting to engineer herbicide resistance using genes coding for altered proteins with no catalytic function would be ineffective because these genes are not dominant (Comai and Stalker, 1984). For example, efforts to engineer resistance in tobacco to the photosynthesis inhibiting herbicide, atrazine, have met with little success using a gene from *Arabidopsis thaliana* which encodes an insensitive form of this herbicide's target; a thylakoid membrane polypeptide component of the photosystem II complex known as the quinone binding or Q_β protein. The reason that this gene provided only a marginal improvement in resistance in transgenic plants was that these plants produced both the sensitive native and the insensitive foreign forms of the Q_β protein. It was probable, therefore, that a substantial proportion of the photosystem II reaction centres in transgenic plants contained the sensitive native form of Q_β. In the presence of atrazine these reaction centres would have become inoperative once the Q_β they contained became bound to molecules of the herbicide (Cheung *et al.*, 1988).

Another factor when considering the altered target mechanism approach to engineered resistance is that the exact cellular location of herbicide targeted enzymes is significant. Because of compartmentalisation within eukaryotic cells it will often be necessary when

working with altered target resistance genes to splice them into DNA sequences encoding transit peptides which will direct their enzyme products to the correct cellular compartments (Cheung *et al.*, 1988; Della-Cioppa *et al.*, 1987; Guerineau *et al.*, 1990). In this regard, the quality of the glyphosate resistance in transgenic tobacco achieved by Comai *et al.* (1985) was relatively poor since the introduced gene's product, EPSPS, was cytoplasmically active while the native enzyme functioned within chloroplasts. *In vitro* studies carried out by Della-Cioppa *et al.* (1987) demonstrated that the transcription of a mutant *Escherichia coli* gene encoding a glyphosate resistant EPSPS which had been attached to the transit sequence of the petunia (*Petunia hybrida*) EPSPS, yielded a stable herbicide insensitive enzyme which was proteolytically processed to release the transit peptide following its transportation into chloroplasts. In order to improve on the degree of glyphosate resistance achieved by Comai *et al.* (1985), Larson-Kelley *et al.* (1988) constructed a chimeric gene containing a mutant *S. typhimurium aroA* gene, the first 72 base pairs of the spinach (*Spinacia oleracea*) ribulosebisphosphate carboxylase (RuBisCo) small subunit gene and the RuBisCo small subunit gene's chloroplast transit sequence. Upon insertion of the construct into tobacco, glyphosate resistance improved due to successful importation of the foreign EPSPS into the plant's chloroplasts (Larson-Kelly *et al.*, 1988). In instances where it is exceedingly difficult to direct altered herbicide insensitive enzymes to their correct cellular locations (such as when the enzymes occur in two different cell compartments), the only practical means of engineering resistance will be through the use of either detoxifying genes or altered plant target genes already carrying the appropriate transfer sequences (Haughn *et al.*, 1988). The appeal of the detoxification approach under such circumstances is that herbicide resistance is achievable through detoxification in the cytosol irrespective of where the herbicide's target resides (Comai and Stalker, 1984).

A final consideration when altered plant target genes are to be used for engineering herbicide resistance is that it may be necessary to determine whether their expression is tissue specific. Due to the occurrence in eukaryotes of multiple copies of certain genes encoding isoenzymes, the expression of all but one of the genes may be suppressed in any particular tissue. During efforts to introduce glufosinate resistance into tobacco using an altered glutamine synthetase (GS) gene from alfalfa (*Medicago sativa*), it was found that

transgenic plants were only glufosinate resistant if the herbicide was applied to their roots and not their foliage (H. M. Goodman, unpublished data; cited by Mazur and Falco, 1989). Mazur and Falco (1989) have proposed that this differential resistance could be explained if, as is the case with *Phaseolus vulgaris*, there are multiple copies of GS genes within the tobacco genome. In this case it might have meant that because the regulatory region of the transferred alfalfa gene resembled that of the gene encoding a GS isozyme expressed in tobacco roots but not tobacco leaves, resistance afforded by the gene was restricted to the root tissues. In order to obtain whole plant resistance in this system it would therefore be necessary to either transfer several different GS genes or create an altered constitutively expressed alfalfa GS gene.

As is the case with the use of genes encoding altered herbicide targets, when planning the incorporation of genes encoding herbicide degradation into plants there are also a number of factors which require consideration. One of these being that the toxicological properties of metabolites produced during herbicide breakdown need to be taken into account. In some cases it may be observed that the primary breakdown products in a herbicide degradation pathway are either toxic themselves or they cannot be properly metabolised and therefore accumulate to toxic levels in plant cells. When use is made of a gene encoding the first step of such a pathway, these initial degradation products, by virtue of either their intrinsic toxicity or the toxic effects of their accumulation in plant tissues, will greatly diminish the gene's resistance value. In such cases either the gene must be discarded and a more desirable one sought, or the gene should be transferred in conjunction with genes encoding further steps in the degradation pathway (Comai and Stalker, 1984).

Another point which should be kept in mind when use is made of detoxification genes for engineering herbicide resistance, is that foreign degradative enzymes may possibly interfere with native plant metabolic processes (Comai and Stalker, 1984). In this regard, Stalker *et al.* (1988) took preemptive steps to avoid any likelihood that a bromoxynil resistance gene from *Klebsiella ozaenae* would interfere with the native metabolism of transgenic tobacco plants expressing the gene. These researchers linked a gene encoding bromoxynil degradation to a specifically inducible promoter. Because bromoxynil is a

photosystem II inhibiter it was correctly predicted that in order to achieve bromoxynil resistance, degradation of the herbicide need only occur in cells containing chloroplasts. The promoter selected by Stalker *et al.* (1988) was that of the tobacco light-inducible, tissue-specific RuBisCo small subunit gene. In herbicide resistant transgenic plants the promoter effectively restricted the production of bromoxynil degrading enzymes to the photosynthetic tissues.

A final factor which should be considered when attempting to use genes encoding herbicide detoxification as herbicide resistance genes, is that the rate at which detoxifying enzymes are able to degrade herbicide molecules may be of importance. Because of the low affinities between herbicides and many of their degradative enzymes, in many instances a substantial proportion of herbicide molecules will only be detoxified after extensive lag periods (Comai and Stalker, 1984). An essential variable determining the degree of tolerance displayed by plants to many herbicides is the rate at which their natural detoxifying systems are able to function (Hatzios, 1987). Comai and Stalker (1984) have suggested that unless the affinities of many degradative enzymes for their corresponding herbicides are improved through mutagenesis they will be unable to function efficiently in the provision of resistance. It has, however, been demonstrated during the engineering of bromoxynil resistance (Stalker *et al.*, 1988) and glufosinate resistance (DeGreef *et al.*, 1989) in tobacco, that very low levels of expression of detoxifying genes were sufficient to provide plants with herbicide resistance at rates recommended for agricultural application. While complete resistance to bromoxynil at its recommended application rate was conferred by genes giving rise to detoxifying enzyme levels between 0.01 and 0.0007 percent of total cell protein (Stalker *et al.*, 1988), transgenic tobacco containing glufosinate degrading enzymes at levels between 0.1 and 0.001 percent of total cell protein were resistant to glufosinate at four times its recommended application rate (DeGreef *et al.*, 1989). This indicates that there may be a permissable degree of flexibility regarding the herbicide degradation rates that will provide plants with herbicide resistance.

The consensus among most workers using recombinant DNA technology for the improvement of herbicide resistance in crops, is that the introduction of herbicide degrading genes is the most promising approach to engineering agriculturally useful levels

of herbicide resistance. Because of the various considerations discussed, it will in most cases be more difficult to successfully engineer resistance to herbicides when using genes encoding altered herbicide targets than when using genes encoding herbicide degrading enzymes (Comai and Stalker, 1984). Also the degree of resistance afforded transgenic plants by altered target genes has generally been lower than that achieved by introducing detoxifying genes. Although significant improvements in herbicide tolerance have been achieved using both types of resistance gene, the use of detoxifying genes has been the more successful with regard to the production of transgenic plants with resistance to herbicides applied at their maximum recommended dosages (Table 1.3).

TABLE 1.3. Improvement in herbicide resistance in crops achieved through recombinant DNA technology

Mechanism of Resistance	Herbicide	Transgenic Plant	Improvement in Resistance (fold)	Recommended Application Rate (kg.hectare^{-1})	Tolerance[a] (kg.hectare^{-1})	References
Altered Target	Glyphosate	Tobacco	-[b]	0.3-2.2	0.6	Comai *et al.*, 1985
		Tomato	-	0.3-2.2	0.8	Fillatti *et al.*, 1987
		Petunia	4.0	0.3-2.2	0.9	Shah *et al.*, 1986a
		Tobacco	-	0.3-2.2	1.3	Larson-Kelly *et al.*, 1988
	Chlorsulfuron	Tobacco	1000	0.004-0.026	0.032	Mazur *et al.*, 1987a
		Flax	-	0.004-0.026	0.012	McHughen and Holm, 1991
			260	0.004-0.026	0.020	McSheffrey *et al.*, 1992
	Atrazine	Tobacco	1.3	1.5-2.6	-	Cheung *et al.* ,1988
	Glufosinate	Tobacco	20	1.0-2.0	<2.0	Eckes *et al.*, 1989
Detoxification	Glufosinate	Tobacco	-	1.0-2.0	4.0	DeBlock *et al.*, 1987
		Alfalfa	>40	1.0-2.0	1.6	D'Halluin *et al.*, 1988
		Sugar beet	-	1.0-2.0	1.2	D'Halluin *et al.*, 1990
		Poplar	-	1.0-2.0	4.8	DeVillard, 1992
	Bromoxynil	Tobacco	>100	0.4-0.6	4.5	Stalker *et al.*, 1988
			-	0.4-0.6	7.0	Freyssinet *et al.*, 1989
	2,4-D	Tobacco	>30	0.3-2.3	10.0	Streber and Willmitzer, 1989
			10	0.3-2.3	0.7	Lyon *et al.*, 1989
		Cotton	900	0.3-2.3	1.5	Bayley *et al.*, 1992

[a] Highest measured herbicide dosage at which the growth and development of transgenic plants remains unaffected.
[b] No information available.

1.5.2 Isolation of Herbicide Resistance Genes

Of primary importance in any project aimed at the improvement of herbicide tolerance in plants is the availability of a suitable source of resistance genes. These may originate from plants or microbes either with preexisting resistance or which, through prolonged exposure to herbicides, have developed a degree of herbicide tolerance (Comai and Stalker, 1984). Possibly because of the relative ease with which their genes can be manipulated, microorganisms have provided most of the foreign herbicide resistance genes currently expressed in genetically engineered crops (Table 1.2). However, the production and processing of certain complex bacterial proteins (such as oligomeric enzymes) by plant cells may be problematic. In bacteria, for example, the enzyme acetolactate synthase (ALS) which is targeted by sulphonylurea and imidoazolinone herbicides, comprises at least two individually translated subunits (Eoyang and Silverman, 1984; Schloss *et al.*, 1985). It has been suggested that achieving the correct expression and assembly of active microbial ALS's in transgenic plants would be incredibly difficult (Mazur and Falco, 1989). Therefore, from the perspective of engineering sulphonylurea tolerant crops, the value of genes from bacteria encoding herbicide insensitive forms of ALS could be very limited (Mazur and Falco, 1989). It is for this reason that Mazur *et al.* (1987a) and Haughn *et al.* (1988) made use of mutant ALS genes from plant sources for the development of sulphonylurea resistant tobacco.

The procedures followed for the isolation of herbicide resistance genes have depended upon whether the genes coded for altered target or detoxifying enzymes. In order to indicate some of the techniques involved in the isolation or construction of herbicide resistance genes, the methods employed in obtaining most of the genes successfully transferred into, and expressed in, crops (Table 1.2) will be briefly discussed beginning with the altered target resistance genes.

While altered herbicide target genes have been isolated from naturally occurring herbicide resistant organisms (Guerineau and Mullineaux, 1989; Hirschberg and McIntosh, 1983; Misawa *et al.*, 1990), they have also been isolated from normally sensitive plants or microbes which have been specifically selected for improved herbicide resistance (Comai

et al., 1983; Haughn *et al.*, 1988). As an alternative to their isolation, altered herbicide target resistance genes have also been constructed *in vivo*. This has been achieved by coupling genes encoding herbicide sensitive enzymes to promoters which have ensured their overexpression in transgenic plants (Eckes *et al.*, 1989; Shah *et al.*, 1986a).

Naturally occurring genes encoding herbicide insensitive target enzymes have been isolated for use in the transfer of asulam, atrazine and norflurazon resistance into crops. Asulam, a sulphonamide herbicide, functions by inhibiting dihydropterate synthase (DHPS), an enzyme involved in the synthesis of folic acid. The *sul1* gene, encoding a sulphonylurea resistant DHPS, was obtained from the antibiotic resistance plasmid R46. During efforts to produce a physical and genetic map of R46 the gene was localised to a 1.4 kilobase (Kb) region of DNA (Brown and Willetts, 1980). A 2.8-Kb fragment of R46 containing the *sul1* gene was first cloned into pBR322 and then sequenced (Guerineau and Mullineaux, 1989).

An atrazine resistance gene, *psbA*, encoding a Q_B protein insensitive to this herbicide was obtained from the chloroplast genome of a naturally atrazine resistant *Amaranthus hybrius* strain. The chloroplast DNA of *A. hybrius* was digested with EcoRI, electrophoresed and blotted onto a nitrocellulose membrane. Following identification of a 3.68-Kb restriction fragment carrying the *Amaranthus psbA* gene using the maize *psbA* gene as a probe, the fragment was cloned in pBR322 (Hirschberg and McIntosh, 1983).

The *Erwinia uredovora* gene encoding a naturally norflurazon insensitive carotenoid biosynthetic enzyme, phytoene desaturase, has been transferred into tobacco and shown to provide elevated levels of norflurazon resistance in transgenic plants (Misawa *et al.*, 1993). The gene, *crtI*, was isolated during the course of a study to elucidate the carotenoid biosynthetic pathway of *E. uredovora*. An *E. uredovora* genomic library was constructed in *E. coli* DH-1 using the cosmid vector, pJB8. *E. coli* cells infected with recombinant pJB8 DNA carrying the entire carotenoid biosynthetic gene cluster were identified by virtue of the fact that expression of the carotenoid biosynthetic genes resulted in colonies with yellow pigmentation. One of the plasmids isolated from cells containing the complete gene cluster was partially digested with Sau 3A and recloned into pUC19.

Recombinants containing the entire compliment of carotenoid biosynthetic genes where again identified according to the yellow pigmentation of their colonies. Of 50 positive recombinant plasmids encoding carotenoid biosynthesis which were examined, the smallest insert found which enabled transformants to synthesise the full complement of carotenoids produced by *E. uredovora*, was 8.2-Kb in size. Subcloning localised the gene cluster to a 6.9-Kb HindIII-KpnI fragment. The 6.9-Kb fragment was sequenced to reveal six open reading frames. By first constructing 14 different plasmids containing deletions in one or more of these open reading frames and then examining the end products of carotenoid biosynthesis produced by these deletion mutants, the *crtI* open reading frame was identified (Misawa *et al.*, 1990).

Mutant organisms have been isolated in which binding of the herbicides glyphosate and sulfometuron methyl (a sulphonylurea herbicide) to their respective target enzymes, is prevented. These mutants have been used as sources of glyphosate and sulfometuron methyl resistance genes. Comai *et al.* (1983) were able to isolate a mutant *Salmonella typhimurium* strain which was highly resistant to glyphosate by subjecting a sensitive strain to two cycles of chemical mutagenesis interspersed with selection at high concentrations of the herbicide. The mutant strain's glyphosate insensitive EPSPS gene, *aroA*, was then isolated by constructing an *S. typhimurium* genomic library in an *aroA* deficient mutant *E. coli* strain and selecting for the mutant *aroA*'s presence by complimentation on an aromatic amino acid deficient medium. Following selection of *aroA* positive transformants it was possible to show that they possessed an elevated degree of glyphosate tolerance. It was demonstrated that two distinct mutations were responsible for the resistant phenotype. The first mutation, providing a low level of glyphosate resistance, was traced to the *aroA* promoter and was responsible for increasing the gene's expression. The second mutation, producing a substitution of serine by proline at residue 101 of the EPSPS enzyme, decreased the ability of glyphosate to bind with the enzyme (Comai *et al.*, 1983).

Mutations in the acetolactate synthase (ALS) genes of *E. coli*, *S. typhimurium* and the yeast, *Saccharomyces cerevisiae*, have been extensively studied with regard to the resistance they provide to the sulphonylurea herbicides (Falco and Dumas, 1985; LaRossa and Schloss, 1984; Yadav *et al.*, 1986). The yeast gene encoding ALS was isolated from

a genomic library constructed in a high copy number yeast plasmid cloning vector. Following transformation of yeast cells with recombinant plasmids, cells harbouring amplified ALS genes were isolated by selecting for their elevated resistance to low concentrations of sulfometuron methyl (Falco and Dumas, 1985). Using the yeast ALS gene as a heterologous probe it was possible to isolate the *Arabidopsis thaliana* and tobacco wild type ALS genes (Mazur *et al.*, 1987b). Two approaches were used to obtain mutants of these genes which would encode sulphonylurea resistance. The first, employed the wild type plant ALS genes as a probe for the detection of mutant ALS genes from the genomic libraries of mutant sulphonylurea resistant tobacco and *A. thaliana* strains (Falco *et al.*, 1989; Lee *et al,* 1988). The second approach drew on information obtained during the study of resistance mutations in yeast ALS genes, and concentrated on the production of mutant herbicide resistant tobacco ALS genes by site directed mutagenesis (Falco *et al.*, 1989).

The *in vitro* construction of glyphosate and glufosinate resistance genes has been accomplished by coupling genes encoding enzymes sensitive to these herbicides to promoters which will ensure their over-expression. In a three-stage isolation procedure Shah and co-workers (1986a) were able to isolate a petunia (*Petunia hybrida*) EPSPS gene for use in the construction of a glyphosate resistance gene. Since it was known that cultured plant cells selected for glyphosate resistance overproduced EPSPS (Amrhein *et al.*, 1985), the first stage of the isolation involved the selection of a glyphosate resistant petunia cell line containing EPSPS enzyme concentrations 20 times higher than normal. The EPSPS enzyme produced by the cell line was purified and its N-terminal amino acid sequence was determined by microsequencing. In the second step of the isolation procedure three sets of oligonucleotide probes were synthesised using the microsequencing data and tested for their ability to hybridise with northern blotted RNA isolated from the EPSPS overproducing cell line. The oligonucleotide displaying the highest degree of homology was selected as a probe and used to identify a partial EPSPS cDNA clone. In the last isolation step this partial clone was used as a probe for the identification of a complete petunia EPSPS cDNA clone and its 226 base pair chloroplast transit sequence. Glyphosate resistance was obtained by coupling this gene to the Cauliflower Mosaic virus 35s promoter which, upon expression in transgenic petunia plants, resulted in a 20 fold

increase in active EPSPS concentrations within the plant's chloroplasts (Shah *et al.*, 1986a). Additional herbicide resistance has since been achieved through mutation of the overexpressed wild type petunia enzyme to a glyphosate insensitive form (Eichholtz *et al.*, 1989).

The successful use of an overexpressed gene encoding a herbicide sensitive target as a herbicide resistance gene has also been accomplished in the engineering of plants resistant to the herbicide glufosinate (Eckes *et al.*, 1989). Overproduction of glutamine synthetase (GS), the target of glufosinate, was found to occur in alfalfa cell lines selected for tolerance to this herbicide. As was the case with the isolation of petunia EPSPS, a cell line overproducing GS was used to simplify isolation of the enzyme so that part of its amino acid sequence could be determined (Donn *et al.*, 1984). During experiments to demonstrate the tissue specific expression of pea (*Phaseolus vulgaris*) GS genes, immunopurified GS mRNA from pea root nodule polysomes (which contain GS as two percent of their total protein; Cullimore and Miflin, 1983) was used to construct a probe for the isolation of pea GS cDNA clone from a pea root nodule cDNA library (Cullimore *et al.*, 1984). This clone was subsequently used by Donn *et al.* (1984) as a probe for the isolation of a partial alfalfa GS cDNA clone. The identity of the clone was verified by comparing the amino acid sequence it encoded with that of the alfalfa GS enzyme which they had previously determined. Using information on the sequence of the partial GS cDNA clone, Tischer *et al.* (1986) produced a 27 base pair DNA probe to facilitate the isolation of a complete GS cDNA clone from an alfalfa cDNA library. As was the case with the petunia EPSPS gene which was used for the production of glyphosate resistance, glufosinate tolerance in transgenic tobacco plants containing the complete alfalfa GS gene was afforded by the high level of the gene's expression achieved as a result of its attachment to the Cauliflower Mosaic Virus 35s promoter (Eckes *et al.*, 1989).

Unlike the procedures adopted for isolating altered herbicide target genes, the isolation of herbicide detoxifying genes for use in the engineering of herbicide resistance in crops has been exclusively from microbial sources. The herbicide degrading microorganisms which have yielded useful herbicide resistance genes have been either naturally occurring strains (McBride *et al.*, 1986; Murakami *et al.*, 1986; Pemberton *et al.*, 1979; Stransky and

Amberger, 1973; Wohlleben *et al.*, 1988) or laboratory produced mutants (Senior *et al.*, 1976).

The glufosinate resistance gene, *bar*, was first isolated during an experiment designed to demonstrate the close linkage of the glufosinate resistance gene to the glufosinate biosynthetic gene cluster in *Streptomyces hygroscopicus*. Two partial genomic libraries of the wild type *S. hygroscopicus* strain ATCC21705 were constructed by individually inserting complete PstI and BamHI digests of genomic DNA into the streptomycete vector pIJ61. Recombinant plasmids were used to transform individuals of the glufosinate sensitive host species *Streptomyces lividans*. Transformants were then grown to the sporulation stage before being tested for resistance to the herbicide. While all the resistant clones from the BamHI partial library contained the *bar* gene on an insert 2.0-Kb in size, those from the PstI partial library all contained the gene on a 1.7-Kb insert (Murakami *et al.*, 1986). Another glufosinate degradative gene, *pat*, isolated for use in the engineering of herbicide resistance in crops has since been obtained from another known glufosinate producer, *Streptomyces viridochromogenes* (Wohlleben *et al.*, 1988).

While the genes mediating glufosinate detoxification have been obtained from two actinomycete species known to produce glufosinate, the cloning of bromoxynil, cyanamide, 2,4-D and dalapon resistance genes has depended on the initial isolation of microbial strains with the capacity to use these herbicides as nutrient sources. McBride *et al.* (1986) obtained bromoxynil degrading soil microorganisms from soils which had a history of contamination with this herbicide. The process of isolating the microbes began with an enrichment for species capable of growth in minimal media supplemented with bromoxynil as the main nitrogen source. This was followed by a screening program during which pure cultures of the strains surviving enrichment were tested individually for their ability to degrade bromoxynil. Using high performance liquid chromatography to monitor herbicide transformations, nineteen distinct microbial strains capable of carrying out bromoxynil degradation were obtained. Of these nineteen isolates a bacterial strain, identified as *Klebsiella ozaenae*, was found to very rapidly transform the herbicide and was thus selected for further study. It was found that the *K. ozaenae* strain could, by virtue of a single step reaction catalysed by a nitrilase specific for bromoxynil, utilise the

herbicide as its sole source of nitrogen. This reaction, which involved the conversion of the nitrile group of bromoxynil to a carboxylate group, was coupled to the release of ammonia (Fig. 1.1; McBride *et al.*, 1986). The *K. ozaenae* isolate was found to contain a plasmid, designated pBrx1, which was estimated to be approximately 82-Kb in size. After transforming *E. coli* M294 with pBrx1 and successfully selecting transformants capable of using bromoxynil as their sole source of nitrogen, it was demonstrated that the gene encoding the bromoxynil specific nitrilase, *bxn*, was located on this plasmid. Mapping the location of the *bxn* gene on pBrx1 was simplified by the observation that growth of transformed *E. coli* M294 cells on rich media simultaneously resulted in both the irreversible loss of their ability to utilise bromoxynil as a nitrogen source and the spontaneous excision of a fragment, 14-Kb in size, from the pBrx1 plasmids they contained. Using the detection of cellular nitrilase activity in the presence of bromoxynil as an assay for the presence of the *bxn* gene, a partial pBrx1 library was constructed in an *E. coli* plasmid cloning vector and, following a series of subcloning steps, the *bxn* gene was isolated on a 2.6-Kb DNA fragment (Stalker and McBride, 1987).

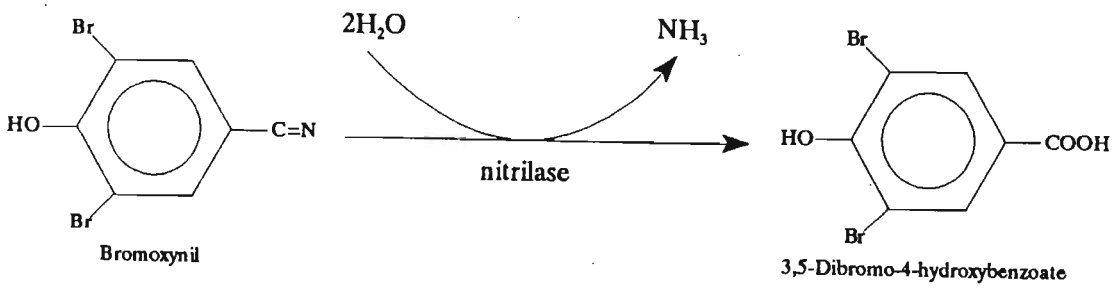

FIG. 1.1. The conversion of the nitrile group of bromoxynil to a carboxylate group by the *K. ozaenae* strain isolated by McBride *et al.* (1986). The reaction, catalysed by a nitrilase specific for bromoxynil, is coupled to the release of ammonia which is utilised by the bacterium.

Stransky and Amberger (1973) observed that *Myrothecium verucaria* was capable of utilising the herbicide cyanamide as its sole nitrogen source. These researchers demonstrated that this fungal species transformed cyanamide to urea by virtue of an inducible cyanamide specific hydratase. The ammonia released upon breakdown of the urea was used by the fungus as a source of nitrogen. In order to isolate the gene encoding cyanamide hydratase Maier-Greiner *et al.* (1991b) constructed a total *M. verucaria* genomic library in λEMBL3. From the partial amino acid sequence of the cyanamide hydratase enzyme, a mixed 17 base pair oligonucleotide probe for the cyanamide hydratase gene was constructed. Using the probe six independent clones were isolated from the genomic library. By subcloning one of the phage inserts into pUC19 and probing with the mixed oligonucleotide probe, a 1.104-Kb HpaII restriction fragment, which hybridised strongly with the probe, was obtained. A *M. verucaria* cDNA library was constructed in λgt10 using mRNA from cells grown in the presence of cyanamide. Using the 1.104 HpaII fragment as a probe, a complete cyanamide hydratase gene was isolated from the cDNA library on a 900 base pair DNA fragment.

The isolation of both 2,4-D degrading microbes and their degradative genes were initially carried out in the course of investigations of, firstly, the environmental fate of this herbicide and, secondly, the extent to which this fate was determined by plasmid encoded degradative enzymes. Pemberton *et al.* (1979) used a three-step selection procedure in order to isolate microorganisms containing plasmids with genes encoding the complete degradation of 2,4-D. The first step involved three cycles of enrichment for 2,4-D degrading soil microbes in Loos' (1975) bromocresol purple indicator medium. Positive enrichments in which 2,4-D degradation was occurring were identified by a colour change in the indicator medium (due to the release of chloride ions during 2,4-D degradation). In the second selection stage microbial strains surviving enrichment were individually tested for their ability to dechlorinate 2,4-D on agar plates containing an eosin-methylene blue indicator dye and the herbicide as a main carbon source. As a final selection step, isolates were screened for loss of their 2,4-D degrading capacity during growth in a rich medium without 2,4-D. Those microbes which retained their 2,4-D degradative capacity after 15-20 generations of growth in the rich medium were selected for further study (Pemberton *et al.*, 1979). One of the isolates, identified as a strain of *Alcaligenes*

eutrophus, was shown to contain a plasmid approximately 80-Kb in size that encoded genes involved in 2,4-D transformation (Don and Pemberton, 1981). A physical and partial genetic map of the plasmid, given the name pJP4, was then constructed (Don and Pemberton, 1985). Amy *et al.* (1985) succeeded in localising the *tfdA* gene (encoding the first step of the 2,4-D degradation pathway) to a 21-Kb HindIII fragment of an independently isolated *A. eutrophus* plasmid similar to pJP4. In order to isolate the corresponding 21-Kb HindIII fragment of pJP4, the plasmid was digested with HindIII and cloned into the mobilisable, broad host range plasmid, pVK101 (Streber *et al*, 1987). The plasmid DNA obtained was used to transform *E. coli* S17-1 which was subsequently used for the conjugative transfer of the plasmid to an *A. eutrophus tfdA* deficient transposon insertion mutant. The presence of the transferred *tfdA* gene was detected by its ability to confer 2,4-D resistance to the mutant receptor strain. A restriction map of the 21-Kb HindIII fragment containing the *tfdA* gene was constructed and SacI fragments of the PVK101 plasmid containing the 21-Kb HindIII insert were shotgun cloned into pGSS33 (another broad host range plasmid vector). *E. coli* LE392 was used as a host strain for the selection of transformants containing recombinant plasmids. Recombinant plasmids were isolated for use in transforming *E. coli* S17-1 which, in turn, was used for the conjugative transfer of these plasmids to the *tfdA* deficient *A. eutrophus* transposon mutant. Following the selection of recipient bacteria able to utilise 2,4-D as a sole carbon and energy source, the *tfdA* gene was isolated on a 3-Kb DNA fragment (Streber *et al.*, 1987).

The *Pseudomonas putida* gene encoding the degradation of dalapon is the only detoxification gene from a laboratory produced mutant that has been used to engineer herbicide resistance in crops. During the course of observing a stable, defined mixed microbial community capable of growth on the herbicide dalapon Senior *et al.* (1976) isolated a mutant strain of *Ps. putida* capable of utilising the herbicide as its sole carbon and energy source. The mutant strain, P3, was believed to have arisen from a parent *Ps. putida* strain, S3, which was incapable of utilising dalapon in pure culture. S3 had been added to the community as a secondary dalapon utiliser capable of survival on dalapon degradation products and other nutrients released into the growth medium by primary dalapon utilisers. Weightman *et al.* (1979) demonstrated that P3 possessed the ability to

dehalogenate both dalapon and the related herbicide, 2MCPA. Thomas *et al.* (1992a) observed that the gene responsible for dalapon dehalogenation, *deh1*, resided on P3's chromosome and was carried on what appeared to be a transposon-like element, designated DEH. The 6.0-Kb DEH element was noted to preferentially insert itself into a 5.6-Kb EcoRI fragment of the chromosome-mobilising TOL plasmid pWWO. pWWO and recombinant derivatives of this plasmid were conjugatively transferred to *Ps. putida*. Selection of recombinant pWWO derivatives carrying the DEH element was carried out on media with dalapon present as a sole carbon and energy source. The 11.0-Kb EcoRI fragment of a recombinant pWWO derivative plasmid containing DEH was cloned into both the *E. coli* vector pHG327 and the broad host range vector pKT231 and transformed into *E. coli* C600 and JM107. pKT231 was conjugatively transferred to *Ps. putida* KT440. Analysis of subcloned fragments for their ability to provide *E. coli* and *Ps. putida* with the capacity to dehalogenate 2MCPA was used to discover the exact location of the *deh1* containing DEH element (Thomas *et al.*, 1992b).

1.5.3 Alternative Uses for Herbicide Resistance Genes

Apart from their use in the production of crop varieties which tolerate herbicides, another incentive behind efforts to isolate and transfer genes encoding herbicide resistance is that they may eventually be as useful in plant genetic engineering as antibiotic resistance genes have proved to be in bacterial genetic manipulations (Mazur and Falco, 1989). In this regard the main purpose behind the construction of herbicide resistant transgenic tobacco plants by Guerineau *et al.* (1990) and Buchanan-Wollaston *et al.* (1992) was to demonstrate that genes encoding resistance to asulam and dalaphon respectively, could be used as stable dominant selectable genetic markers in plant transformation experiments. For example, Guerineau and co-workers were able to demonstrate that the successful transformation of tobacco leaf explants co-cultivated with *Agrobacterium tumefaciens* harbouring a pBIN19 derived plasmid containing the asulam resistance gene, *sul1*, was possible using asulam as the sole selective agent. The *bar* gene of *Streptomyces hygroscopicus* encoding glufosinate resistance and the *csr1* gene of *Arabidopsis thaliana* encoding sulphonylurea herbicide resistance, have also been investigated with regard to their potential use as plant transposon excision reporters (Cardon *et al.*, 1993; Fedoroff

and Smith, 1993) and as selectable markers in both gene fusion (Botterman *et al.*, 1991) and plant transformation experiments (Becker *et al.*, 1992; Li *et al.*, 1992; Rathore *et al.*, 1993; Weeks *et al.*, 1993; White *et al.*, 1990). However, not all herbicide resistance genes will be suitable as selectable markers. For example, the *tfdA* gene encoding 2,4-D resistance cannot be used effectively as a selectable marker because 2,4-D, due to its auxin-like hormonal properties, poses problems when used as a selective agent in plant cell and tissue culture (Streber and Willmitzer, 1989).

As an alternative to the production of herbicide resistance through the insertion of altered target or detoxification genes into plants, protection of crops from herbicides using recombinant DNA technology may be achievable via another route. In many cases it may be possible to transfer herbicide degrading genes into microbes which specifically colonise the roots, seeds or internal organs of herbicide sensitive crops. By actively taking up herbicides and degrading them these microorganisms could effectively shield the plants they have colonised from herbicide exposure (Hatzios, 1987; Cork and Kreuger, 1991). Kreuger *et al.* (1991) have shown that the use of herbicide degrading microbes for protecting crops from herbicide damage is possible. By using three natural dicamba degrading soil isolates to inoculate soils containing this herbicide at its recommended dosage, it was found that sufficient microbe mediated detoxification occurred to protect soybean (a crop sensitive to dicamba) from herbicide injury. However, because in this system microbes uniformly degraded dicamba throughout a plot they could not be used to specifically protect dicamba sensitive crops without also protecting weed species. An effective strategy for providing exclusive protection for crops from herbicide damage might require the use of microbes genetically engineered to degrade herbicides which naturally associate specifically with crop varieties and not their weeds. The feasibility of using genetically engineered microbial species which specifically associate with particular crop species in this way has been demonstrated with the development of a bioinsecticide for the control of corn root feeding insects. The gene encoding the proteinaceous *Bacillus thuringiensis* endotoxin was cloned into the corn root and seedling colonising bacterium, *Pseudomonas fluorescens*. Protection of corn from lepidopteran pests was then achievable by growing plants in the presence of the transgenic *Ps. fluorescens* (Lindow *et al.*, 1989). A herbicide detoxification system of this nature would be particularly desirable if it was

impractical to attempt the insertion of a resistance gene into a crop species.

Another potentially useful function that the transfer of herbicide detoxification genes into microbes might provide is in the remediation of agricultural soils and irrigation water contaminated with high herbicide concentrations. Microbes containing highly mobile plasmids carrying genes enabling the use of recalcitrant herbicide molecules as nutrient sources could be used to shorten the time required for natural soil and water detoxification to occur (Kreuger *et al.*, 1991; Smith *et al.*, 1994). The accelerated degradation of 2,4-D has been demonstrated in native, unamended agricultural soils inoculated with a genetically altered *Pseudomonas putida* strain harbouring a derivative of the *Alcaligenes eutrophus* 2,4-D degradative plasmid, pJP4. Despite the fact that only between 0.1 and 1 % of the inoculum was found to survive a twenty day test period, the rate at which 2,4-D disappeared from inoculated soils was two to three times faster than that from uninoculated soils (Short *et al.*, 1990). Being a feasible method of environmental decontamination, it would therefore be reasonable to envisage the production of genetically engineered microbes for the cleanup of many other agricultural and industrial pollutants.

1.6 Acetochlor

2-chloro-N-ethoxymethyl-6′-ethylacet-o-toluide, also known as acetochlor, is a member of a group of structurally related xenobiotic chemicals called the chloroacetanilides. The chloroacetanilides marketed for use as herbicides (Fig 1.2) are, together with 2,4-D and the triazine herbicides, the most extensively used pesticides in the world today (Gressel, 1992). Unlike 2,4-D and the triazines, however, no attempts have as yet been made to isolate and transfer chloroacetanilide resistance genes into crops. It seems only logical therefore, that the potential should be examined for engineering resistance in crops to a chloroacetanilide herbicide such as acetochlor.

Of the chloroacetanilides the most widely used and thoroughly researched are definitely alachlor and metolachlor. While internationally the frequency of use and, subsequently, the level of interest in acetochlor is not very high, it is the third most important chloroacetanilide used in South Africa (Fig. 1.3). Because it lacks international

popularity, information on acetochlor is scarce relative to that available for either alachlor or metolachlor. Therefore, where information on acetochlor relevant to the present study could not be found in the literature, use was made of analogous information relating to alachlor and metolachlor.

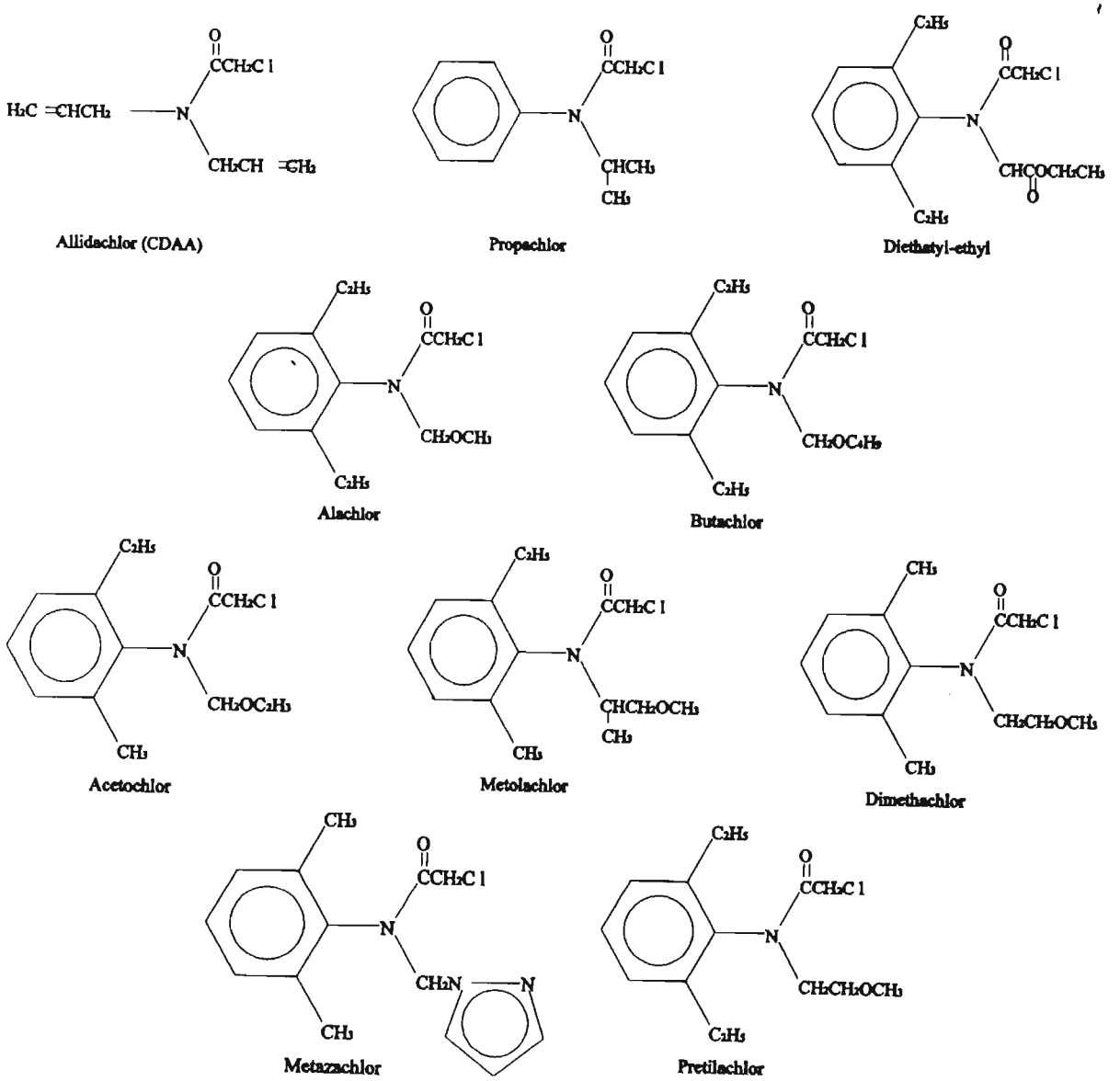

FIG. 1.2. Chloroacetamides which are marketed for use as herbicides. Note that while allidachlor is a chloroacetamide it is not a chloroacetanilide.

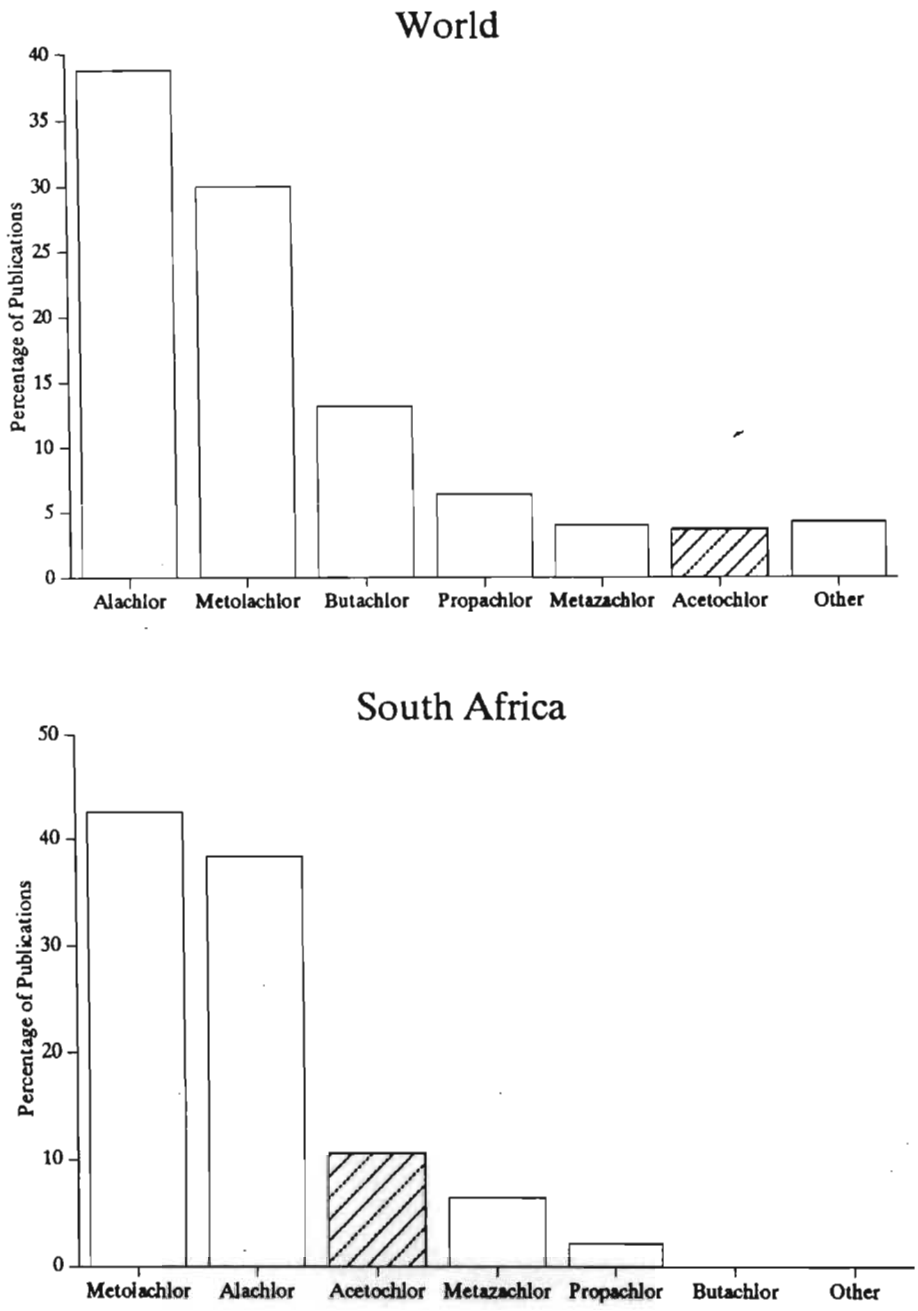

FIG. 1.3. The relative importance of the various chloroacetanilide herbicides in both a South African and a worldwide context. This is expressed as the percentage of the total number of papers dealing with chloroacetanilide herbicides published between 1983 and 1993 which have concentrated specifically on each of the herbicides presented here. Included in the "other" category are pretilachlor, dimethachlor and diethatyl-ethyl. It is interesting to note that while on this scale acetochlor (shaded) ranks only sixth in the world, in South Africa it is the third most researched chloroacetanilide. The apparent absence of interest in butachlor, pretilachlor, dimethachlor and diethatyl-ethyl among South African researchers is because these herbicides are not registered for use in South Africa. This graph was compiled from information gathered by the present author using a CD-ROM based version of CAB Abstracts (an agricultural database published by CAB International in association with Silver Platter Information). The search protocols used were "PY=x AND y" for world-wide publications and "SOUTH* AND AFRICA* AND PY=x AND y" for South African publications (where"x" is the publication year and "y" is one of the specific chloroacetanilides presented in Fig 1.3.).

Acetochlor is sold as an emulsifiable concentrate which, although usually applied to fields either preemergence or preplant, may also be applied postemergence, for controlling annual grasses, certain annual broadleaf weeds, and yellow nutsedge (Cairns, 1993; personal communication[2]; Worthing and Hance, 1991). In South Africa its use in the control of these weeds is recommended on fields which are to be used in the cultivation of maize, groundnuts, potatoes, sugarcane and sweetcorn (Vermeulen *et al.*, 1991). The main site of acetochlor uptake in germinating plants is via the shoots (Jackson *et al.*, 1986). The chloroacetanilides function as general growth inhibitors affecting root elongation and the emergence of susceptible seedlings. While germination occurs at recommended application rates, early growth, probably due to inhibition of mitosis and cell enlargement, is prevented (Ashton and Crafts, 1981). On the cellular level the chloroacetanilides effect protein (Sharp, 1988), lipid (Weisshaar *et al.*, 1988), anthocyanin and lignin synthesis (Molin *et al.*, 1986), acetylation of DNA associated proteins (Weisshaar and Boger, 1991), gibberellic acid induced reactions (Wilkinson, 1982), respiration and photosynthesis (Van Rensberg *et al.*, 1990; LeBaron *et al.*, 1988; Sharp, 1988). While the primary site of chloroacetanilide action has not yet been identified (Gronwald, 1991), it is thought that these herbicides may have multiple targets (Gressel, 1992; LeBaron *et al.*, 1988; Sharp, 1988).

The basis of acetochlor's selective phytotoxicity is the inefficiency of its metabolic detoxification in susceptible plants. In virtually all resistant plants acetochlor is rapidly detoxified following its glutathione transferase (GST) mediated conjugation with either glutathione (GSH) or homoglutathione (hGSH; Fig. 1.4). While plant species which are not resistant to acetochlor have the capacity to deactivate acetochlor via this route, it appears that either the catabolic efficiency of their GST enzymes is not sufficient or their GST, GSH or hGSH contents are not high enough to afford rapid enough herbicide detoxification to provide them with tolerance (Breux, 1987; Scarponi *et al.*, 1991). Chemicals called safeners, which either induce GSH production or stimulate the activity of GST enzymes, have been developed and are able to elevate levels of chloroacetanilide tolerance in plants to which they are applied (Breux *et al.*, 1987; Cottingham and Hatzios, 1991).

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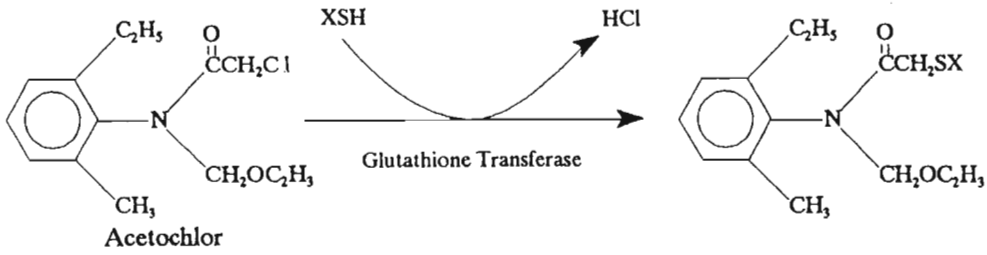

FIG. 1.4. Acetochlor detoxification in plants. "XSH" represents either glutathione or homoglutathione (after Breux, 1987).

1.6.1 Environmental Contamination

While no comprehensive information is available on the extent of any chloroacetanilide contamination in South Africa, environmental monitoring programs in various parts of the world have detected the presence of acetochlor and other chloroacetanilides in surface and ground waters (Chesters *et al.*, 1989). Of all the chloroacetanilides the environmental fate of alachlor has been most extensively studied. The preplant or preemergence use of this and other chloroacetanilides during spring and their subsequent leaching from bare cropland soils in heavy rains, is seen as the major cause of their occurrence in surface waters (Gressel, 1992). The amount of alachlor ultimately reaching drinking water supplies is dependent upon both its dilution during transport and its recalcitrance (Chesters *et al.*, 1989) and in a number of instances in the USA and Canada alachlor residues have been detected in tap water (Chesters *et al.*, 1989; Frank *et al.*, 1991). When contamination of groundwater with alachlor occurs it is usually thought to be due to the improper handling or disposal of the herbicide. While groundwater contamination is significantly less than that of surface water, a finding of some concern in the USA is that alachlor together with other pesticides, even when correctly handled, sometimes contaminate public wells. This is significant since public wells, due to the depths of the aquifers from which they draw water and the quality of their construction, were thought to be impregnable to contamination by properly applied agricultural chemicals (Chesters *et al.*, 1989).

The most significant concern with widespread chloroacetanilide contamination is that it may pose a threat to both humans and other organisms in the environment. Because of their moderately acute and chronic toxicities to mammals, birds, fish and insects (Table 1.4), the chloroacetanilides registered for use in South Africa are all classified as class III chemicals (they should be handled with caution; Vermeulen *et al.*, 1991). While no chloroacetanilides have been found to have any teratogenic, reproductive or immunosuppressive effects in mammals, certain (and possibly all) of the herbicides in this group are genotoxic (Flaherly *et al*, 1992; Chesters *et al*, 1989). Alachlor is now recognised as a carcinogen and evidence exists which suggests metolachlor may also possess oncogenic properties (Table 1.4; Chesters *et al.*, 1989). Potential carcinogenicity as indicated by herbicide induced genetic aberrations has also been observed in studies involving the chloroacetanilides, butachlor and propachlor (Leckevicius *et al.*, 1992; Wang, 1987). While there is no evidence in the literature which suggests that acetochlor is genotoxic, it seems reasonable to extrapolate results from investigations of the other chloroacetanilides to indicate this herbicides potential carcinogenicity.

TABLE 1.4. Toxicological properties of chloroacetanilides marketed for use in South Africa [after Worthing and Hance (1991) and Chesters *et al.* (1989)].

	Alachlor	Metolachlor	Acetochlor	Propachlor	Metazachlor
Acute Toxicity					
Mammals (rat)					
Oral LD_{50} ($mg.kg^{-1}$)	930-1350	2780	2148	550-1700	2150
Dermal LC_{50} ($mg.kg^{-1}$)	>13300[a]	>3170	4166[a]	>20000[a]	>6810
Birds (bobwhite quale)					
Oral LD_{50} ($mg.kg^{-1}$)	1536	non toxic	1260	91	-[b]
Dermal LC_{50} ($mg.kg^{-1}$)	>5620	non toxic	>5620	5620	-
Fish (rainbow trout)					
LC_{50} ($mg.l^{-1}$)	1.8	2.0	0.5	0.17	-
Insects (*Daphnia maga*)					
LC_{50} ($mg.l^{-1}$)	10	-	16	7.8	-
Chronic Toxicity					
Mammals (rat)					
NOEL (2y - $mg.l^{-1}.d^{-1}$)	2.5	90[c]	12[d]	0.65	-
Oncogenicity					
CD-1 mice	260	>3000	-	-	-
Rats	42	3000	-	-	-

[a] Calculated using rabbits.
[b] No information available
[c] 90 day NOEL.
[d] 1 year NOEL calculated using dogs.

While the discovery of widespread chloroacetanilide contamination and the potential health risks it may pose have been disconcerting enough for the use of alachlor to be banned in Italy (Rapparini, 1991) and Canada (Fox, 1987), it has also prompted the development of new techniques, both preventative and remedial in nature, for reducing the possible environmental and health impacts of chloroacetanilide usage. The prevention of chloroacetanilide entry into surface and ground-waters is achievable either by adopting farm management practices which reduce herbicide runoff (Bengston *et al.*, 1990; Felsot *et al.*, 1990) or through the use of lignin and starch encapsulated herbicide formulations which have been shown to significantly reduce the leaching of these herbicides from soils (Wilkins, 1989; Wing *et al.*, 1990). Various water and soil treatment methods for the remediation of both high and low levels of chloroacetanilide contamination are presently under development. These methods include the use of granular activated carbon absorber systems for removing traces of alachlor from large volumes of drinking water (Marcomini *et al.*, 1992; Pirbazari *et al*, 1991), the mineralisation of chloroacetanilides in small volumes of heavily contaminated agricultural waste waters by a combination of ozonation, ultraviolet irradiation and treatment with biologically active soil (Somich *et al.*, 1988) and the bioremediation of herbicide contaminated soils and waters through the introduction of specifically isolated herbicide degrading microbes (Liu *et al.*, 1990).

The bioremediation approach to the cleanup of chloroacetanilide contaminated soils has thus far centred upon the isolation, screening and reintroduction into the environment of microorganisms which are capable of degrading alachlor and metolachlor. Although a number of microbes have been isolated which have the capacity to rapidly transform these herbicides in controlled soil systems under laboratory conditions (Gizzarelli *et al.*, 1993; Liu *et al.*, 1990), attempts to inoculate natural herbicide polluted soils with these isolates in order to accelerated natural detoxification processes, have met with failure (Liu *et al.*, 1990). Bartha (1990) has insisted that it is futile to simply isolate naturally occurring xenobiotic metabolising microbial strains from soils, bulk their numbers in pure culture and then reintroduce them into contaminated soils in the hope that they will increase the rate of natural xenobiotic degradation. The reason for this is that the isolate, as a naturally occurring soil inhabitant, will almost certainly already be present in soils contaminated with the xenobiotic. While under suitable environmental conditions enrichment of these

organisms will occur without the help of inoculation, unsuitable conditions will seriously impede the degradative capacity of a microbial inoculum even at very high doses. Bartha (1990) has claimed that in order to obtain microbes capable of enhancing natural degradation processes it will be necessary to in some way manipulate the microbes mediating these processes in order to improve their natural degradative capabilities. Examples of such manipulations are the use of recombinant DNA techniques for the production of novel 2,4-D (Short *et al.*, 1990) and pentachloroethane (Wackett *et al.*, 1994) degrading *Pseudomonas putida* strains and the employment of "*in vivo* genetic engineering" or "accelerated evolution" enrichment techniques for the production of microbial strains capable of utilising the extremely recalcitrant chemicals 2,4,5-trichlorophenoxyacetate (Kilbane *et al.*, 1982), 3,5-dichlorobenzoate (Hartman *et al.*, 1979) and 1,4-dichlorobenzene (Krockel *et al.*, 1987) as their sole carbon and energy sources.

Instead of merely isolating chloroacetanilide degrading microbes from the environment, the focus of research aimed at obtaining microorganisms useful in the remediation of chloroacetanilide contaminated water and soil should possibly be the isolation and characterisation of chloroacetanilide degradative genes. By first isolating mutant genes involved in chloroacetanilide degradation, upregulating their expression, improving the reaction kinetics of the enzymes they encode and then incorporating them into highly mobile plasmid constructs, it may be possible to create a variety of new bacterial strains that have the ability to enhance natural chloracetanilide degradation rates under a range of environmental conditions.

1.6.2 Reasons and Prospects for Engineering Resistance to Acetochlor

There are certain practical justifications for the chloroacetanilide herbicides being among the most popular groups of chemicals employed in pest control. Being relatively cheap and with a fairly broad spectrum of weed control, the benefits of their use on tolerant crops is almost without parallel. Because of their toxicity to virtually all annual grasses, grass crops such as wheat would benefit enormously from engineered resistance to the chloroacetanilides. With wheat in particular, due to the rapid evolution of grassy weeds

with multiple and cross-resistance to all of the selective herbicides presently used during its cultivation, there is very serious need of new selective herbicides (Gressel, 1992). Resistance to chloroacetanilides amoung wheat strains would be of value in this regard since the extremely widespread use of these herbicides has, as yet, not been associated with the emergence of resistant weed strains.

For various reasons acetochlor is one of the chloroacetanilide herbicides against which genetically engineered resistance in crops would be of great agronomic value. A comparison between acetochlor and the two most widely used chloroacetanilide herbicides, metolachlor and alachlor, indicates that acetochlor has a number of characteristics which make it at least as good as the latter two compounds. Notwithstanding having a mammalian toxicity half that of alachlor (Table 1.4) and being degraded in soils twice as fast as metolachlor (Peterson *et al.*, 1988), acetochlor is applied at rates half those recommended for either metolachlor or alachlor (Vermeulen *et al.*, 1989). One possible drawback associated with acetochlor's high phytotoxicity, which might be overcome with genetically engineered resistance to this herbicide, is that its application at high doses resulted in injury to a number of crop species with moderate chloroacetanilide tolerance (Bewick *et al.*, 1988; Van Rensberg *et al.*, 1988; Veiger and Eberlein, 1986).

Acetochlor, unlike the predominantly root absorbed chloroacetanilides such as alachlor and metolachlor, is readily absorbed via the foliage of plants (Jackson *et al.*, 1986). Foliar adsorption of acetochlor is significant in that it permits the use of this herbicide postemergence (Cairns, 1993; personal communication[3]). The chloroacetanilide residues detectable in surface waters have been linked to the preemergence or preplant use of these herbicides on fields with insufficient ground-cover to prevent their leaching in rainwater runoff (Gressel, 1992). The postemergence use of acetochlor on fields containing a moderate ground-cover should, therefore, eliminate some of the pollution problems associated with chloroacetanilide usage. Also, post emergence herbicides generally require lower application rates than root absorbed preemergence herbicides because foliar

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absorption avoids problems of herbicide loss through adsorption by soil colloids (Burnside, 1992). Thus, while engineered resistance to acetochlor in crop species such as wheat could be instrumental in providing new markets for this herbicide, improved tolerance in crop species to which this herbicide could then be applied postemergence, would contribute significantly to making acetochlor usage an attractive alternative to the use of less environmentally benign chloroacetanilides.

The feasibility of engineering plants resistance to any herbicide is dependent upon the factors presented in section 1.5.1. Due to the likelihood that chloroacetanilide herbicides have multiple target sites (LeBaron *et al.*, 1988; Sharp, 1988), it would be impractical to attempt the isolation of genes encoding altered target enzymes for genetically engineering crop resistance to these herbicides. Since detoxification is the mechanism of resistance naturally employed by chloroacetanilide resistant plant species, it is logical that an attempt to isolate chloroacetanilide resistance genes should concentrate on obtaining genes which encode the detoxification of these herbicides. Success in obtaining genetically engineered crops which tolerate high doses of acetochlor without suffering any ill effects will be strongly dependent on the detoxification genes selected for transfer. Any individual gene which encodes a detoxifying enzyme that is able to identify the parent acetochlor molecule and rapidly convert it to a form which is both non-toxic and metabolisable by a transgenic plant will fulfil the criteria required of an effective resistance gene.

It should be possible to obtain chloroacetanilide resistance genes from both plant and microbial sources. The maize genes encoding the GST isozymes which are responsible for triazine and chloroacetanilide detoxification (Fig 1.4) have been isolated and characterised (Moore *et al.*, 1986; Shah *et al.*, 1986b). However, apart from an attempt in the late 1980's to transfer one of these genes into tobacco plants in order to achieve resistance to the triazine herbicide, atrazine (Mazur and Falco, 1989), there have been no further reports in the literature to suggest whether or not this has been achieved.

Because the major routes of chloroacetanilide degradation in soils are mediated by microorganisms (Chesters *et al.*, 1989), microbial sources of genes encoding acetochlor degradation should be plentiful. While no information is available on the specific nature

of acetochlor degradation by soil microbes, certain soil inhabiting fungal and bacterial strains which are able to transform metolachlor, alachlor, propachlor and diethatyl-ethyl have been isolated and the exact transformations they are responsible for have been determined in both pure and mixed cultures (Figs 1.5.1, 1.5.2, 1.5.3 and 1.5.4 respectively). The study of these isolates has demonstrated that although there are many microorganisms capable of transforming chloroacetanilides, the ability amoung soil bacteria to utilise these herbicides as sole sources of carbon or nitrogen is relatively rare: Only one bacterial isolate, a propachlor degrading *Moraxella* sp. has been shown to be capable of utilising a chloroacetanilide as its sole source of carbon and energy (Villarreal *et al.*, 1991). This finding indicates that it may be difficult to obtain acetochlor degrading soil bacteria by employing techniques aimed at the isolation of microbes capable of utilising the herbicide as a nutrient source.

From the study of microbial degradation products it is apparent that dechlorination reactions play a major part in the initial stages of chloroacetanilide breakdown in soils (metabolite 1 in Figs 1.5.1, 1.5.2 and 1.5.3). There are two important reasons why genes encoding the dechlorination of acetochlor may be ideal for use in providing crops with resistance to this herbicide. Firstly, they encode an initial step in the herbicide's degradation pathway which is significant since it indicates that the dechlorinating enzymes are able to recognise and transform parent acetochlor molecules. Secondly, because non-halogenated acetanilides are herbicidally inactive (Hamm *et al.*, 1974), acetochlor dechlorinating enzymes independently encode the detoxification of this herbicide. In addition, because the detoxification of acetochlor in tolerant plants occurs by means of the nucleophilic displacement of the herbicide's chlorine atom by the thiol group of either GSH or hGSH (Fig. 1.4; Breux, 1987), it is conceivable that the dechlorinated acetochlor degradation product, as a possible intermediate in a naturally occurring plant detoxification reaction, will in some way be metabolisable by plants.

An important problem which may be encountered with the transfer of microbial acetochlor detoxifying genes into plants is that the activity of the enzymes they encode may not be high enough to afford herbicide sensitive recipient plants sufficient resistance. As the level of a plant's acetochlor tolerance is strongly correlated with the rate at which it is able to

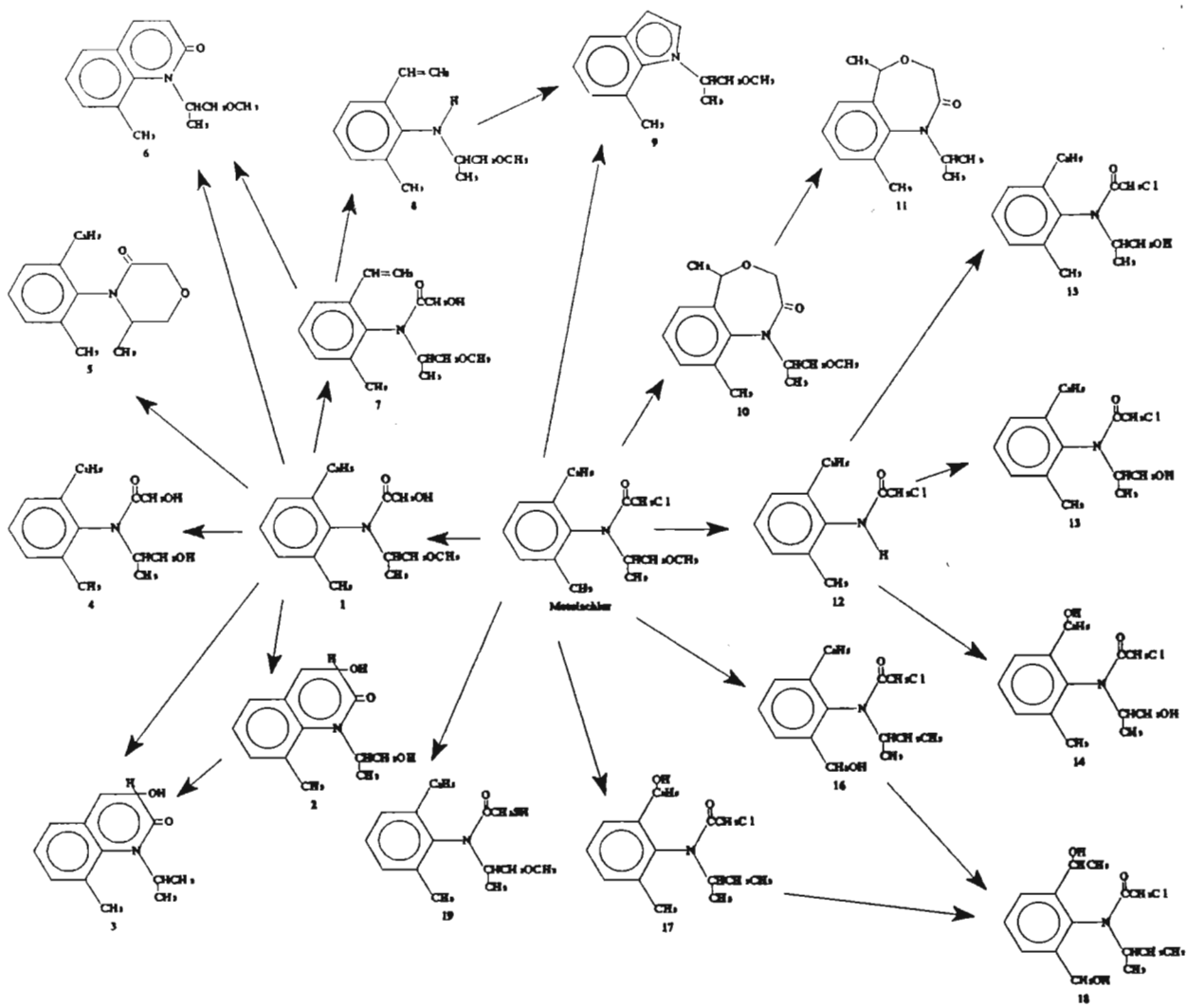

FIG. 1.5.1. Proposed pathways of metolachlor degradation which are mediated by soil microorganisms. Degradation products: 1, 4, 5, 10, 11, 12, 13, and 18 by a *Streptomyces* sp. and *Syncephalostrum racemosum* (Liu *et al.*, 1991); 1, 4, 5, 10, 11, 12, 13, and 16 by *Phanerochaeta chrysosporium* and *Rhizoctonia praticola* (Liu *et al.*, 1991); 13 and 16 by an actinomycete and *Mucor racemoces* (Bollag, 1990; Saxena *et al.*, 1987); 13 by *Bacillus circulans* and *B. megaterium* (Bollag, 1990; Saxena *et al.*, 1987); 13, 14, 15, 16, 17, and 18 by an actinomycete (Krause *et al.*, 1985); 1, 2, 3, 7, 8, 9, 12, 13, and 14 by *Chaetomium globossum* (McGahen and Tiedje, 1978); 5, 7, and 19 by a stable mixed bacterial community (Liu *et al.*, 1989).

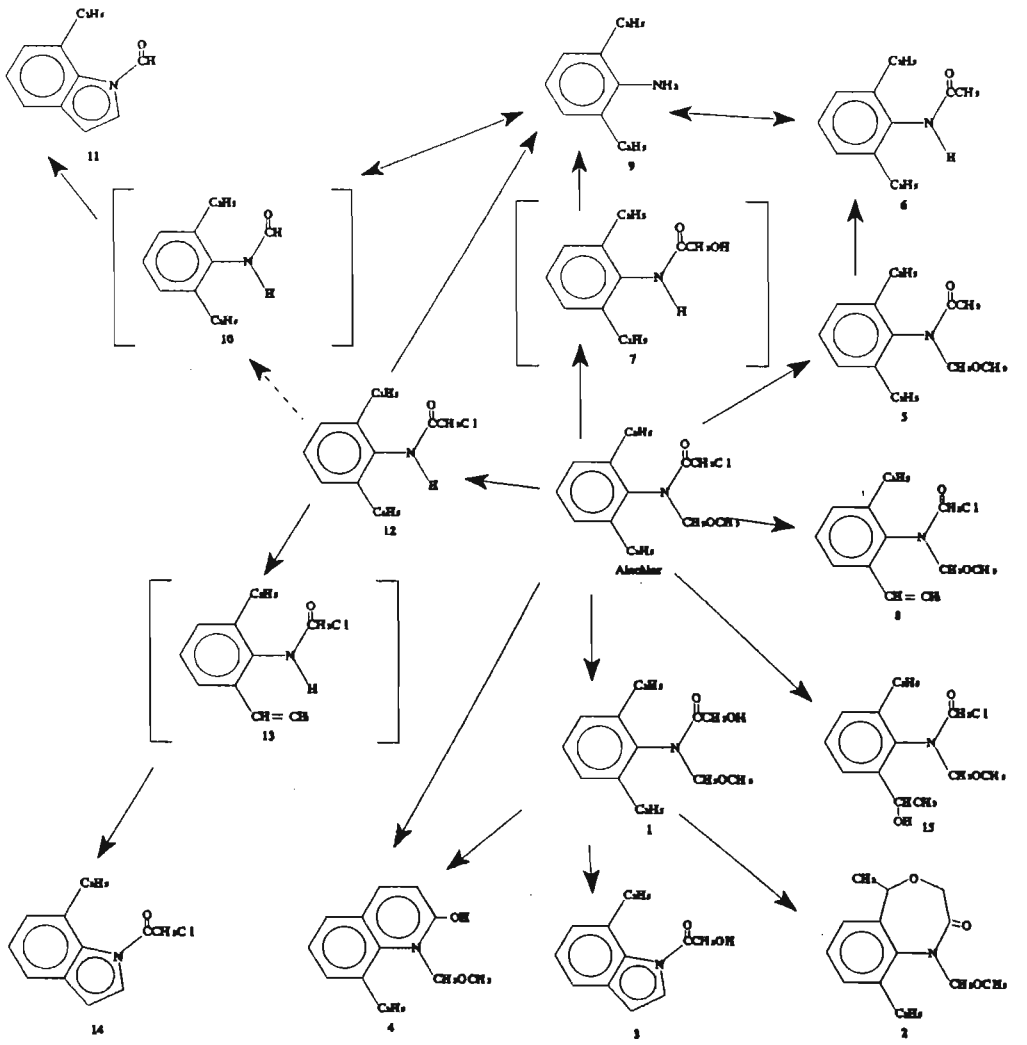

FIG. 1.5.2. Proposed pathways of alachlor degradation which are mediated by soil microorganisms. Degradation products: 7, 8, 9, 12, 13, and 14 by *Chaetomium globossum* (Tiedje and Hagedorn, 1975); 1 by *Streptomyces lavendale*, *Arthrobacter suliflex*, and *Bacillus subtilis* (Lee, 1986 cited by Chesters *et al.*, 1989 and Pothuluri *et al.*, 1993); 2, 3, 4 by *Rhizoctonia solani* (Lee, 1986 cited by Chesters *et al.*, 1989 and Pothuluri *et al.*, 1993); 8, 12, 15 by *Cunninghamella elegans* (Pothuluri *et al.*, 1993); 1, 2, 3, 4, 5, 6, 8, 9, 10, 11, 12 by unidentified soil microbes (Lee, 1984 and Lee, 1986 cited by Chesters *et al.*, 1989 and Pothuluri *et al.*, 1993). Bracketed compounds are proposed intermediates.

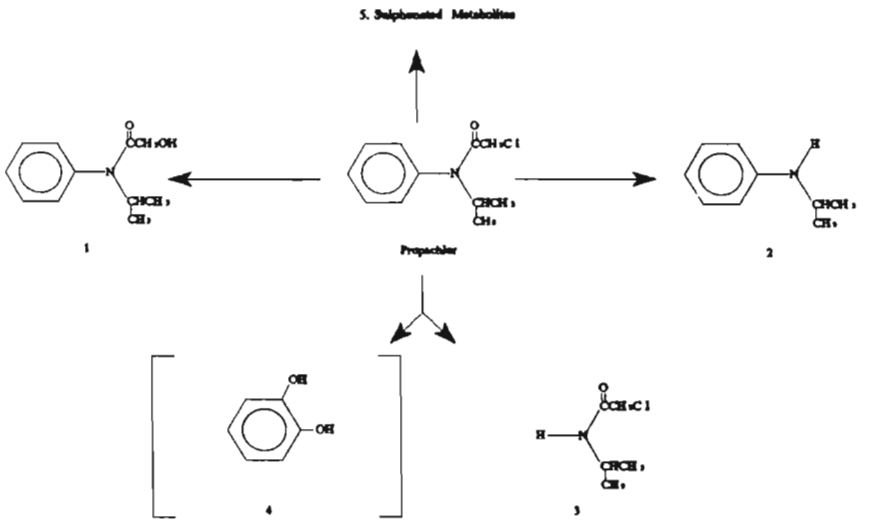

FIG. 1.5.3. Proposed pathways of propachlor degradation which are mediated by soil microorganisms. Degradation products: 1 by *Fusarium oxysporum* (Kaufman *et al.*, 1971 cited by Tiedje and Hagadorn, 1975); 2 in a defined mixed culture (Novick *et al.*, 1986); 3 and 4 by a *Moraxella* sp. (Villarreal *et al.*, 1991). 5 by mixed cultures (Lee *et al.*, 1982 cited by Bollag, 1990). Bracketed compounds are proposed intermediates.

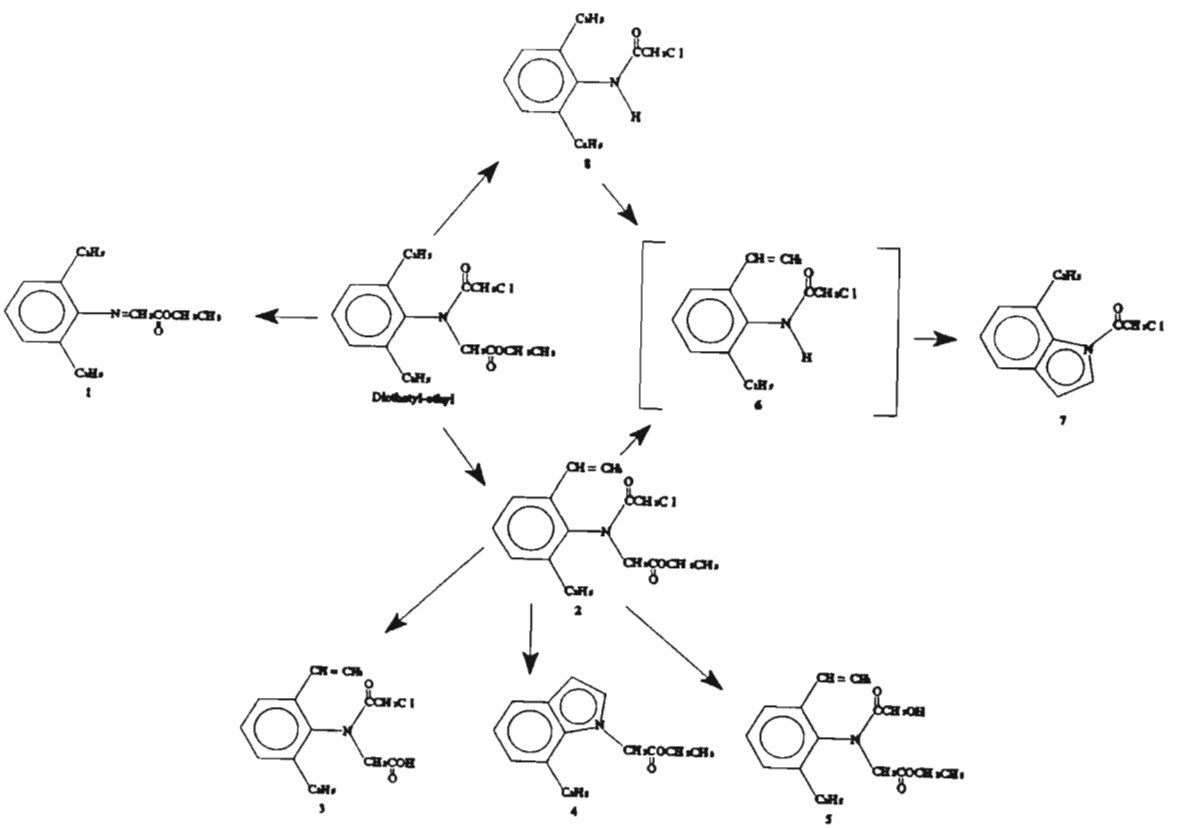

FIG. 1.5.4. Proposed pathways of diethatyl-ethyl degradation mediated by *Chaetomium globossum* (from McGahen and Tiedje, 1978). The bracketed compound is a proposed intermediate.

detoxify this herbicide (Breux, 1987), the reaction kinetics of enzymes encoded by anyprospective resistance gene may be very important in determining the quality of resistance that it is able to provide. However, it is also possible that because most crops have an innate (although often insufficient) ability to detoxify acetochlor (Scarpori *et al.*, 1991), any auxiliary acetochlor degrading genes may augment the effectiveness of a susceptible plant's native detoxification processes sufficiently to provide it with considerably improved resistance. Thus, by mediating an increase in the detoxification rate of parent herbicide molecules, microbial genes encoding the single step dechlorination of acetochlor may be both amenable to transfer into plants using currently available recombinant DNA techniques and capable of providing engineered crops with dominant, monogenically inherited herbicide resistance.

2 GENERAL MATERIALS AND METHODS

During the course of the present study use was made of various procedures which were common to more than one area of the research carried out. For the sake of clarity general methods will be discussed in this chapter while details of techniques pertaining exclusively to specific experiments will be described in the materials and methods of the relevant chapters.

2.1 Chemicals

Unless otherwise indicated, all chemicals used were either reagent or analytical grade and purchased from BDH, Saarchem or Merck. Technical grade acetochlor with a 90.5% purity (9.5% inert ingredients) was a gift from Saarchem. Acetochlor in formulation (900 $g.l^{-1}$ active ingredient), with the tradename Harness® (marketed by Monsanto) was a gift from Professor A.L.P. Cairns in the Agronomy Department of the University of Natal in Pietermaritzberg. Wherever the form of the herbicide is unspecified, technical grade acetochlor was used. Both formulation and technical grade acetochlor were passed through a sterile 0.4 μm nylon filter (from Micron Separations Inc.) before use. Tetracycline was obtained from Sigma® Chemical Company. Ampicillin was obtained from Boehringer Mannheim. The different grades of agar used were bacteriological agar (from Oxoid), purified agar (from Oxoid) and noble agar (from Difco). Ultra pure Millipore Milli Q Plus water was supplied by the Biochemistry Department of the University of Natal in Pietermaritzberg.

2.2 Growth Media

A mineral salts medium (MS) based on that described by Kaufman and Kearney (1965) contained the following per litre of double distilled water: K_2HPO_4, 0.8 g; KH_2PO_4, 0.2 g; $MgSO_4$, 0.2 g; $(NH_4)_2SO_4$, 5.0 g; $(NH_4)_6Mo_7O_{14}$, 0.001 g. MSA was MS containing 0.18 $g.l^{-1}$ acetochlor (active ingredient). MS and MSA media were solidified with 10 g of either purified agar or noble agar per litre. A full strength nutrient medium (NM) consisted of MS medium together with 0.2 g glucose and 0.05 g yeast extract per litre.

NMA was NM containing 0.18 $g.l^{-1}$ acetochlor. NM and NMA media were solidified with 10 g bacteriological agar per litre. A one tenth strength nutrient medium (NM.1) comprised MS medium together with 0.02 g glucose and 0.005 g yeast extract per litre. Nutrient broth (NB) and nutrient agar (NA) powder were both bought from Oxoid. NB contained the following per litre: "Lab-Lemco" powder (Oxoid), 1.0 g; yeast extract (Oxoid), 2.0 g; peptone (Oxoid), 5.0 g; NaCl, 5 g. NA contained the same nutritional components as NB but was solidified with 1.5 g agar per litre. Luria broth (LB) contained the following per litre: NaCl, 10 g; bacto-tryptone, 10 g; yeast extract, 5 g. Luria agar (LA) was solidified with 1.5 g bacteriological agar. M9 mineral salts medium contained the following per litre: Na_2HPO_4, 6.78 g; KH_2PO_4, 3 g; NaCl, 0.5 g; NH_4Cl, 1 g; bacteriological agar, 15 g. When required tetracycline (tet) and ampicillin (amp) were included in media at a concentration of 10 $mg.l^{-1}$ and 50 $mg.l^{-1}$ respectively.

2.3 Plasmids, Bacteriophages, Bacterial Strains and Maintenance of Bacterial Cultures

Plasmids and bacteriophages used in this study are listed and described in Table 2.1. Helper phage R408(f1) from Stratagene were obtained from W. A. Cress in the Botany Department of the University of Natal in Pietermaritzberg, South Africa and was used with his permission. pBluescript®SK+ was obtained from P. Hare in the Botany Department of the University of Natal in Pietermaritzberg, South Africa. pUC18 was obtained from Clontech Laboratories Inc. Bacterial and fungal strains used in the present study and their growth conditions are given in Table 2.2. *E. coli* cells in possession of both recombinant and non-recombinant pUC18 and pBluescript SK+ were maintained on NA-amp. In those experiments involving microbial strains aseptic techniques were used wherever possible. While the *E. coli* strains used were obtained from the culture collection of Dr W. A. Cress, the *Ps. fluorescens* strain was obtained from the culture collection of Professor F. M. Wallis in the Department of Microbiology and Plant Pathology of the University of Natal in Pietermaritzberg, South Africa. *E. coli* strains, *Ps. fluorescens* and the soil isolates were stored for periods of up to 2 months on the appropriate solidified growth media in sealed petri dishes at 4°C. For long term storage of *E. coli* strains, *Ps. fluorescens* and important soil isolates, freezer stocks were made. This was achieved using mid log phase cultures (OD_{A600} 0.4 - 0.6) grown at either 30°C in NB (*Ps. fluorescens* and

TABLE 2.1. Description of the bacteriophages and plasmids used during the present study

Bacteriophage or Plasmid	Genotype (Description)	Source or Reference
Bacteriophages:		
R408(f1)	(f1 helper phage)	Stratagene
Plasmids:		
pUC18	Ap^r, lacZ′ (2.69-Kb)	Clontech
pR1	(3.8-Kb. pUC18 with a 1.1-Kb A108 DNA insert)	This Study
pR2	(3.9-Kb. pUC18 with a 1.2-Kb A108 DNA insert)	This Study
pR3	(3.6-Kb. pUC18 with a 0.9-Kb A108 DNA insert)	This Study
pR5	(5.9-Kb. pUC18 with a 3.3-Kb A108 DNA insert)	This Study
pR51	(4.4-Kb EcoRI fragment of pR5)	This Study
pR52	(4.0-Kb PstI fragment of pR5)	This Study
pR53	(4.5-Kb SalI fragment of pR5)	This Study
pR511	(3.8-Kb PstI fragment of pR511)	This Study
pBluescript®SK+	Ap^r, lacZ′, f1IG (2.95-Kb)	Sambrook *et al.*, 1989
pR7	(4.7-Kb. pBluescript®SK+ with 1.7-Kb EcoRI-HindIII fragment of pR51)	This Study
pR71-pR77	(4.6 to 3.2-Kb nested pR7 deletion series)	This Study

TABLE 2.2. Bacterial strains, their descriptions and the conditions under which they were maintained

Strain	Phenotype/Genotype	Maintenance	Reference or Source
Soil Isolates			
A1-A16, A33-A36, A41-A56, A73-A76, A81-A96, A113-A116	Acetochlor resistant	30°C on NMA agar	This Study
A17-A32, A37-A40, A57-A72, A77-A80, A97-A112, A117-A120	Acetochlor resistant	30°C on MSA agar	This Study
Pseudomonas fluorescens		30°C on NM agar	
Bioassay Fungus	Acetochlor sensitive	Room Temp. on NM agar	This Study
E. coli		37°C on M9 agar	
JM109	*recA*1 *supE*44 *endA*1 *hsdR*17 *gyrA*96 *relA*1 *thi*Δ (*lac-proAB*) F′ [*traP*36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	37°C on NA-tet	Sambrook *et al.*, 1989
XL1Blue	*supE*44 *hsdR*17 *recA*1 *endA*1 *gyrA*46 *thi relA*1 *lac*$^-$ F′ [*proAB*$^+$ *lacI*^{q} *lacZ*ΔM15 Tn10(*tet*)]		Sambrook *et al.*, 1989

Soil isolates) or 37°C in LB (*E. coli* strains) with shaking at 200 rpm. Two ml of these cultures were mixed with 2 ml sterile glycerol in a 5 ml disposable plastic test tube, frozen in liquid nitrogen for 40 seconds and stored at -70°C. Cells were revived by scraping an inoculating loop over the frozen surface of the freezer stock, streaking cells which adhered to the loop onto the appropriate solidified medium and incubating the cells at either 37°C for *E. coli* or 30°C for *Ps. fluorescens* and soil isolates. The bioassay fungus was stored at room temperature on NM agar for up to two months in a sealed plastic bag. It was subcultured every 4 to 8 weeks.

2.4 Spectrophotometric Determination of Acetochlor Concentration

A spectrophotometric technique was developed which allowed the measurement of precise acetochlor concentrations. An important feature of this technique was that the spectrophotometer was air blanked. Once acetochlor had been added to a solvent and its absorbance measured, the background absorbance (comprising the combined absorbances of the solvent and the particular quarts cuvette being used) was subtracted mathematically from the acetochlor solution's absorbance. Air was used for blanking the spectrophotometer since it provided a fixed standard against which the spectrophotometer could be blanked repeatedly during a series of measurements. This was important because acetochlor did not absorb very strongly in the concentration range over which it was soluble in water. Thus small fluctuations in absorbance measurements affected the precision with which acetochlor concentrations could be measured spectrophotometrically. It was found that the absorbance of any particular medium upon which the spectrophotometer was blanked, would gradually drift away from zero and such drifting of measured background absorbance contributed to significant errors when attempting to spectrophotometrically measure acetochlor concentrations. To avoid such errors it was necessary to constantly blank the spectrophotometer using a medium, such as air, the absorbance of which did not change over time. Consistency between the technique used in the construction of a standard acetochlor absorbance curve and the technique used for obtaining the absorbances of acetochlor solutions meant that the standard curve could be validly used to calculate acetochlor concentrations using absorbance data.

2.4.1 Construction of a Standard Curve

A standard curve was constructed which related the absorbance of acetochlor containing solutions at 264.9 nm and 272.6 nm to the herbicide concentrations present in the solutions. The optical density (OD) of acetochlor at its two absorption peaks (Fig. 2.1) was measured using a Beckman DU®640 spectrophotometer.

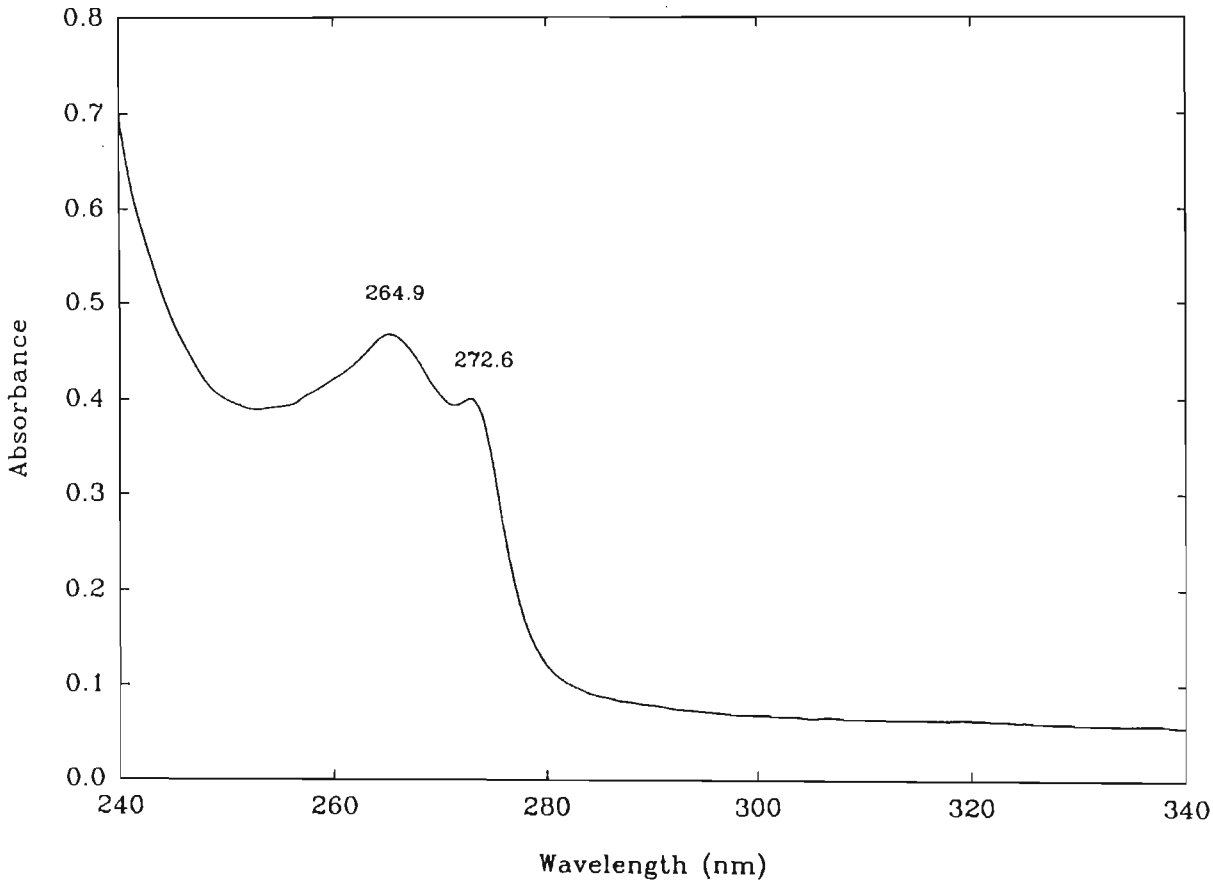

FIG. 2.1. The absorbtion pattern of an acetochlor solution (0.1 $g.l^{-1}$) over the wavelength range 240 nm to 340 nm. In producing this particular plot distilled water was used to both make up the acetochlor solution and to blank the DU®640 spectrophotometer over the range of wavelengths scanned.

101.0 ml (determined by mass) of distilled water was added to each of 45 numbered 100 ml bottles. Using the DU®640's multiple wavelength scan function the spectrophotometer was simultaneously blanked on air at 264.9 nm, 272.6 nm and 330 nm. Three measurements of the absorbance of 1 ml of the distilled water from each of the 45 bottles were taken at 264.9 nm, 272.6 nm and 330 nm using a clean semi-micro quarts cuvette. The three measurements taken at these three wavelengths were averaged to give the values of $A^{d}_{264.9}$, $A^{d}_{272.6}$ and A^{d}_{330} respectively. Three acetochlor concentration series were constructed by adding 0.002, 0.004, 0.006, 0.008, 0.010, 0.012, 0.014, 0.016, 0.018, 0.020, 0.022, 0.024, 0.026, 0.028 and 0.030 g of acetochlor (active ingredient) to the 100.0 ml of distilled water remaining in the 45 bottles. Because of the herbicide's high viscosity care was required when pipetting to ensure the transfer of accurate volumes. Immediately prior to their absorbances being measured the acetochlor solutions were each vigorously hand shaken for two minutes to ensure that a maximum concentration of the herbicide was in solution. For each acetochlor solution fifteen unadjusted absorbance readings at 264.9 nm, 272.6 nm and 330 nm were taken. The averages of the fifteen readings at each of these three wavelengths ($A^{u}_{264.9}$, $A^{u}_{272.6}$ and A^{u}_{330} respectively) were calculated. Between measuring the absorbance of different solutions the cuvette was thoroughly rinsed with the solution about to be measured. In all instances after every three readings the spectrophotometer was reblanked on air, the measured sample discarded and a new sample added to the cuvette.

For each of the 45 acetochlor solutions a background absorbance factor for the 264.9 nm ($K_{264.9}$) and 272.6 nm ($K_{272.6}$) absorption peaks was calculated. For any given solution this was achieved using the $A^{d}_{264.9}$, $A^{d}_{272.6}$ and A^{d}_{330} values of the pure distilled water that had originally been used for preparing that solution in the following formulae:

$$K_{264.9} = A^{d}_{264.9} \div A^{d}_{330} \qquad (1)$$

$$K_{272.6} = A^{d}_{272.6} \div A^{d}_{330} \qquad (2)$$

The actual, adjusted, absorbance of the acetochlor present in the various solutions at 264.9 nm ($A_{264.9}$) and 272.6 nm ($A_{272.6}$) could then be determined using the following equations:

$$A_{264.9} = A^{u}_{264.9} - A^{u}_{330} \text{ X } K_{264.9} \qquad (3)$$

$$A_{272.6} = A^{u}_{272.6} - A^{u}_{330} \text{ X } K_{272.6} \qquad (4)$$

The two adjusted absorbance values thus obtained for each of the different acetochlor solutions were plotted against the herbicide concentrations they represented. Over the full concentration range tested (Fig. 2.2.1) the heights of the two acetochlor absorbance peaks were described by the following binomial equations:

$$A_{264.9} = -2.155[acet]^2 + 2.640[acet] - 0.0055 \quad (R = 0.9980) \qquad (5)$$

$$A_{276.9} = -1.835[acet]^2 + 2.197[acet] - 0.0047 \quad (R = 0.9980) \qquad (6)$$

where [acet] is the concentration of acetochlor in $g.l^{-1}$. It should be noted that the shape of these plots differ from the shape of absorbance versus concentration plots predicted by the Labert-Beer Law. One implication of this law is that the intensity of monochromatic light transmitted by a solvent containing an absorbing solute decreases exponentially in relation to a linear increase in the solute's concentration (Burris, 1964). This indicates that the absorbance of a solution should increase exponentially with increasing concentrations of the absorbing solute. The opposite of this was observed in the case of the plot of absorbance against acetochlor concentrations presented in Fig. 2.2.1. Over the lower concentration ranges represented in this plot (0 $g.l^{-1}$ to 0.12 $g.l^{-1}$), approximate linearity occurred which conformed to the Lambert-Beer Law. However, at the higher acetochlor concentrations plotted (0.20 $g.l^{-1}$ to 0.30 $g.l^{-1}$) the plateauing of absorbance values ran contrary to what should, according to this law, have been expected. There are two possible reasons why this deviation from the Lambert-Beer Law was observed. The first is that technical grade acetochlor has a high viscosity which contributes to significant pipetting errors when attempting to produce solutions with a high concentration of the herbicide. A second possible reason is that the solubility of acetochlor in water at room

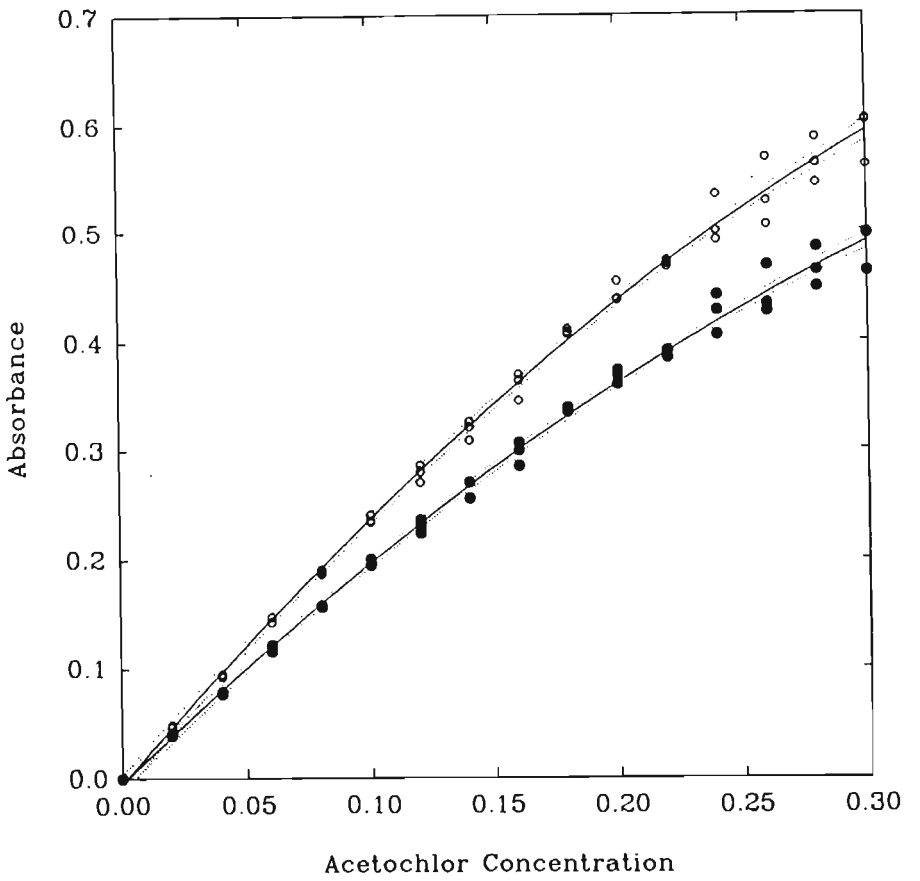

FIG. 2.2.1. Standard curves for relating the absorbance of acetochlor at 264.9 nm (○) and 272.6 nm (●) to the concentration of the herbicide in solution over the range of 0.00 to 0.30 $g.l^{-1}$. The two curves are described by second order polynomials (equations [5] and [6]) in the text). (···) the 95% confidence intervals.

temperature is only 0.223 $g.l^{-1}$ (Worthing and Hance, 1991). This suggests that as herbicide concentrations increased to above 0.223 $g.l^{-1}$, so too did the amount of acetochlor failing to go into solution. The plots of absorbance versus the lower, more accurately measurable range of acetochlor concentrations between 0 and 0.12 $g.l^{-1}$ (Fig. 2.2.2), were described by the following linear equations:

$$A_{264.9} = 2.335[acet] + 0.00088 \quad (R = 0.9993) \qquad (7)$$

$$A_{272.6} = 1.933[acet] + 0.00098 \quad (R = 0.9993) \qquad (8)$$

Only the linear sections of the standard curves, encompassing the acetochlor concentrations between 0 $g.l^{-1}$ and 0.12 $g.l^{-1}$, that did not significantly contradict the expectations of the Lambert-Beer Law, were used in the conversion of absorbance values to estimates of acetochlor concentrations.

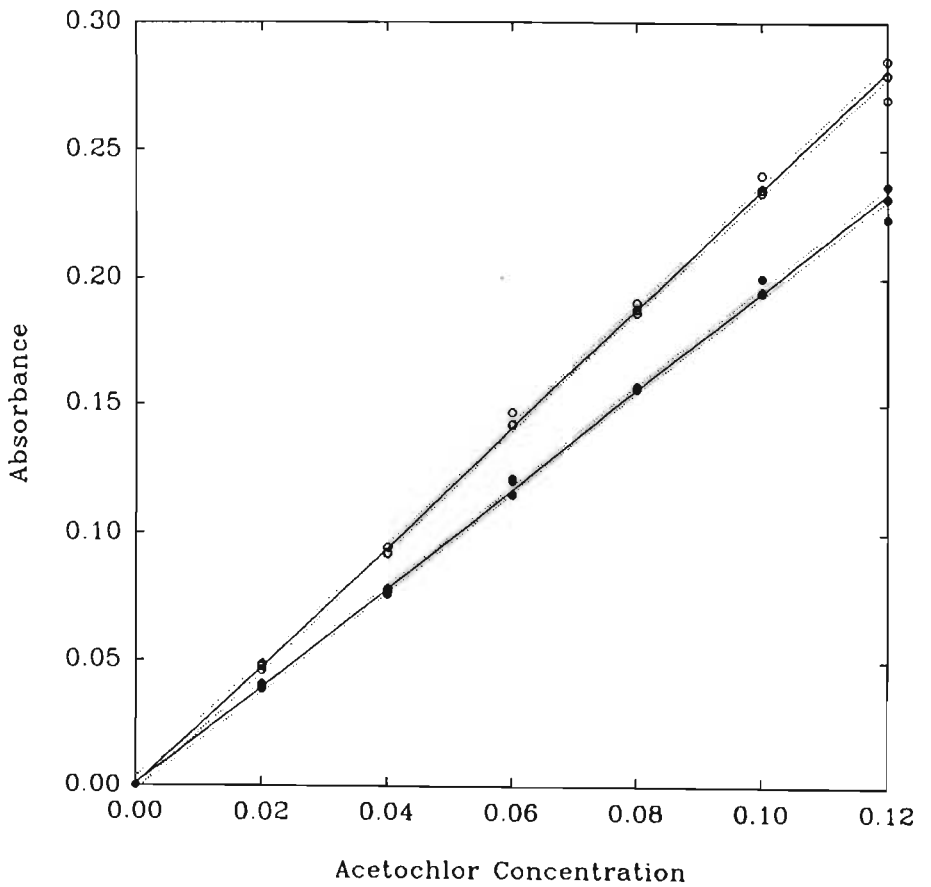

FIG. 2.2.2. Standard curves for relating the absorbance of acetochlor at 264.9 nm (○) and 272.6 nm (●) to the concentration of the herbicide in solution over the range of 0.00 to 0.12 $g.l^{-1}$. The two curves are described by linear equations [7] and [8] in the text. (···) 95 % confidence intervals.

2.4.2 Spectrophotometric Determination of Acetochlor Concentrations in Various Solutions

Relatively accurate estimations of acetochlor concentrations between 0.02 and 0.12 $g.l^{-1}$ in clear solutions could be achieved using spectrophotometry (section 2.4.1). Calculation of background absorbances were carried out in a similar manner to those calculated during the construction of the acetochlor standard curve. Thus following blanking of the DU®640 spectrophotometer the absorbances of the solutions to which acetochlor was to be added were simultaneously measured at 265.9, 272.6 and 330 nm using the spectrophotometer's fixed, multiple wavelength scan function. The background absorbance constants $K_{264.9}$ and $K_{272.6}$ of the particular solutions were calculated in a similar way as was described for the standard curve. Fifteen readings of each solution's absorbance at 264.9 nm ($A^s_{264.9}$), 272.6 nm ($A^s_{272.6}$) and 330 nm (A^s_{330}) were taken. The background absorbance constants of the solutions were then calculated using the following equations:

$$K_{264.9} = A^s_{264.9} \div A^s_{330} \qquad (9)$$

$$K_{272.6} = A^s_{272.6} \div A^s_{330} \qquad (10)$$

Following the addition of acetochlor fifteen readings of the solution's unadjusted absorbance at 264.9 nm ($A^u_{264.9}$), 272.6 nm ($A^u_{272.6}$) and 330 nm (A^u_{330}) were made using the same procedures and precautions described in section 2.4.1.

The actual absorbances of the solutions at 264.9 nm ($A_{264.9}$) and 272.6 nm ($A_{272.6}$) measured by each of these fifteen readings were calculated using equations (3) and (4) respectively. The means ($\bar{x}_{264.9}$ and $\bar{x}_{272.6}$) and standard errors ($s_{264.9}$ and $s_{272.6}$) of the absorbance values obtained at each wavelength were calculated and the 95% confidence intervals of their means determined using the Student's t-test with 14 degrees of freedom (t-value of 2.145; Clarke, 1987). The maximum ($\bar{x}^U_{264.9}$ and $\bar{x}^U_{272.6}$) and minimum ($\bar{x}^L_{264.9}$ and $\bar{x}^L_{272.6}$) possible values of $\bar{x}_{264.9}$ and $\bar{x}_{272.6}$ could be calculated with a 95% degree of certainty using the following equations:

$$\bar{x}^{U}_{264.9} = \bar{x}_{264.9} + (s_{264.9} \div \sqrt{15}) \text{ X } 2.145 \qquad (11)$$

$$\bar{x}^{L}_{264.9} = \bar{x}_{264.9} - (s_{264.9} \div \sqrt{15}) \text{ X } 2.145 \qquad (12)$$

$$\bar{x}^{U}_{272.6} = \bar{x}_{272.6} + (s_{272.9} \div \sqrt{15}) \text{ X } 2.145 \qquad (13)$$

$$\bar{x}^{L}_{272.6} = x_{272.6} - (s_{272.9} \div \sqrt{15}) \text{ X } 2.145 \qquad (14)$$

The average acetochlor concentration ($[acet]^{av}$) together with the 95% probable upper ($[acet]^{U}$) and lower ($[acet]^{L}$) acetochlor concentrations in solution were calculated using the following formulae derived from equations (7) and (8):

$$[acet]^{av} = \{[(\bar{x}_{264.9} - 0.00088) \div 2.335] + [(\bar{x}_{272.6} - 0.00098) \div 1.933]\} \div 2 \qquad (15)$$

$$[acet]^{U} = \{[(\bar{x}^{U}_{264.9} - 0.00088) \div 2.335] + [(\bar{x}^{U}_{272.6} - 0.00098) \div 1.933]\} \div 2 \qquad (16)$$

$$[acet]^{L} = \{[(\bar{x}^{L}_{264.9} - 0.00088) \div 2.335] + [(\bar{x}^{L}_{272.6} - 0.00098) \div 1.933]\} \div 2 \qquad (17)$$

If the difference between the solution's calculated $[acet]^{U}$ and $[acet]^{L}$ was greater than 0.0025 $g.l^{-1}$ the absorbance of the solution was remeasured.

2.5 Determination of Acetochlor Toxicity by Bioassay

The development of an effective acetochlor bioassay, including the isolation of a bioassay organism (a fungus) from the environment and the examination of parameters which effect the accuracy of the bioassay, are described in Chapter 3. Since the bioassay was to play a central role in identifying the capacity of both isolated soil bacteria and transformed *E. coli* strains to detoxify acetochlor, the procedures employed in carrying out the bioassay will be discussed in this chapter. Because the assay was developed over a period of a year, the qualitative procedure used initially for the detection of acetochlor detoxifying soil bacteria was far less precise than the more refined assay subsequently used for quantifying degrees of acetochlor detoxification carried out by either an isolated herbicide degrading

soil bacterium or a recombinant *E. coli* strain with one of the selected soil isolate's degradative genes inserted.

2.5.1 Qualitative Detection of Diminished Acetochlor Toxicity

An outline of the procedure for detecting the detoxification of acetochlor is given in Fig. 2.3. For each bacterium tested for its ability to detoxify acetochlor, two 250 ml Erlenmeyer flasks, containing 50.0 ml MS medium (measured by mass), were prepared. For each detoxification experiment an additional two flasks containing 50.0 ml MS medium were prepared and would serve as uninoculated controls. The flasks were all stoppered with cotton wool capped with aluminium foil and sealed with masking tape. They were weighed before and after autoclaving at 121°C for 15 minutes in order to determine the volume of water lost through evaporation during autoclaving. Care was taken when aseptically pipetting acetochlor to ensure that volumes measured were as exact as possible. Acetochlor was added to half of the flasks to obtain a acetochlor concentrations of 0.15 $g.l^{-1}$. The flasks were shaken vigorously by hand for five minutes. Using an inoculating loop a single colony from a pure culture of each of the bacteria being tested was transferred to each of two flasks; one with (test flask) and one without acetochlor (inoculated control flask). The uninoculated control flasks consisted of one flask containing the herbicide and another without the herbicide. The flasks were resealed with masking tape and incubated in the dark at 30°C for 18 days with constant rotary shaking at 120 rpm. Following incubation the flasks were reweighed in order to estimate the volume of liquid remaining in them. One or two 20 ml samples from each flask (depending on how much liquid was left remaining in the flasks) were pipetted into sterile 20 ml bottles containing 0.2 g bacteriological agar. 200 μl samples of a solution containing 5 g yeast extract and 20 g glucose per litre, were added to each of the bottles to give a final concentration of 0.2 $g.l^{-1}$ glucose and 0.05 $g.l^{-1}$ yeast extract. The bottles were heated to 100°C until all of the agar they contained had properly dissolved. The bottles were vigorously hand shaken for two minutes before decanting their contents into petri dishes. The plates were left to dry for 24 hours before inoculating them with a combination of spores and hyphal segments (cfu's) from three to five week old colonies of the bioassay fungus which had been maintained at room temperature on NM agar.

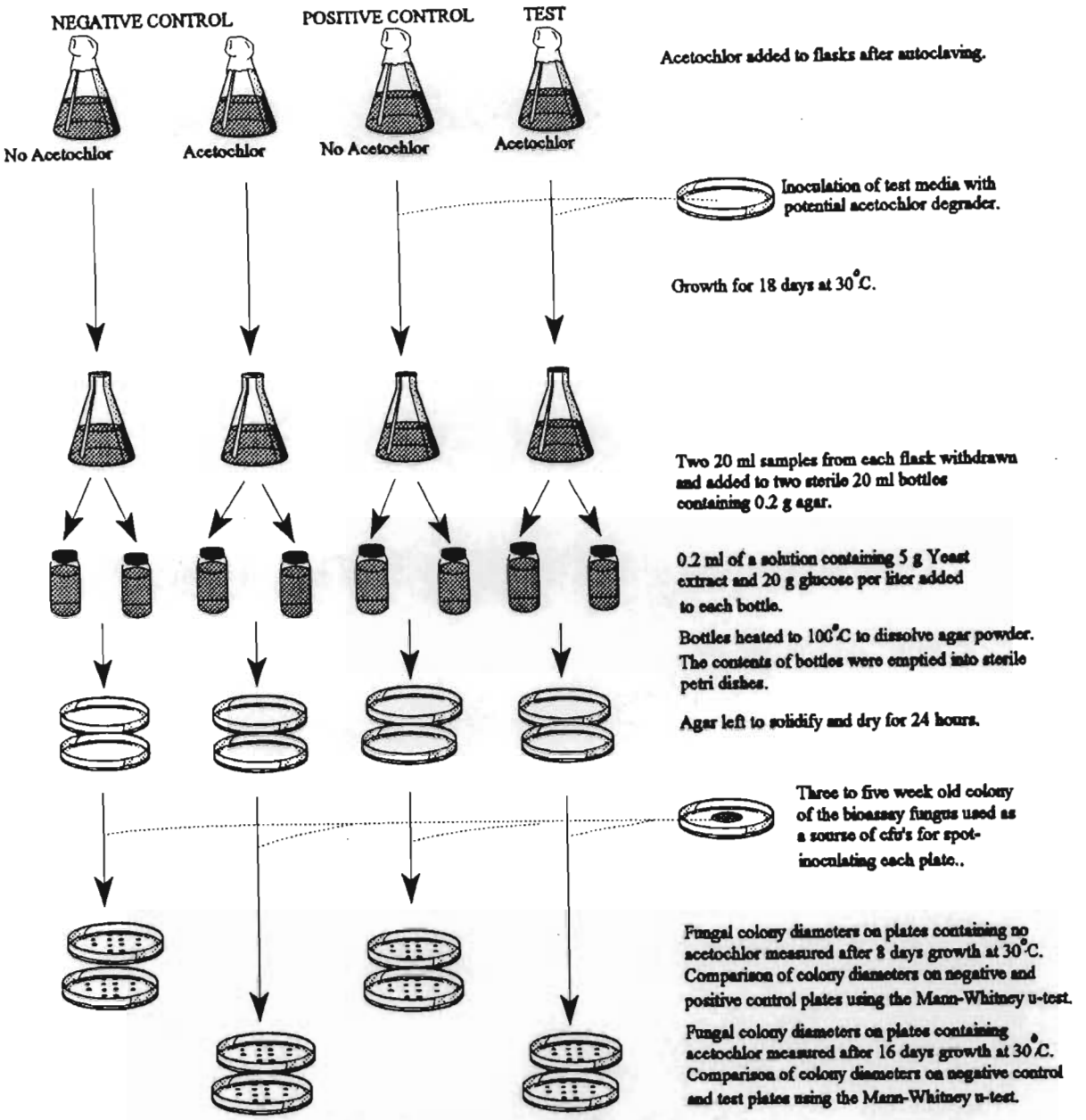

FIG. 2.3. Diagrammatic outline of the experimental procedure followed during the bioassay to detect diminished acetochlor toxicity.

Sterile micropipette tips were used to transfer inoculum and spot inoculate each plate at twelve separate positions (Fig. 2.4). The plates were incubated in the dark at 30°C for up to 16 days. During the second day of incubation the plates were sealed with masking tape to prevent excessive drying out. After 8 days of growth the diameters of the fungal colonies appearing on the plates inoculated and uninoculated control plates containing no acetochlor were measured to the nearest 0.25 mm. The diameter of fungal colonies occurring on the plates containing acetochlor were measured on day 16. Only the

diameters of well separated single fungal colonies were measured. The mean fungal colony diameter on each plate was calculated. If two plates had been poured from the same bottle of medium the sets of colony diameter measurements from both plates were combined for further statistical analysis. The fungal growth on acetochlor containing media originating from the test flasks was compared with that originating from the acetochlor containing control flasks using the Mann-Whitney u-test (Neave and Worthington, 1988). A comparison, employing the Mann-Whitney u-test, was also made between the fungal growth on media containing no acetochlor originating from the inoculated control flasks and the growth on media originating from the uninoculated control flasks containing no acetochlor. The results of the Mann-Whitney test were obtained using the Statgraphics statistical analysis software package (produced by Graphics Software Systems, Inc.). Three criteria had to be met before it could be assumed that any bacterium being tested was detoxifying the herbicide. The first was that the mean diameters of the fungal colonies on plates containing the herbicide were greater on test plates than on control plates. The second criterion was that, according to the u-test, differences in colony diameters on acetochlor containing control and test plates should occur with a probability of greater than 95%. The final criterion was that the probability

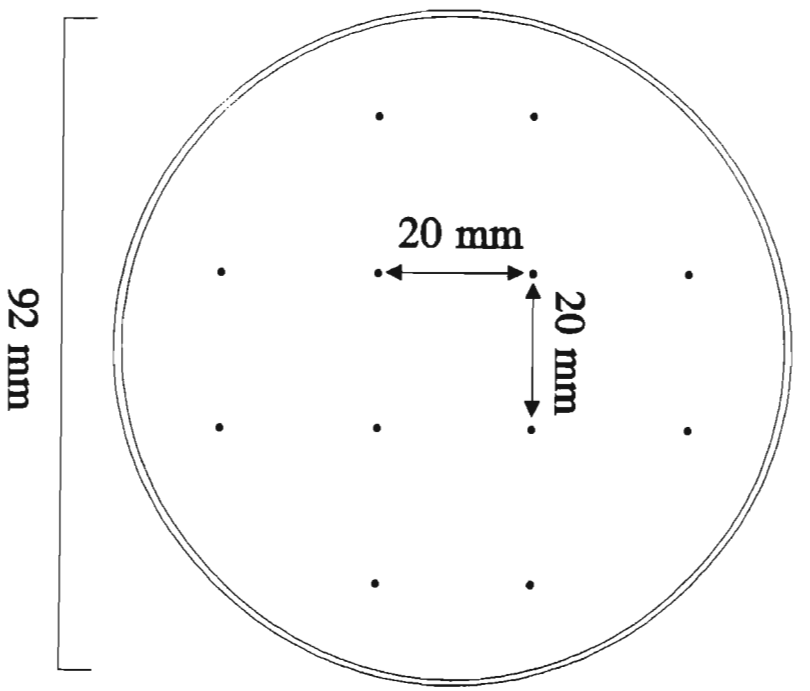

FIG. 2.4. Pattern in which plates were inoculated with the bioassay fungus.

that the colony diameters on media originating from the inoculated and uninoculated control flasks were not different from one another was greater than 5%. The purpose of this last criterion was to guarantee that the test bacterium had not, independently of the herbicide, contributed anything to the test medium to stimulated growth of the bioassay fungus.

2.5.2 Construction of a Standard Curve for the Determination of Active Acetochlor Concentrations

Three separate 1 litre batches of MS medium were made up and decanted immediately in 100.0 ml aliquots (measured by mass) into 30 sterile 100 ml bottles. All the bottles were numbered according to the batch of MS medium from which they originated. The bottles were separated into 3 groups (representing the three original batches of medium) comprising 7 bottles each were made. The 9 remaining bottles were kept in reserve. To five of the seven bottles in each group was added either 2, 4, 6, 8 or 10 mg of acetochlor (to obtain respective acetochlor concentrations of 0.02, 0.04, 0.06, 0.08 and 0.10 $g.l^{-1}$). Before adding acetochlor, 0.5 ml samples from each bottle to which the herbicide was to be added were withdrawn for use in background absorbance determinations. The exact concentrations of acetochlor added to the bottles was verified spectrophotometrically using a single 0.5 ml sample from each bottle. From each of the two bottles containing no acetochlor in each group, 1 ml was withdrawn and discarded. Exactly 1 g of bacteriological agar and 1 ml of a solution containing 20 $g.l^{-1}$ glucose and 5 $g.l^{-1}$ yeast extract was added to each bottle. The lids of the bottles were all tightly sealed and each of their masses was determined before and after autoclaving at 100°C for 30 minutes. If after autoclaving the mass of a bottle had decreased by more than 1% of its initial mass it was discarded and one of the back-up bottles were prepared in its place. While still hot from autoclaving, both the bottles of media containing acetochlor at various concentrations and those without any added acetochlor were kept for between 10 and 50 minutes in a 55°C water bath. Immediately prior to dispensing the media into plates the bottles were vigorously hand shaken for two minutes. The 100 ml of medium in each bottle was aseptically pipetted (20.0 ml volumes) into five petri dishes positioned on a level surface. The thirty five plates thus obtained for each of the three groups were left to dry for 24h.

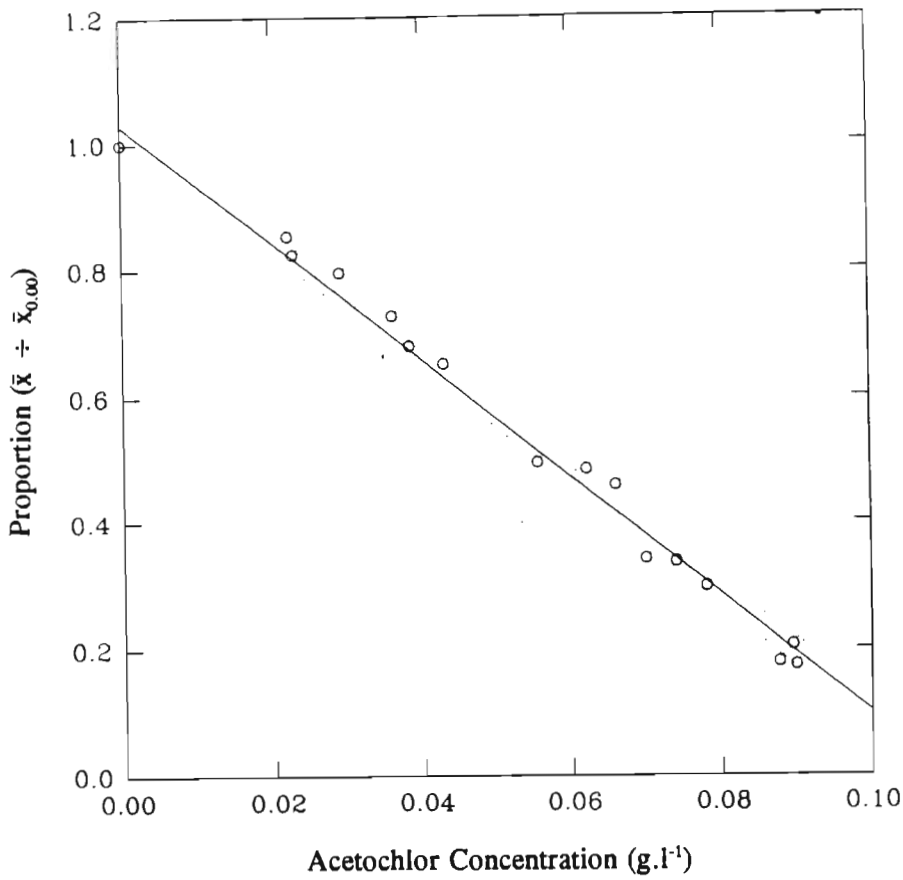

FIG. 2.5. Standard curve for the determination of active acetochlor concentrations in NM agar. Herbicide concentrations were related to the fraction of bioassay fungal growth occurring on acetochlor containing NM agar relative to the growth occurring on acetochlor-free NM agar. (···) 95% confidence intervals.

The inoculum used to seed each bioassay plate in a single group was obtained from the perimeter of a single fungal colony which had been maintained at 30°C for three to eight weeks on NM agar. All plates were inoculated with the fungus at twelve sites using one sterile micropipette tip per plate (Fig. 2.4). Stacked plates were placed in plastic bags which were then sealed and incubated at 30°C for 9 to 10 days by which time the mean diameters of the colonies on the plates containing no acetochlor were between 9 and 11 mm.

The diameters of the fungal colonies on all plates were then measured to the nearest 0.25 mm. On each plate only single colonies situated on one of the twelve inoculation sites were considered. For slightly non-circular colonies the smallest diameter was measured. All the plates in a group were measured within a 2.5 hour period.

In each of the three groups the mean diameter of the colonies on the five plates containing different concentrations of acetochlor ($\bar{x}$) was expressed as a fraction of the mean diameter of the colonies on the 10 plates containing no acetochlor ($\bar{x}_{0.00}$). These fractions were plotted against the acetochlor concentrations they represented (Fig. 2.5) and the regression obtained was described by the linear equation:

$$\bar{x} \div \bar{x}_{0.00} = -9.289[\text{acet}] + 1.029 \quad (R = 0.996) \qquad (18)$$

2.5.3 Quantitative Detection of Acetochlor Degradation Using the Bioassay

An outline of the experimental procedure followed is given in Fig. 2.6. For every bacterium tested for its ability to detoxify acetochlor four 2 litre Erlenmeyer flasks, each containing approximately 400 ml of either MS medium or NM.1, were prepared. Prior to autoclaving the flasks were all stoppered with non-absorbent cotton wool and sealed with aluminium foil and masking tape. They were autoclaved at 121°C for 15 minutes. Between 20 and 28 mg of acetochlor (to give an approximate herbicide concentration between 0.05 and 0.07 $g.l^{-1}$) was added to two flasks for which background absorbances had already been measured and these were then shaken vigorously for five minutes. The precise concentration of acetochlor in these two flasks was determined spectrophotometrically in the manner described in section 2.4.2. Two of the flasks, one with and one without acetochlor, were inoculated with the bacterium being tested for acetochlor degradative capacity. Inoculation was carried out by transferring half of a single large colony (3-5 mm in diameter on NM containing acetochlor at 0.2 $g.l^{-1}$) to each of the flasks using an inoculating loop. The Erlenmeyer flasks were resealed with masking tape and incubated at 30°C for 28 days with constant rotary shaking at 120 rpm. At 7, 14, 21 and 28 days after inoculation either 79.2 ml (for tests carried out using MS medium) or 79.3 ml (for tests carried out using NM.1) volumes of medium (determined by mass)

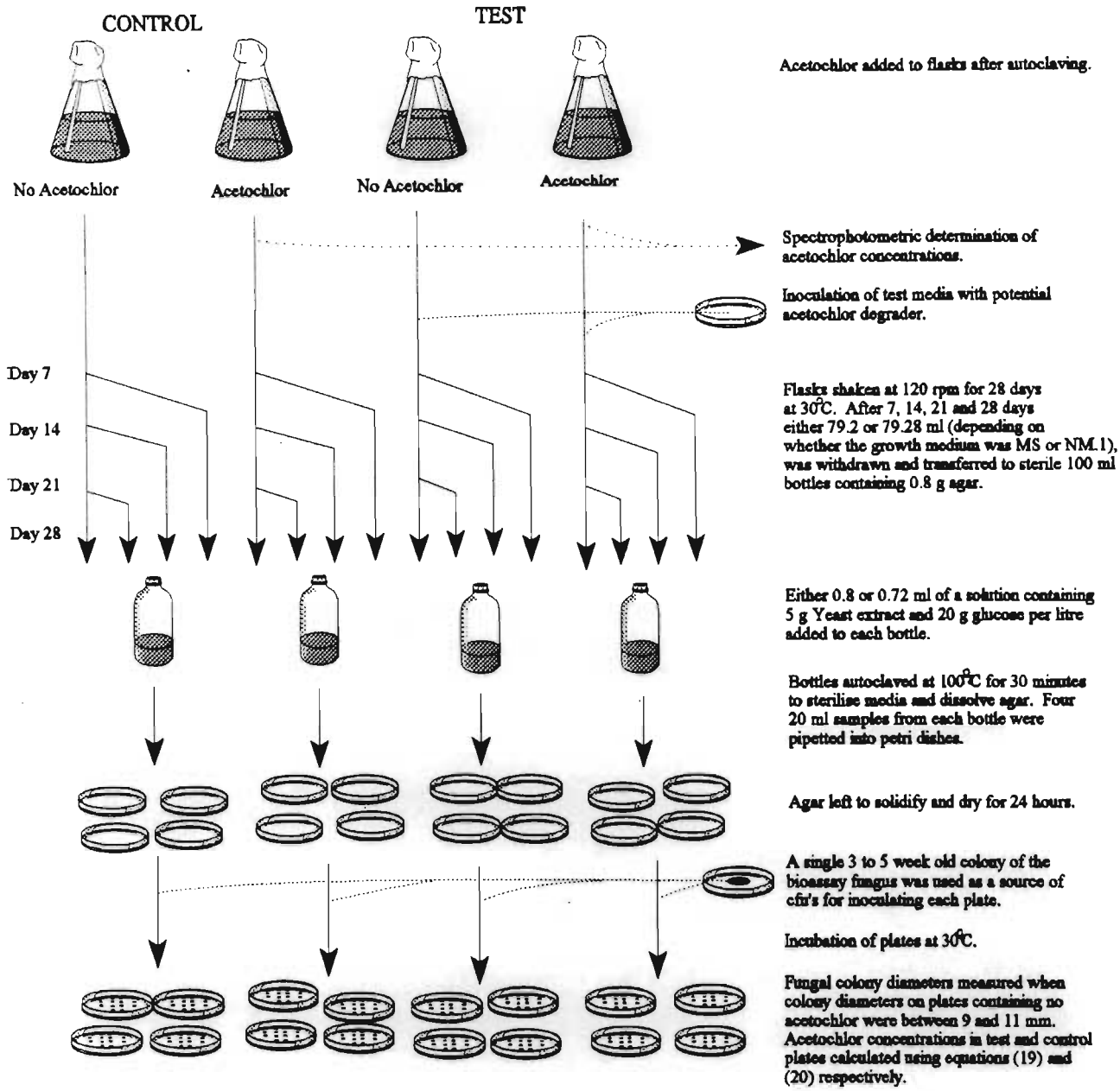

FIG 2.6. Diagrammatic outline of the experimental procedure followed during the bioassay to quantify the amount of acetochlor degraded by a test bacterium.

from each flask were transferred as aseptically as possible to sterile 100 ml bottles containing 0.80 g bacteriological agar. The concentration of acetochlor in the uninoculated control flask containing the herbicide was remeasured using spectrophotometry. Either 0.8 ml (for tests carried out using MS medium) or 0.72 ml (for tests carried out using NM.1) of a sterile solution containing 5 g yeast extract and 20 g glucose per litre, was added to each of the bottles containing media withdrawn from the 4 flasks. The bottles, each tightly sealed with a screw-cap, were weighed before and after autoclaving at 100°C for 30

minutes. Following autoclaving, any bottles which had lost more than 1% of their initial mass were noted. Media were kept melted at 55°C for between 10 and 40 minutes until they were used for pouring plates. The bottles were vigorously hand shaken for two minutes. From each bottle four 20.0 ml volumes of medium were pipetted into petri dishes on a level surface. The sixteen agar plates thus obtained were dried for one day on a laminar flow bench. Plates were inoculated with the bioassay fungus, incubated and analysed as has been described for the construction of the standard curve (section 2.5.2).

The mean diameters of fungal colonies on plates containing acetochlor that originated in inoculated test ($\bar{x}^{TA}$) and uninoculated control ($\bar{x}^{CA}$) flasks were expressed as fractions of the mean fungal colony diameters on plates containing no acetochlor from inoculated test ($\bar{x}^{T}$) and uninoculated control ($\bar{x}^{C}$) flasks respectively. Using an adaptation of equation (18) the concentration of acetochlor in the inoculated and uninoculated flasks could be determined:

$$[acet]^{T} = (\bar{x}^{TA} \div \bar{x}^{T} + 98.35) \div -658.7 \qquad (19)$$

$$[acet]^{C} = (\bar{x}^{CA} \div \bar{x}^{C} + 98.35) \div -658.7 \qquad (20)$$

This measurement of the uninoculated control's active herbicide content was compared with the pre- and post-incubation spectrophotometric measurements of its acetochlor concentration in order to verify that no significant loss in toxicity of the herbicide had occurred during preparation of the bioassay. The bioassay derived estimation of acetochlor concentration was compared to the initial herbicide concentration which had been measured spectrophotometrically. The amount of acetochlor remaining was expressed as a percentage of the initial herbicide concentration prior to detoxification.

On each of the days on which samples were withdrawn from flasks for bioassays, medium from the flasks was screened for possible contamination with non-test microbes. From each flask 1 μl of medium was transferred and spread onto a NA plate. If growth occurred on plates inoculated with medium from the control flasks the test was abandoned. Bacterial growth obtained on NA plates inoculated with media from the test flasks was

examined for contamination according to whether the bacterium being tested was the acetochlor degrading soil isolate or recombinant *E. coli*. In both cases cells from colonies on the NA plates were checked for their morphology and gram staining characteristics. For those flasks meant to contain only the soil isolate the test for contaminants involved both the detection of growth at 42°C (A temperature at which the soil isolate was unable to grow) and scanning the plates for colonies which lacked the distinctive fluorescent pigmentation of the soil isolate. In assays involving recombinant *E. coli* strains the presence of characteristic antibiotic resistance markers and the inability to grow in the absence of thiamine was tested for.

A scaled down version of the quantitative acetochlor bioassay was also used. This scaling down involved the use of initial media volumes of 100 ml in 500 ml Erlenmeyer flasks. After 21 days of exposure to the bacterium being tested for herbicide detoxification capacity either 79.20 or 79.28 ml of the medium (depending upon whether MS medium or NM.1 was being used) was processed for analysis with the bioassay using the methods described.

3 DEVELOPMENT OF AN ACETOCHLOR BIOASSAY

3.1 Introduction

Of primary importance in studies involving the isolation of pesticide metabolising microbes is the establishment of suitable assays permitting the detection of pesticide transformation reactions. Whereas many assays are qualitative and merely indicate whether or not pesticidal compounds have been metabolised, ideally they should also be quantitative and provide estimates of the numbers of pesticide molecules which have undergone transformation.

In order to identify microbes capable of degrading the chloroacetanilide herbicide, acetochlor, a reliable assay for the herbicide was required. A number of qualitative and quantitative assays have been employed for the detection of microbial chloroacetanilide degradation. Generally the qualitative assays used have been simpler than the quantitative ones. At their simplest, attempts to qualitatively detect chloracetanilide degradation among microbial strains has centred around testing strains for their ability to grow on these herbicides as sole carbon or nitrogen sources (Liu *et al.*, 1989; Novick and Alexander, 1985; Saxena *et al.*, 1987; Villarreal *et al.*, 1981). More complex qualitative assays have aimed at the detection of $^{14}CO_2$ (Novick *et al.*, 1986) and highly water soluble ^{14}C-labelled metabolites (Novick and Alexander, 1986) produced during the microbial degradation of ^{14}C-labelled herbicide molecules.

The quantitative assays most commonly used to monitor the microbial degradation of chloroacetanilide herbicides have been based on gas-liquid chromatography (GLC) (Krause *et al.*, 1985; Liu *et al.*, 1989; Liu *et al.*, 1991; McGahen and Tiedje, 1978; Novick *et al.*, 1986; Saxena *et al.*, 1987; Smith and Phillips, 1975; Tiedje and Hagedorn, 1975; Villarreal *et al.*, 1991). Other chromatography-based quantitative chloroacetanilide assays have employed high performance liquid chromatography (HPLC) (Pothuluri *et al.*, 1993); column chromatography with Waters Associates' Sep-Pak® C_{18} cartridges (Novick and Alexander, 1985); and thin layer chromatography (TLC) (Bailey and Coffey, 1986; Liu *et al.*, 1989; Novick and Alexander, 1985). Many of these chromatographic assays (particularly those utilising GLC and HPLC) have been used to not only precisely quantify

the degradation of chloracetanilide molecules but also to both facilitate the isolation of specific degradation products and to provide information on the rates at which these products are formed (Bailey and Coffey, 1986; Krause *et al.*, 1985; Liu *et al.*, 1989; Liu *et al.*, 1991; Novick *et al.*, 1986; Mcgahen and Tiedje, 1978; Pothuluri *et al.*, 1993; Saxena *et al.*, 1986; Tiedje and Hagedorn, 1975; Villarreal *et al.*, 1991). Non-chromatographic quantitative assays which have been used in the detection of microbial chloroacetanilide transformation have included the measurement of $^{14}CO_2$ (Bailey and Coffey, 1986; Novick *et al.*, 1986) or ^{14}C-labelled metabolites arising through the breakdown of ^{14}C-labelled herbicide molecules (Bailey and Coffey, 1986; Liu *et al.*, 1991; Pothuluri *et al.*, 1991; Saxena *et al.*, 1987), the colorimetric determination of chloride ions released from degraded herbicide molecules (Liu *et al.*, 1991; Tiedje and Hagedorn, 1975) and the use of bioassays to monitor decreases in herbicidal activities (Bailey and Coffey, 1985; Bailey and Coffey, 1986).

Bailey and Coffey (1984) have described a cheap, simple and precise agar plate based bioassay for the determination of active metalaxyl (a fungicide chemically related to acetochlor) concentrations in soils. These researchers have also applied the bioassay to the measurement of active soil concentrations of the chloroacetanilide herbicide metolachlor (Bailey and Coffey, 1985). It was realised that a similar bioassay for the measurement of active acetochlor concentrations in growth media, would be extremely valuable in the present study since it would facilitate not only the isolation of acetochlor detoxifying microorganisms, but also the isolation of a gene instrumental in the herbicide's detoxification. Once isolated such genes could potentially be employed to genetically engineer resistance to acetochlor in crops.

The development of an agar plate based quantitative acetochlor bioassay is described here. From amoung 43 cultures isolated from the environment that were tested for their usefulness as bioassay organisms, a fungal strain was selected because of its suitability with regard to the measurement of acetochlor concentrations. The sensitivity of the selected bioassay fungus was quantified under controlled nutritional and environmental conditions and an effective bioassay procedure was developed. Certain factors affecting the precision of the bioassay were also examined.

3.2 Materials and Methods

3.2.1 Isolation of an Acetochlor-Sensitive Bioassay Organism

43 cultures isolated from soil and from water obtained from a stream beside the Faculty of Agriculture buildings of the University of Natal in Pietermaritzberg, were tested for their susceptibility to acetochlor. 39 bacterial and 4 fungal isolates for which pure cultures had been obtained, were each streaked in triplicate onto NM agar (nutrient medium described in section 2.2) containing 0.00, 0.05, 0.10 and 0.15 $g.l^{-1}$ acetochlor. Each plate was sealed with masking tape and incubated at 30°C. After 7 days, isolates were examined for a reduction in growth which corresponded to an increase in herbicide concentrations. Fungal herbicide sensitive isolates were also streaked in triplicate onto MS agar (mineral salts medium described in section 2.2) and incubated at 30°C for ten days.

The effect of different acetochlor concentrations on the growth of a selected fungal isolate was determined over a 56 day period. 500 ml of sterile NM agar was melted and 20 ml aliquots aseptically transferred to 25 sterile 20 ml bottles. The bottles were kept at 50°C in a water bath for up to 30 minutes and divided into 5 groups of 5 bottles each. To each of the bottles in four of the groups was added either 1, 2, 3 or 4 mg acetochlor (to give acetochlor concentrations of 0.05, 0.10, 0.15 and 0.20 $g.l^{-1}$ respectively). Following vigorous shaking for two minutes each bottle was emptied into a petri dish. The plates once set were left to dry for 48 hours. Hyphae from the border of a 45 day old colony of the fungus on NM agar at room temperature (fluctuating between approximately 8°C and 21°C) were suspended in 1 ml sterile distilled water with vigorous shaking by hand. 1 μl of the hyphal suspension was pipetted into the centre of each dry plate. Plates were stacked, sealed with masking tape and incubated at 30°C for 8 weeks. At weekly intervals the diameters of the fungal colonies which appeared on the plates were measured to the nearest 0.25 mm.

The effect of acetochlor concentrations between 0.00 and 0.165 $g.l^{-1}$ on colony diameters of the selected fungal isolate growing on NM agar was investigated. The same procedure used for constructing the standard curve for the determination of active acetochlor

concentrations (section 2.5.2) was followed with the following exceptions: batches of MS agar were made up, decanted in 98 ml aliquots into six 100 ml bottles and autoclaved at 121°C for 15 minutes; 1 ml of both glucose and yeast extract stock solutions were added to each bottle following autoclaving to give glucose and yeast extract concentrations of 0.2 $g.l^{-1}$ and 0.05 $g.l^{-1}$ respectively; acetochlor was added in carefully pipetted quantities (ranging from between 0.02 to 0.165 $g.l^{-1}$) to medium with no spectrophotometric verification made of the amount added; instead of pipetting 20 ml aliquots when making plates, 100 ml quantities of medium was transferred in approximately equal volumes into five sterile petri dishes.

The calibration curve which was used in this study for relating the growth of the fungus to acetochlor concentrations between 0.00 and 0.1 $g.l^{-1}$, was constructed in the manner described in section 2.5.2.

3.2.2 The Effect of Heating on Acetochlor Activity

An 800 ml batch of MS medium was made up using freshly sterilised distilled water. Half of the MS medium in a batch was decanted in 99.0 ml aliquots into four 100 ml sterile bottles each containing 1.00 g bacteriological agar. Approximately 22 g acetochlor was added to the 400 ml of MS medium remaining undecanted. Following mixing of the acetochlor into the MS medium (2 minutes of vigorous shaking by hand), the exact concentration of the acetochlor dissolved was determined spectrophotometrically (section 2.4.1). The MS medium containing acetochlor was decanted in 99.0 ml aliquots into sterile 100 ml bottles each containing 1.00 g bacteriological agar. 1 ml of a solution containing 20 $g.l^{-1}$ glucose and 5 $g.l^{-1}$ yeast extract was added to each bottle. Bottles containing acetochlor were paired with bottles containing no acetochlor. Each pair of bottles was autoclaved separately in a Tomy SS-320 autoclave using one of the following four sets of parameters: 120°C for 15 minutes; 110°C for 15 minutes; 100°C for 15 minutes; 100°C for 30 minutes. Immediately prior to pouring plates the bottles were vigorously hand shaken for two minutes. The medium in each bottle was decanted by pipetting 20.0 ml volumes into five petri dishes positioned on a level surface. The 10 plates thus obtained for each pair of bottles were left to dry for 24 hours. The bioassay

fungus inoculum used to seed each of the plates poured from a single pair of bottles was obtained from the perimeter of a single fungal colony which had been grown at 30°C for between 3 and 5 weeks on NM agar. All plates were inoculated with the fungus at twelve sites (Fig. 2.3) using one sterile micropipette tip to transfer inoculum per plate. Plates were stacked in groups of ten, placed in plastic bags (which were subsequently sealed) and incubated at 30°C until the mean diameter of the colonies developing on plates containing no acetochlor was between 9 and 10 mm. For each set of plates, after an eight to ten day incubation period, the diameters of all the single, non overlapping fungal colonies growing at the sites of inoculation were measured to the nearest 0.25 mm. The average bioassay fungus colony diameters on the five plates poured from single bottles were obtained. The concentration of active acetochlor remaining in the media containing the herbicide was determined using equation (18) described in section 2.5.2. This was achieved using the mean of colony diameters obtained for the 5 plates with herbicide ($\bar{x}$) and the 5 plates without herbicide ($\bar{x}_{0.00}$) poured from bottles comprising a pair. The proportion of active acetochlor remaining after each treatment was calculated relative to the concentration of the herbicide measured spectrophotometrically prior to autoclaving. The procedure was repeated a further two times.

3.2.3 The Effect of Nutrient Availability on Acetochlor Toxicity

The susceptibility of the bioassay organism to acetochlor was tested on media based on NM medium which were supplimented with different concentrations of $(NH_4)_2SO_4$, glucose and yeast extract (Table 3.1). For each of the media tested 600 ml NM minus the variable nutrient was made up and dispensed in 100.0 ml quantities into six bottles each containing 1.00 g bacteriological agar. Six stock solutions of $(NH_4)_2SO_4$ (50 $g.l^{-1}$), glucose (20 $g.l^{-1}$) and yeast extract (5 $g.l^{-1}$) were made up in MS medium from which the standard amount of $(NH_4)_2SO_4$ was omitted. Depending upon the nutrient being tested six samples of either $(NH_4)_2SO_4$, glucose, yeast extract or both glucose and yeast extract were individually either weighed out or aliquoted from the appropriate stock solutions and added to each of the six bottles. The lids of the bottles were tightly sealed and their masses were measured before and after autoclaving (at 120°C for 15 minutes) to verify that no significant volume of liquid (in excess of 1 ml) was lost from them. While still warm acetochlor was carefully

pipetted into five of the six bottles to obtain approximate herbicide concentrations of 0.02, 0.04, 0.06, 0.08 and 0.10 $g.l^{-1}$. Each bottle was vigorously hand shaken for two minutes immediately prior to pouring five approximately equal volumes into sterile petri dishes. After drying for 24 hours the plates were inoculated with the bioassay fungus in the fashion mentioned previously (section 2.5.2). Plates containing the same medium were grouped, stacked and incubated together in sealed plastic bags at 30°C. All plates were incubated until the borders of adjacent fungal colonies on plates containing no acetochlor were separated from one another by approximately 10 mm. Within a three hour period the smallest diameters of all discreet, non-overlapping fungal colonies on the 35 plates in each group were measured to the nearest 0.25 mm. Data on the mean fungal colony diameters measured on those plates containing the same test medium were analysed in the same way as the data used for the construction of the calibration curve relating bioassay fungus colony diameters to acetochlor concentrations (section 2.5.2).

TABLE 3.1. The composition of the media used to test the effect of various nutrient concentrations on the toxicity of acetochlor.

Variable	Concentration ($g.l^{-1}$)		
	$(NH_4)_2SO_4$	Glucose	Yeast Extract
None	5.00	0.20	0.05
$(NH_4)_2SO_4$	0.50 0.05	0.20 0.20	0.05 0.05
Glucose	5.00 5.00	0.02 0.00	0.05 0.05
Yeast Extract	5.00 5.00	0.20 0.20	0.01 0.00
Glucose and Yeast Extract	5.00	0.00	0.00

3.2.4. The Effect of Other Selected Parameters on the Precision and Accuracy of the Bioassay

The procedure for constructing the standard curve for the determination of effective acetochlor concentrations (section 2.5.2) was followed with the following modifications: 3 separate 600 ml batches of MS agar were made up, decanted in 98 ml aliquots into six 100 ml bottles and autoclaved at 121°C for 15 minutes; 1 ml of heat sterilised concentrated glucose and yeast extract stock solutions (made up in MS medium) were added to each bottle following autoclaving to give final glucose and yeast extract concentrations of 0.2 $g.l^{-1}$ and 0.05 $g.l^{-1}$ respectively; acetochlor was added in carefully pipetted quantities to five of the six bottles with no spectrophotometric verification made of the amount added; instead of pipetting 20 ml aliquots when making plates, 100 ml quantities of media were simply poured in approximately equal volumes into five sterile petri dishes and allowed to solidify.

3.3 Results

3.3.1 Isolation of an Acetochlor-Sensitive Bioassay Organism

Of the 43 isolates examined 3 displayed a marked reduction in growth over the herbicide concentration ranges tested. It was found that 50% of the fungi (2 out of 4) and only 2.6 % of the bacteria (1 out of 39) tested were sensitive to acetochlor at concentration of 0.15 $g.l^{-1}$. None of the organisms found to be sensitive to acetochlor were unable to grow at the highest concentration of the herbicide tested. The isolate which was most severely affected by the herbicide was one of the fungi (Figs 3.1, 3.2 and 3.3). On NM agar hyphal inoculum from this fungus gave rise to rapidly growing, uniformly sized colonies with well defined borders. The fungus was probably agarolytic as it could grow on MS agar and pitted the agar surface immediately surrounding the colonies. This fungus was selected as the bioassay organism to function as an indicator of active acetochlor in this study.

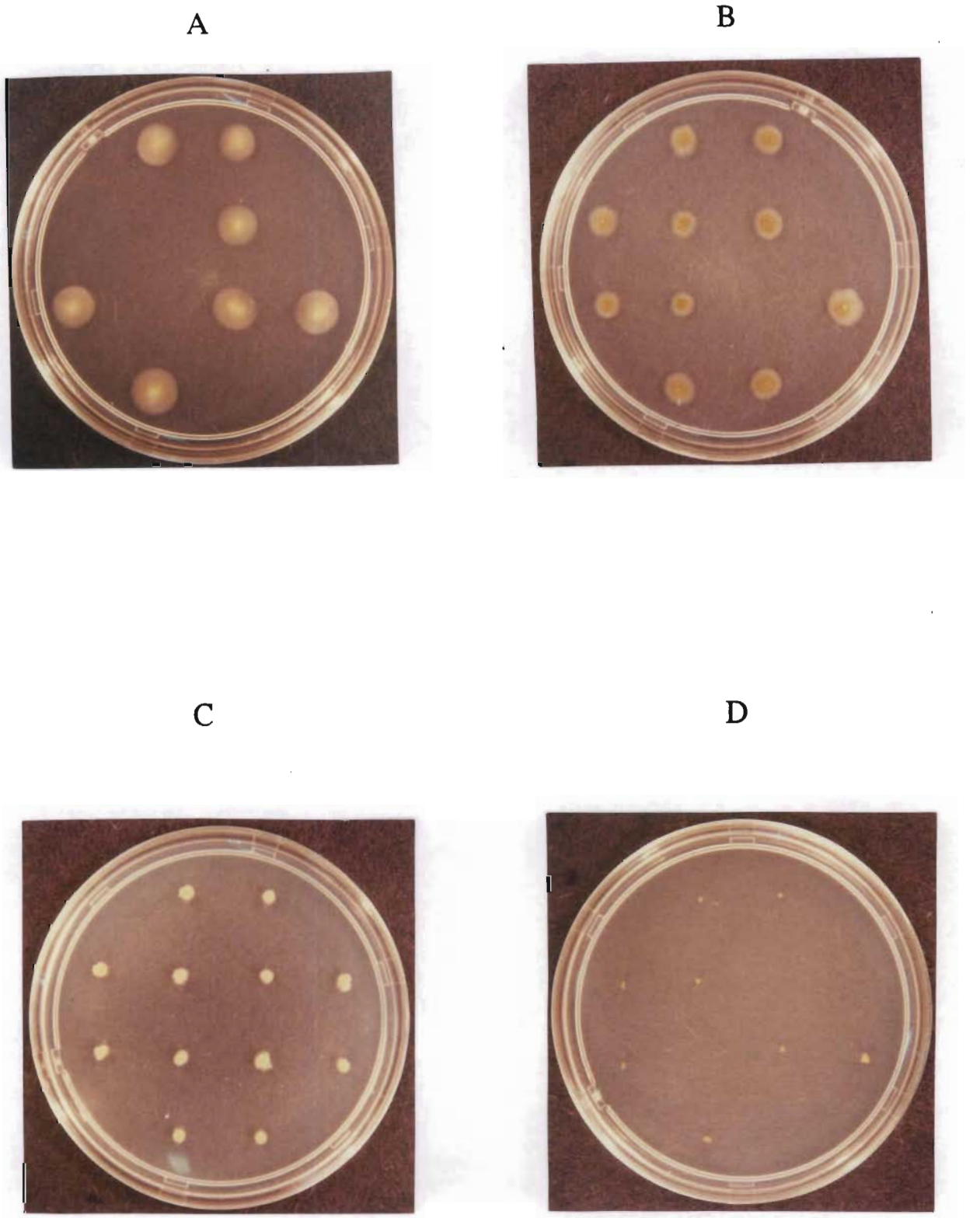

FIG. 3.1. The effect of acetochlor at approximate concentrations of 0.00 $g.l^{-1}$ (A), 0.05 $g.l^{-1}$ (B), 0.1 $g.l^{-1}$ (C) and 0.15 $g.l^{-1}$ (D) in NM agar on the growth, after 9 days, of the fungal strain selected as an acetochlor sensitive bioassay organism.

Growth of the bioassay fungus on NM agar containing 0, 0.05, 0.10, 0.15 and 0.20 $g.l^{-1}$ acetochlor was monitored over a 56 day period (Fig. 3.2). Growth on plates containing no acetochlor occurred at a uniform rate from the onset of inoculation until fungal colonies had reached a diameter of approximately 55 mm. After reaching this size the borders of colonies became increasingly ill defined and the rate of colony expansion decreased. On plates containing acetochlor a lag period was observed which lasted between 7 and 14 days during which period the rate of colony diameter increase was lower than that observed over the remainder of the growth period.

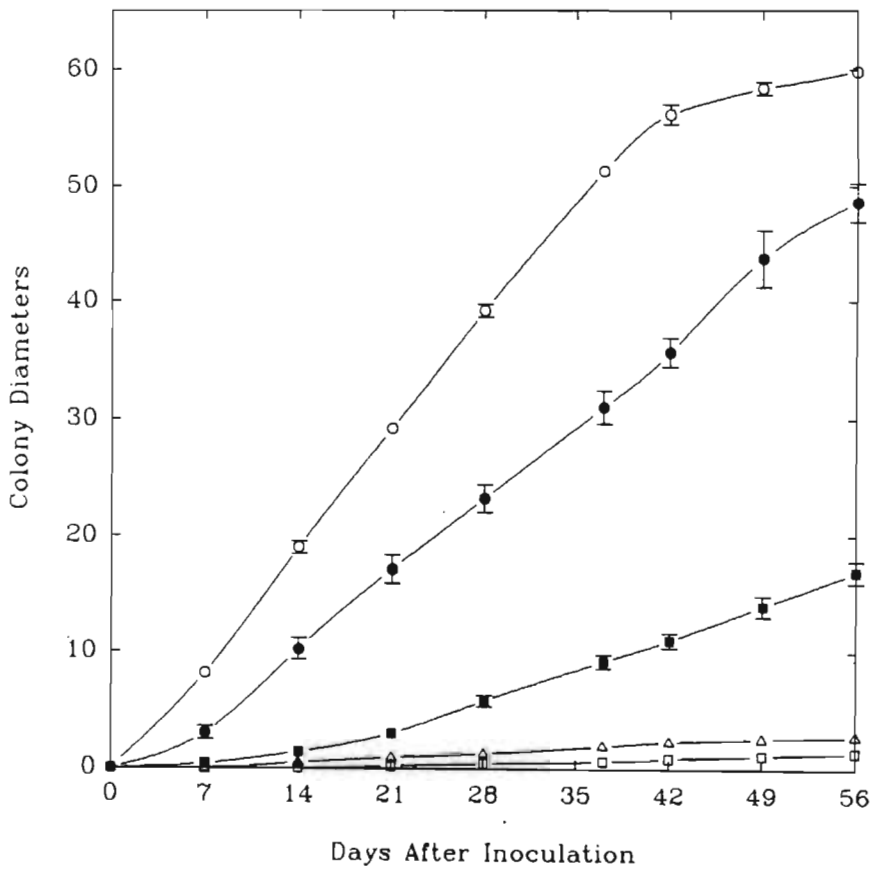

FIG. 3.2. Colony diameters (in mm) of the selected acetochlor sensitive bioassay fungus following growth on NM agar at 30°C over a 56 day period. (○) growth in the absence of acetochlor. Growth in the presence of: (●) approximately 0.05 $g.l^{-1}$ acetochlor; (■) approximately 0.10 $g.l^{-1}$ acetochlor; (△) approximately 0.15 $g.l^{-1}$ acetochlor; (□) approximately 0.20 $g.l^{-1}$ acetochlor. Error bars represent the standard deviations of five fungal colony diameters.

The curve best representing the response of the bioassay fungus to concentrations of acetochlor between 0.00 and 0.165 $g.l^{-1}$ was described by a second order polynomial (Fig. 3.3). However, the growth of fungal mycelia decreased linearly with increasing acetochlor concentrations between 0.00 and 0.1 $g.l^{-1}$ (Fig. 2.5; section 2.5.2). The concentration of acetochlor inhibiting mycelial growth by 50% (EC_{50}) was determined from the linear response curve (Fig. 2.5) to be 0.057 $g.l^{-1}$.

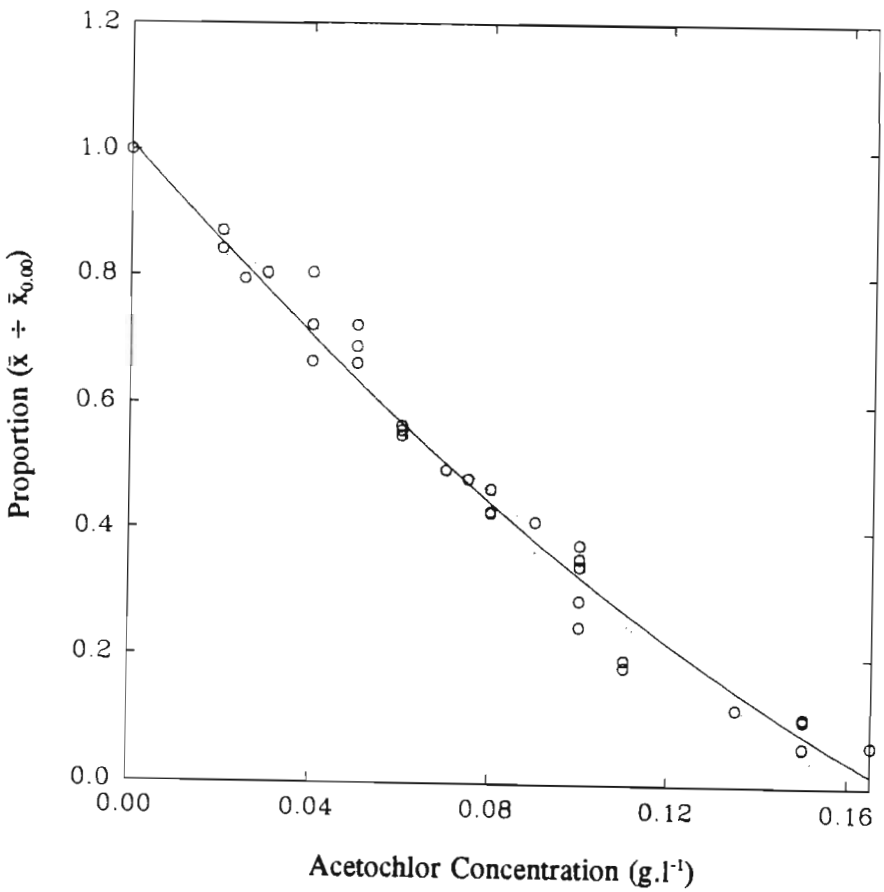

FIG. 3.3. Fungal growth occurring on NM agar containing between 0.00 and 0.165 $g.l^{-1}$ acetochlor. Growth is expressed as the proportions of the mean fungal colony diameters which occurred at the various acetochlor concentrations tested relative to mean colony diameters which occurred in the herbicide's absence ($\bar{x} \div \bar{x}_{0.00}$). (···) 95 % confidence intervals.

3.3.2 The Effect of Heating on Acetochlor Activity

During the course of the present study it was observed that autoclaving acetochlor solutions at 121°C for 15 minutes resulted in the dissolved herbicide being less toxic to the bioassay fungus than was anticipated. While boiling acetochlor solutions for 20 to 30 minutes did not noticeably effect the activity of the herbicide, it was not as effective at sterilising solutions as autoclaving at 121°C for 15 minutes. The effect autoclaving had on the activity of acetochlor was thus examined in order to find a suitable set of autoclaving parameters which could be employed in the sterilisation of solutions of this herbicide. Relative to autoclaving at 100°C for either 15 or 30 minutes autoclaving for 15

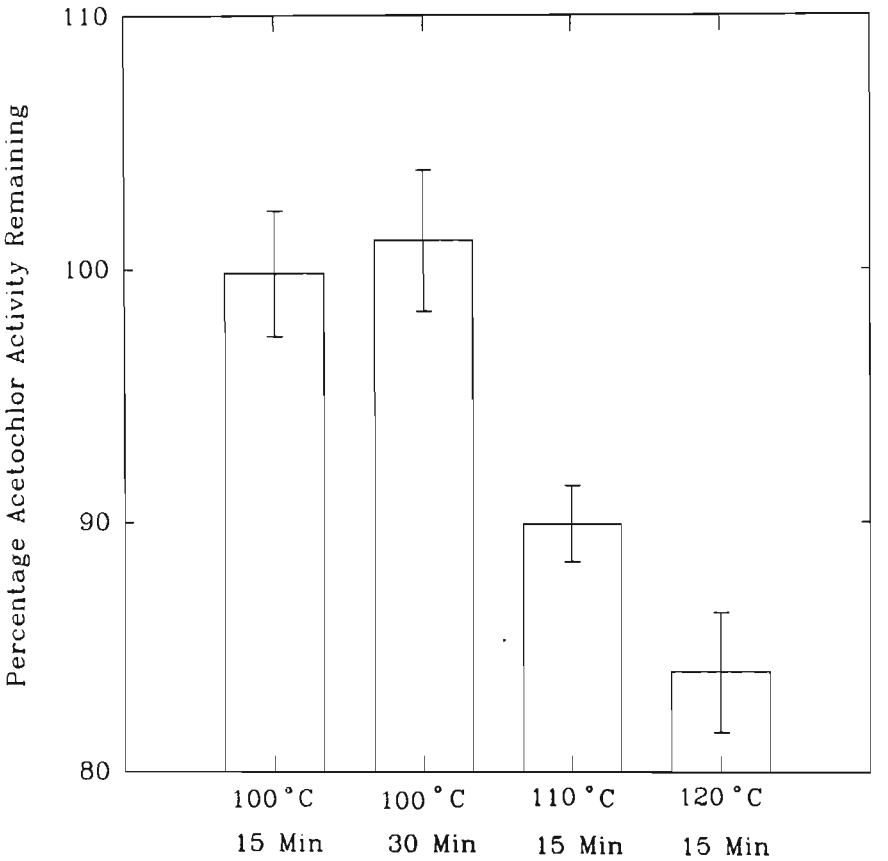

FIG. 3.4. The percentage of active acetochlor remaining in an approximately 0.05 $g.l^{-1}$ solution of the herbicide in NM agar following autoclaving for 15 minutes at 100, 110 and 120°C and for 30 minutes at 100°C. Error bars indicate standard deviations.

minutes at either 110°C or 120°C significantly reduced the activity of a 0.05 $g.l^{-1}$ acetochlor solution (Fig. 3.4). Because no significant loss of activity was noted when acetochlor was autoclaved at 100°C for either 15 or 30 minutes, it was assumed that neither of these treatments resulted in degradation of the herbicide. Equation (18) described in section 2.5.2 was used to determine herbicide concentrations remaining in the autoclaved solutions. According to the bioassay, autoclaving for 15 minutes at 110°C or 120°C was found to diminish acetochlor activity by approximately 10 % and 16 % respectively. It was found that while NM agar appeared to be sterile following autoclaving for 15 minutes at 110°C and 120°C and for 30 minutes at 100°C, autoclaving at 100°C for 15 minutes yielded consistently sterile NM agar only when sterile bottles and agar were used to make up the medium prior to autoclaving.

3.3.3 The Effect of Nutrient Availability on Acetochlor Toxicity

During the course of constructing a standard acetochlor response curve for the bioassay fungus, a set of results was obtained which contained readings completely at variance with the data already collected. This anomalous data indicated that the bioassay fungus was far more sensitive to acetochlor than earlier results had indicated. A possible reason for this heightened sensitivity was suggested when it was found that an error had been made while making up the yeast extract stock used for producing the NM agar employed to support growth of the bioassay fungus. This error resulted in the NM containing only 1 % of the intended yeast extract concentration. The influence that different concentrations of various nutrients had on the bioassay fungus' sensitivity to acetochlor was therefore investigated. While $(NH_4)_2SO_4$ concentrations appeared to have no significant effect on the toxicity of acetochlor to the bioassay fungus, lowering the concentration of glucose or yeast extract separately (Figs 3.5.1 and 3.5.2 respectively) or of both concurrently (Fig. 3.5.3) significantly increased the sensitivity of the bioassay fungus to the herbicide.

Growth of the bioassay fungus on nutritionally poor media occurred at a slower rate than on NM. Mycelial growth was particularly slow in the absence of glucose and/or yeast extract. Colonies which arose on media lacking in these nutrients were lighter in colour with less defined borders than colonies grown on NM.

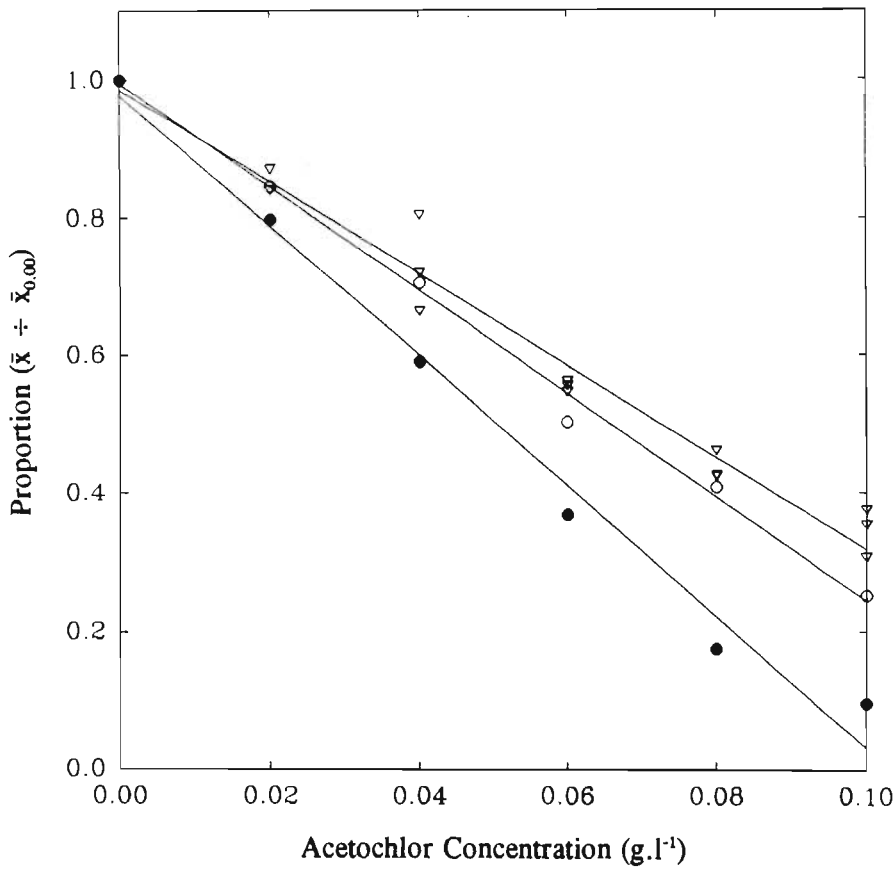

FIG. 3.5.1. The effect of varying glucose concentration on the toxicity of different acetochlor concentrations (in $g.l^{-1}$) to the bioassay fungus. The media used were identical to NM agar except that glucose was present at either 0.20 $g.l^{-1}$ (▽), 0.02 $g.l^{-1}$ (○) or 0 $g.l^{-1}$ (●). The regressions are described by the following equations: Glucose present at 0.20 $g.l^{-1}$, $\bar{x} \div \bar{x}_{0.00}$ = -6.667[acet] + 0.985 (R = 0.987); glucose present at 0.02 $g.l^{-1}$, $\bar{x} \div \bar{x}_{0.00}$ = -7.501[acet] + 0.994 (R = 0.997); glucose present at 0.00 $g.l^{-1}$, $\bar{x} \div \bar{x}_{0.00}$ = -9.439[acet] + 0.976 (R = 0.993). Growth is expressed as the proportions of the mean fungal colony diameters which occurred at the various acetochlor concentrations tested relative to mean colony diameters which occurred in the herbicide's absence ($\bar{x} \div \bar{x}_{0.00}$).

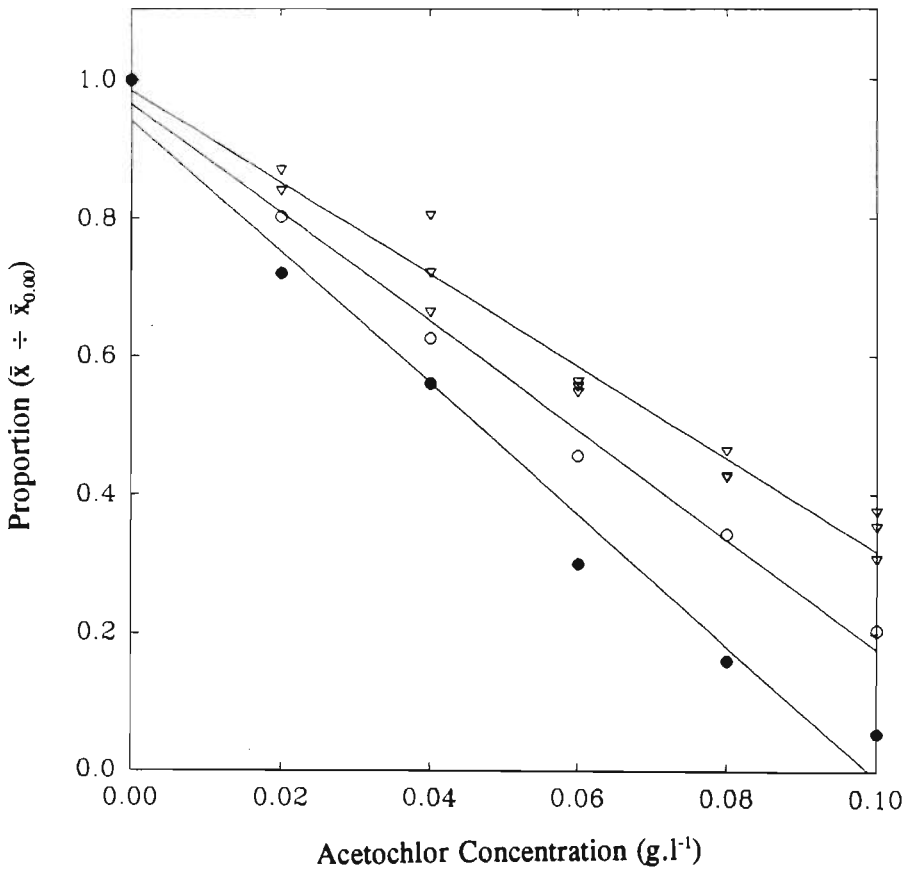

FIG. 3.5.2. The effect of varying yeast extract concentration on the toxicity of different acetochlor concentrations (in $g.l^{-1}$) to the bioassay fungus. The media used were identical to NM agar except that yeast extract was present at either 0.05 $g.l^{-1}$ (▽), 0.01 $g.l^{-1}$ (○) or 0.00 $g.l^{-1}$ (●). The regressions are described by the following equations: Yeast extract present at 0.05 $g.l^{-1}$, $\bar{x} \div \bar{x}_{0.00}$ = -6.667[acet] + 0.985 (R = 0.987); yeast extract present at 0.01 $g.l^{-1}$, $\bar{x} \div \bar{x}_{0.00}$ = -7.896[acet] + 0.966 (R = 0.995); yeast extract present at 0.00 $g.l^{-1}$, $\bar{x} \div \bar{x}_{0.00}$ = -9.517[acet] + 0.989 (R = 0.989). Growth is expressed as the proportions of the mean fungal colony diameters which occurred at the various acetochlor concentrations tested relative to mean colony diameters which occurred in the herbicide's absence ($\bar{x} \div \bar{x}_{0.00}$).

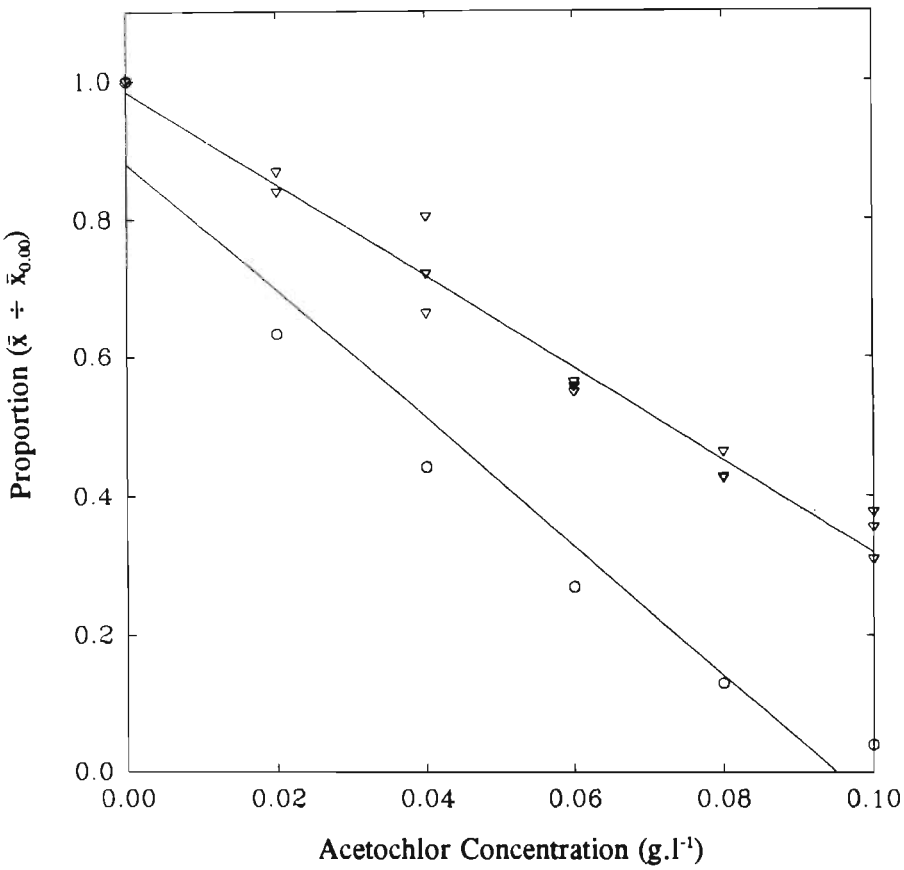

FIG. 3.5.3. The effect of excluding both glucose and yeast extract from NM (ie using MS as growth medium) on the toxicity of different acetochlor concentrations (in $g.l^{-1}$) to the bioassay fungus. (▽) growth of the bioassay fungus on NM agar. (○) growth of the bioassay fungus on MS agar. The regressions are described by the following equations: Glucose and yeast extract present at 0.2 $g.l^{-1}$ and 0.05 $g.l^{-1}$ respectively, $\bar{x} \div \bar{x}_{0.00}$ = -6.667[acet] + 0.985 (R = 0.987); neither glucose nor yeast extract present, $\bar{x} \div \bar{x}_{0.00}$ = -9.263[acet] + 0.882 (R = 0.973). Growth is expressed as the proportions of the mean fungal colony diameters which occurred at the various acetochlor concentrations tested relative to mean colony diameters which occurred in the herbicide's absence ($\bar{x} \div \bar{x}_{0.00}$).

3.3.4 The Effect of Other Selected Parameters on the Precision and Accuracy of the Bioassay

During the course of the year over which the quantitative bioassay was developed a number of modifications were made to the bioassay procedure which were intended to increase its precision and accuracy. Motivation to make these modifications stemmed from the following three observations: (1) Due to acetochlor's high viscosity large pipetting errors were encountered when aseptically transferring small volumes of the herbicide; (2) the rate at which the diameters of the bioassay fungus' colonies increased was visibly lower when plates were thinner than approximately 3.5 mm than when they were thicker than 4 mm; (3) natural variation in mean fungal colony diameters in treatments without any acetochlor (to which all treatments containing the herbicide were compared) probably resulted in inaccuracies in the estimation of acetochlor concentrations. Therefore, procedures used for both the quantitative bioassay and the construction of the acetochlor response curve of the bioassay fungus, were amended to include the spectrophotometric verification of acetochlor concentrations and the growth of the bioassay fungus on agar plates uniformly 4 mm thick. The procedure for constructing the standard curve was further altered to include the use of twice as many replications of treatments without the herbicide as individual treatments with the herbicide. While the individual effects of each of these alterations were not quantified their overall effect was evident from a comparison of the standard curves constructed with and without these procedural modifications (Fig. 3.5.). Whereas the regression coefficient of the calibration curve ultimately used in the present study (curve A) was 0.996, the regression coefficient of the initial curve constructed without the procedural modifications (curve B) was 0.987. It is also noteworthy that curve A had a steeper gradient than curve B.

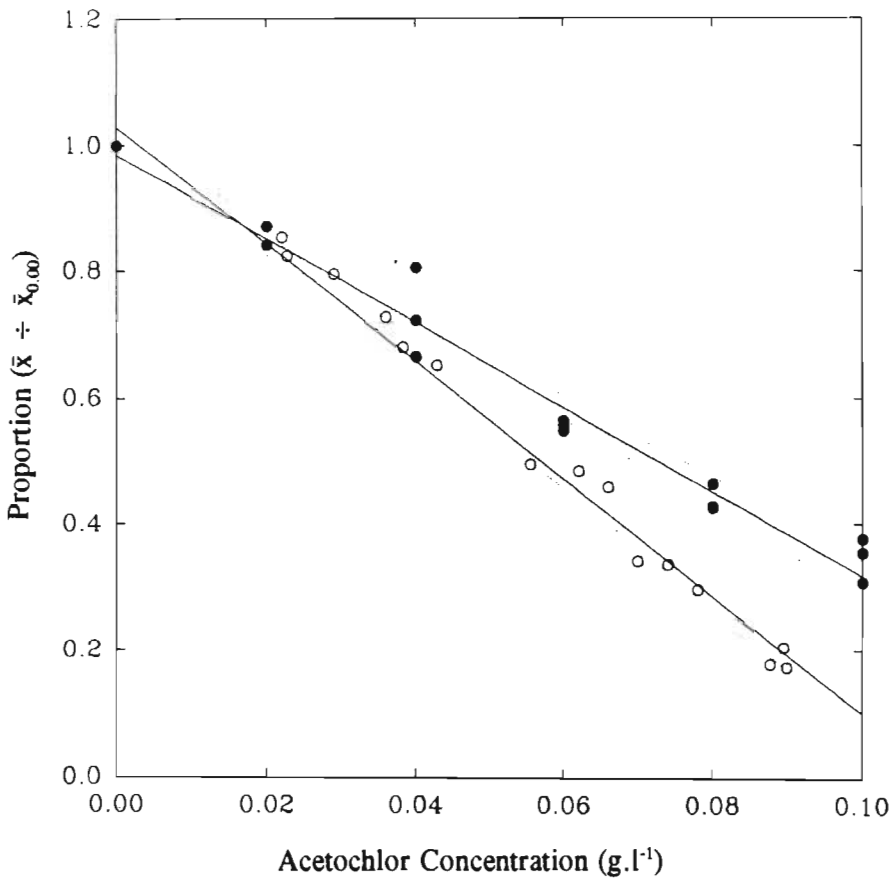

FIG. 3.6. Comparison of curves for relating acetochlor concentrations between 0.00 and 0.1 $g.l^{-1}$, to the growth of the bioassay fungus (represented here as the proportion of mean colony diameters in the herbicides presence relative to the mean colony diameter in the herbicides' absence or $\bar{x} \div \bar{x}_{0.00}$). Curve A (○) is the standard response curve of the bioassay fungus used in this study (also presented as Fig. 2.4.). It was constructed using the procedure outlined in section 2.5.2. Curve B (●) is an acetochlor response curve of the bioassay fungus constructed in a similar manner to curve A with the following exceptions: (1) there was no spectrophotometric verification of acetochlor concentrations; (2) bioassay plates were not of uniform thicknesses; (3) approximately half the number of colony diameter measurements were used to obtain a measure of mean fungal colony diameters in the absence of acetochlor ($\bar{x}_{0.00}$). Curve A is described by the linear equation: $\bar{x} \div \bar{x}_{0.00} = -9.289[acet] - 1.029$ ($R = 0.996$). Curve B is described by the linear equation: $\bar{x} \div \bar{x}_{0.00} = -6.667[acet] - 0.985$ ($R = 0.987$). (···) 95 % confidence intervals.

3.4 Discussion

A bioassay was developed which enabled the quantitative approximation of inhibitory acetochlor concentrations between 0.02 and 0.1 $g.l^{-1}$ in NM agar. The quantitative bioassay (section 2.5.3) evolved from a qualitative bioassay (section 2.5.1) used in the early stages of the present study as a means of identifying acetochlor detoxifying bacteria. The success of both the qualitative and quantitative bioassays depended on certain characteristics of the fungal isolate chosen as a bioassay organism.

3.4.1 Isolation of an Acetochlor-Sensitive Bioassay Organism

It was found that of the microbes examined for sensitivity to acetochlor, bacteria were far more resistant to the herbicide than fungi. Thus whereas 50 % of the fungi examined were obviously inhibited by 0.18 $g.l^{-1}$ acetochlor, over 90 % of the bacteria examined were resistant to this concentration of the herbicide. This finding supports that of Kudjo-Dzantor and Felsot (1991) who have reported that soil fungi are far more sensitive to high dosages of the chloroacetanilide herbicide, alachlor, than are soil bacteria and actinomycetes.

The choice of organism to be used initially for the qualitative bioassay and ultimately for the quantitative bioassay, depended on several factors. Firstly, the organism selected had to be versatile enough for use in detecting the detoxification of acetochlor in media containing a variety of organic nutrients. The procedure employed for isolating a bioassay organism focused on the isolation of microbes sensitive to low concentrations of technical grade acetochlor when grown on NM agar (a medium containing 0.20 $g.l^{-1}$ glucose and 0.05 $g.l^{-1}$ yeast extract). Although many microbes isolated during the initial selection of an acetochlor degrader (section 4.3.2) were sensitive to acetochlor at a concentration of 0.18 $g.l^{-1}$ when grown on MS agar (a medium containing no organic nutrients) (Table 4.1), they were rejected as potential indicators of reduced acetochlor toxicity as they were insensitive to this concentration of the herbicide when grown on NM agar.

Since the solubility of acetochlor in water at room temperature is 0.223 $g.l^{-1}$ (Worthing and Hance, 1991), a second factor influencing the choice of a bioassay organism was that it needed to be strongly inhibited by the herbicide at this concentration. While the selected acetochlor-sensitive fungus strain was not killed in 56 days by the highest concentration of acetochlor tested (0.20 $g.l^{-1}$), its rate of growth was measurably retarded.

The fungus strain chosen from the 43 microbial isolates examined, was initially intended only for use in the qualitative acetochlor bioassay. However, realisation that an adaptation of this qualitative bioassay would also enable its use in the approximation of acetochlor concentrations prompted further investigation of the selected fungus as a possible quantitative acetochlor bioassay organism.

For growth of the selected fungus under controlled conditions to be used as an indicator of not only diminished acetochlor toxicity but also of actual herbicide concentrations, it was necessary that small changes in acetochlor concentrations should cause marked changes in its growth. While the metalaxyl EC_{50} (concentration of metalaxyl resulting in 50 % inhibition of mycelial expansion) of *Phytophthora boehmeriae*, the fungus employed in the quantitative metalaxyl bioassay developed by Bailey and Coffey (1984), was 0.02 $mg.l^{-1}$ (Bailey and Coffey, 1986), the acetochlor EC_{50} of the bioassay fungus used in the present study was 57 $mg.l^{-1}$. This almost three thousand fold difference in sensitivity between these two fungi for the respective chemicals which they have been used to assay, reflects a difference in the exact purpose for which each was required. Bailey and Coffey's bioassay was aimed at the quantification of low concentrations of metalaxyl in soils and was used to examine the fungicide's detoxification in the environment (Bailey and Coffey, 1985; Bailey and Coffey, 1986). The functions of the bioassays developed in the present study, on the other hand, were the detection and/or quantification of acetochlor detoxification involving concentrations of the herbicide between 0.02 and 0.15 $g.l^{-1}$ in a defined growth medium. For the purposes of this study, therefore, it was not necessary to obtain a bioassay organism with a higher sensitivity to acetochlor than that of the fungus ultimately selected.

For the selected fungus to be used in a quantitative acetochlor bioassay it was necessary that some measurable aspect of its growth should vary in a directly proportional relationship to changing concentrations of the herbicide. The fungus was suitable in this regard in that the relationship between its colony diameters and acetochlor concentrations between 0.00 and 0.10 $g.l^{-1}$ was described by a linear regression. Because this linear relationship did not hold for acetochlor concentrations above 0.10 $g.l^{-1}$, all concentrations of the herbicide to be measured using the quantitative bioassay were kept below 0.10 $g.l^{-1}$. The colony radii of the *P. boehmeriae* strain used in the metalaxyl bioassay developed by Bailey and Coffey (1984) also did not vary linearly over the entire range of metalaxyl concentrations examined by these researchers. This deviation from linearity meant that the metalaxyl bioassay was only useful in the measurement of metalaxyl concentrations between 0.002 and 0.03 $mg.ml^{-1}$. However, even within this range of concentrations the effect of metalaxyl on the colony radii of *P. boehmeriae* was best described by a second order polynomial and attempts to transform the data to linearity using a semi-log plot resulted in a poorer curve fit than was obtained in the present study.

The usefulness of the selected fungus in a quantitative acetochlor bioassay was also dependent on the reproducibility of the effect which acetochlor had on its growth. In this regard a problem was encountered when it was found that colony diameter measurements recorded in different experiments could not be directly compared. This was due to slight fluctuations in fungal colony growth within different replications of the experiments which were independent of acetochlor concentration. Two of the factors effecting the rate of mycelial expansion appeared to be the dryness of the NM agar plates inoculated with the fungus and the age of the inoculum used. Inoculating plates which were inadequately dried resulted in moisture droplets at sites of inoculation which appeared to slow down mycelial growth (possibly due to its interference with aeration). By allowing plates to dry for 24 hours before inoculation problems of this type were avoided. Also, inocula obtained from older colonies (particularly those stored at 4°C) generally grew more slowly than inocula obtained from younger colonies (data not presented). A degree of consistency was achieved between data gathered in different experiments by both standardising the fungal innoculum used and expressing means of colony diameters on plates in the presence of the herbicide as proportions of mean colony diameters measured on plates in the absence of

the herbicide. The fungal inoculum was standardised by only using mycelia from the peripheries of single 3 to 5 week old colonies actively growing at 30°C in the centres of NM agar plates. It was found that fungal colonies incubated at 30°C grew at a constant rate on NM agar plates for up to five weeks. If maintained for more than five weeks, however, a decrease in growth rate occurred which was coupled with a loss of colony boundary definition. This loss of definition possibly indicated depletion of nutrients in NM agar essential for growth of the bioassay fungus. To enable the comparison of results from different experiments it was, however, also necessary to take measurements only when mean colony diameters on plates lacking the herbicide were between 9 and 11 mm.

A characteristic of the selected fungus which contributed to its usefulness as a bioassay organism was its agarolytic nature. This may be significant since when fungal growth is used as an indicator of fungicidal chemical concentrations a problem commonly encountered is that fungal colony diameters have a tendency to increase faster in the absence of sufficient nutrients than in the presence of a rich nutrient supply (Rijkenberg, 1993; personal communication[4]). Possibly because it was agarolytic, the colony diameters of the selected fungus did not increase at an accelerated rate upon depletion of nutrients in the growth medium. Thus the borders of two colonies of the bioassay fungus would remain defined even when separated from on another by as little as 8 mm. This permitted up to twelve inoculation sites on each agar plate used in the bioassay. For any particular plate it was assumed that the mean diameter of eight to twelve colonies would accurately reflect the concentration of acetochlor present in the plate.

3.4.2 The Effect of Heating on Acetochlor Activity

During both the qualitative and quantitative bioassay procedures (sections 2.5.1 and 2.5.3 respectively) agar powder was added to media following growth of putative acetochlor degraders. Because heating of media to dissolve the agar was inescapable during the

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bioassays, an assumption was made that boiling media containing acetochlor for approximately 15 minutes [the minimum time required for agar to dissolve at a concentration of 1 % (w/v)], would not contribute measurably to the detoxification of the herbicide. Support for this assumption came from the observation that acetochlor solutions boiled for 20 to 30 minutes were no less toxic to the bioassay fungus than control solutions containing the same acetochlor content but to which the herbicide had been added following cooling. During both the construction of the standard acetochlor response curve of the bioassay fungus (section 2.5.2) and the quantitative acetochlor bioassays carried out in this study, media containing acetochlor were autoclaved for 30 minutes at 100°C. Therefore, had this autoclaving treatment contributed to a slight loss in the herbicide's toxicity, the standard acetochlor response curve of the bioassay fungus would still have been valid for determining acetochlor concentrations as indicated by growth of the fungus during the quantitative bioassay.

In the fungal bioassay developed by Bailey and Coffey (1984) for the measurement of metalaxyl and metolachlor concentrations in soils (Bailey and Coffey, 1985), autoclaving media containing either of these compounds at 120°C for 30 minutes had no effect on their fungicidal activities. The reason these autoclaving conditions could be used when working with metalaxyl and metolachlor was that unlike acetochlor which decomposes at 180°C (Monsanto Acetochlor Technical Safety Data Sheet, 02/86), metalaxyl and metolachlor remain stable at temperatures up to 300°C (Worthing and Hance, 1991).

There were two ways in which autoclaving acetochlor solutions without diminishing their toxicity improved the effectiveness of the quantitative bioassay. Firstly, besides the need for heating to dissolve agar powder added to media to solidify them, the bioassay required that media exposed to putative acetochlor degraders be sterilised prior to using them to support growth of the bioassay fungus. Filter sterilisation would result in the removal from media of herbicide molecules both within and adsorbed to the cell surfaces of putative acetochlor detoxifiers. Although certain researchers have reported a low levels of chloroacetanilide removal from media by microbial sorption (Saxena *et al.*, 1987; Krause *et al.*, 1985), others have reported that certain microbial strains actively accumulate untransformed chloroacetanilide molecules from media (Liu *et al.*, 1989;

Pothuluri *et al.*, 1993). Without measurably influencing the inhibitory concentration of any acetochlor present, autoclaving media at 100°C for 30 minutes both sterilised them and dissolved agar powder added to them.

The second manner in which autoclaving parameters which did not effect acetochlor's activity contributed to the effectiveness of the bioassay related to difficulties encountered in the aseptic measurement of accurate acetochlor volumes. When highly viscous technical grade acetochlor was drawn up into a small bore micropipette tip it adhered to both the inner and outer surfaces of the tip. While acetochlor adhering to the outside of the tip could only be properly removed by wiping the tip with a paper towel or cloth, effective removal of all the herbicide drawn up into the tip required a thorough rinsing process. It was therefore difficult to maintain sterility when attempting to precisely pipette small acetochlor volumes. The non-sterile measurement of precise acetochlor volumes and subsequent sterilisation of acetochlor solutions by autoclaving at 100°C for 30 minutes, was important in the construction of the standard curve for relating growth of the bioassay fungus to acetochlor concentrations (section 2.5.2).

3.4.3 The Effect of Nutrient Availability on Acetochlor Toxicity

While it was shown that $(NH_4)_2SO_4$ concentrations between 0.05 $g.l^{-1}$ and 5.0 $g.l^{-1}$ had no effect on the sensitivity of the bioassay fungus to acetochlor, lowering the concentrations of either glucose or yeast extract in NM agar significantly influenced the sensitivity of the fungus to the herbicide. When using the quantitative bioassay (section 2.5.3) it was important that plates inoculated with the bioassay fungus contained glucose and yeast extract concentrations as close as possible to 0.2 $g.l^{-1}$ and 0.05 $g.l^{-1}$ respectively since these were the concentrations of glucose and yeast extract present in NM during construction of the standard curve (section 2.5.2). Following the first part of both the qualitative and quantitative bioassay procedures (maintenance of putative acetochlor degraders in test media), preparation of the media prior to inoculation with the bioassay fungus involved the addition of glucose and yeast extract solutions to attain the approximate concentrations of these nutrients present NM.

During the bioassays in which putative acetochlor detoxifying microbes were maintained in NM.1 (a medium described in section 2.2 containing 10 % the glucose and yeast extract present in NM) a significant decline in the concentrations of glucose and yeast extract present in the medium would have taken place over the time period permitted for detoxification to occur. This may have marginally effected the accuracy of the bioassay because even after addition of glucose and yeast extract to nutritionally depleted NM.1 media in an attempt to adjust them to resemble NM, the adjusted media would still have contained slightly lower concentrations of these nutrients than NM. The differences in nutrient content between NM and the adjusted NM.1 media did not, however, influence the validity of the bioassays for detecting decreases in acetochlor concentrations. This is because the adjusted NM.1 media contained between 90 and 100 % of the glucose and yeast extract available in NM. Being provided with up to 10 % less glucose and yeast extract the bioassay fungus would have been slightly more sensitive to acetochlor and the assay would have slightly overestimated the herbicide concentration present. If anything this bias would, therefore, have resulted in a slight underestimation of a test microbe's ability to degrade acetochlor in NM.1.

The heightened sensitivity of the bioassay fungus to acetochlor in the presence of lower yeast extract and glucose concentrations could possibly have been used to make the bioassay more sensitive. The effect of this heightened sensitivity would be to increase the gradient of a calibration curve relating growth of the fungus to concentrations of the herbicide. This increased gradient would permit the detection and measurement of smaller fluctuations in herbicide concentrations. The scope of this study was, however, insufficiently broad to fully explore the possibilities of obtaining a more precise bioassay via this route.

Possibly because of the various limitations in the techniques used, the data presented in Figs 3.5.1, 3.5.2 and 3.5.3, was considerably more variable than that obtained for the construction of the standard curve presented in section 2.5.2. The most significant of these limitations are the same as those outlined in section 3.4.4.

3.4.4 The Effect of Other Selected Parameters on the Precision and Accuracy of the Bioassay

The initial procedure used to produce a calibration curve relating growth of the bioassay fungus to acetochlor concentrations contained various shortcomings which compromised both the precision and accuracy of the quantitative bioassay. These shortcomings included the following: there was no reliable means of either aseptically pipetting accurate volumes of acetochlor or of verifying acetochlor concentrations by some means other than the bioassay; media volumes decanted into plates to be inoculated with the bioassay fungus were not kept identical between plates; there were not enough repetitions when determining the growth of the fungus in the absence of the herbicide.

Possibly the most significant omission in the initial procedure for constructing a calibration curve was the absence of an independent verification of acetochlor concentrations. As previously discussed (section 3.4.2) the pipetting of precise acetochlor volumes was hindered by the herbicide's high viscosity. Problems encountered when pipetting the herbicide were such that when aseptic measurement and transfer of small acetochlor volumes was attempted, uncontrollable variation occurred in the actual amount of acetochlor ultimately transferred. The finding that autoclaving acetochlor solutions at 100°C for 30 minutes appeared to have no effect on the toxicity of the dissolved herbicide permitted the non-sterile pipetting of more accurate acetochlor volumes when constructing a standard acetochlor response curve for the bioassay fungus. A reason for the major difference in gradient of the curves in Fig 3.6 could, therefore, be due to curve B (produced without the aid of spectrophotometry) being constructed by plotting fungal growth against intended acetochlor concentrations, while curve A (produced using spectrophotometry) was constructed by plotting fungal growth against actual herbicide concentrations. In the case of the quantitative bioassay (section 2.5.3) spectrophotometric measurement of acetochlor concentrations served to provide a precise means of measuring the amount of acetochlor added aseptically to sterile media prior to inoculation with putative acetochlor detoxifiers.

It was observed that colonies of the bioassay fungus increased in size at a slower rate if agar plates were thinner than 3.5 mm. In the initial procedure used to construct an acetochlor standard curve for the quantitative bioassay, a large degree of variation in mean colony diameters occurred between plates containing the same concentration of the herbicide (data not presented). This between-plate variation was possibly due, in part, to variations in the thickness of the agar layer they contained. It is probable that the different rates of fungal colony expansion observed between plates approximately 4.25 mm thick and plates thinner than 3.5 mm occurred because thinner plates both dried out quicker during incubation and contained fewer nutrients than thicker plates. Therefore, in both the quantitative bioassay procedure and the improved procedure for constructing a standard curve, care was taken to ensure that plates to be used in the bioassay were uniformly approximately 4.25 mm thick.

Because all acetochlor concentrations measured using the bioassay were based on the comparison of fungal colony diameters in the presence and absence of the herbicide, it was important that mean fungal colony diameters measured in the absence of acetochlor be as accurate as possible. In the procedure used initially in the construction of an acetochlor calibration curve equal numbers of plates were used for determining the mean colony diameters of the fungus in treatments without the herbicide as were used for individual treatments containing the herbicide. In the later procedure for constructing a standard curve twice as many plates were used to obtain an estimate of mean fungal colony diameters in treatments without the herbicide as were used in the individual treatments with the herbicide. By providing a more accurate measure of growth on media lacking acetochlor through increased repetition, it was hoped that the accuracy of the bioassay would be increased.

It is also possible that factors which were not examined in this study such as pH of growth media and temperatures at which the bioassay fungus was either incubated or stored, could have affected the sensitivity of the fungus to acetochlor. In this regard it has been found that while the resistance of plants to the chloroacetanilide herbicides is affected by both air (Reinhardt and Nel, 1982) and soil temperatures (Boldt and Barrett, 1989; Van Rensberg *et al.*, 1988), soil pH has not been shown to have any effect on the sensitivity

of plants to these herbicides (Reinhardt and Nel, 1982). It is feasible that if the adjustment of one or both of these parameters increased the toxicity of acetochlor towards the bioassay fungus, then by altering the pH of media and/or incubation temperatures the sensitivity of the bioassay described here could be altered.

4 ISOLATION OF AN ACETOCHLOR DETOXIFYING BACTERIUM

4.1 Introduction

Fears over the potential health and environmental hazards posed by the widespread use of halogenated pesticides in agriculture have prompted a large volume of research centred around obtaining an understanding of the processes whereby these molecules are degraded in the environment. Pivotal to many of these efforts have been the isolation from the environment of microbial strains which, in pure culture, are able to transform these often highly recalcitrant molecules.

Being one of the most extensively used groups of chlorinated pesticides in the world today (Gressel, 1992), much attention has been focused on the environmental fate of the chloroacetanilide herbicides (Chesters *et al.*, 1989). It has been demonstrated that the major agents of chloroacetanilide degradation in soils are microorganisms (Chesters *et al.*, 1989). While there have as yet been no published reports on the isolation of acetochlor degrading microbe, microbial strains which in pure culture are capable of transforming the chloroacetanilides metolachlor (Bailey and Coffey, 1986; Krause *et al.*, 1985; Saxena *et al.*; 1987, Liu *et al.*, 1987), alachlor (Lee, 1984 cited by Chesters *et al.*, 1989; Lee, 1986 cited by Chesters *et al.*, 1989; Novick *et al.*, 1986) and propachlor (Novick and Alexander, 1985; Novick *et al.*, 1986; Villarreal *et al.*, 1991) have been isolated from the environment. However, no microbe or microbial community has yet been discovered that is able to significantly mineralise any of the chloroacetanilide herbicides and it appears that microorganisms with the capacity to utilise chloroacetanilides as their sole source of either carbon or nitrogen are extremely rare. It is likely that the failure of attempts to isolate either utilisers or mineralisers of these herbicides is because chloroacetanilide molecules have both tertiary amine moieties and, with the exception of propachlor, alkyl groups on their aniline rings. While tertiary amines are generally resistant to enzymatic attack, the alkyl groups at ring positions 2 and 6 may further hinder degradation of these molecules by sterically interfering with enzymatic cleavage of bonds to their nitrogen atoms (Saxena *et al.*, 1987).

In studies involving the elucidation of chloroacetanilide degradation pathways mediated by microbes, the transformation reactions discovered have included dechlorination, dehydrogenation, dealkylation, hydroxylation and indoline ring formation (Figs 1.5.1, 1.5.2, 1.5.3, 1.5.4). It is likely that these transformations fall into one of the following three categories: (1) Incidental metabolism during which the herbicide is transformed by generally available enzymes; (2) catabolism during which the herbicide provides nutritional and/or energy benefits for the metaboliser; (3) detoxification metabolism during which the herbicide degrader is sensitive to the herbicide and transforms it merely as a survival mechanism (Matsumura, 1982; cited by Cork and Kreuger, 1991). Because a gene encoding the transformation of acetochlor to a non-toxic metabolite was required in the present study, an attempt was made to isolate microbes which either specifically detoxified acetochlor for survival purposes or utilised it as a nutrient source. It is possible that certain degradative genes (such as those encoding acetochlor dechlorination) isolated from acetochlor transforming microbes would be utilisable as acetochlor resistance genes in the engineering of resistance to this herbicide in crops.

The isolation of an acetochlor detoxifying bacterium which resembles *Pseudomonas fluorescens* is described here. Isolation of the bacterium was facilitated by a multicycle batch culture enrichment procedure aimed at selecting for microbial communities capable of utilising the herbicide as their sole source of carbon and energy. Isolation of the detoxifier from a microbial community possibly capable of utilising acetochlor was achieved by firstly using indicator media to screen individual isolates for their ability to dechlorinate acetochlor and then testing potential dechlorinators for their ability to significantly detoxify the herbicide using a qualitative acetochlor bioassay. The actual degradative capacity of an acetochlor detoxifier thus isolated was determined using of a refined version of the qualitative bioassay which permitted the quantitative detection of active acetochlor concentrations.

4.2 Materials and Methods

4.2.1 Chemicals

Water soluble eosin yellow was purchased from G. T. Gurr. Methylene blue was purchased from Judex. 2,3,5-triphenyl tetrazolium chloride (TTC) was purchased from Saarchem.

4.2.2 Media

EMB (Eosin-Methylene-Blue) agar is an acid/base indicator medium described by Loos (1975) containing the following per litre: eosin, 40 mg; methylene blue, 6.5 mg; yeast extract, 0.1 g; purified or noble agar, 12 g. EMBA is EMB with acetochlor added at a concentration of 0.15 $g.l^{-1}$. MSA-TTC medium is based on an indicator medium described by Bochner and Savageau (1977) and contains MSA agar (mineral salts acetochlor agar described in section 2.2) together with 2,3,5-triphenyl tetrazolium chloride at a concentration of 25 $mg.l^{-1}$.

4.2.3 Enrichment for Acetochlor Degrading Microbes

An *in vivo* enrichment for acetochlor degrading microorganisms similar to that described by Bailey and Coffey (1986) was carried out. A number of different soil types from a variety of locations in the area surrounding the agriculture faculty buildings of the University of Natal in Pietermaritzberg, South Africa, were collected and mixed. Approximately one kilogram of this sample was mixed, in a two litre beaker, with 100 ml of a 0.1 % formulation acetochlor solution in 100ml of tap water. The top of the 2 litre beaker was covered with aluminium foil, partially sealed with masking tape and incubated at 30°C. At weekly intervals over a period of seven weeks 25 ml of a 0.1 % solution of formulation acetochlor was applied to the surface of the soil. After the seventh week the treated soil was mixed and moistened with 100ml of water. Six samples of approximately 50 g each were placed into 100 ml beakers. The tops of the beakers were then sealed with aluminium foil and masking tape and over the next seven week period 3 were incubated

at 30°C and the other 3 left at room temperature. The soil in the six beakers was then added to and mixed with a similar volume of freshly collected soil from the same locations as the original samples. An enrichment procedure similar to that employed for the isolation of s-triazine utilising microbes by Bheki and Kahn (1986) was used. Two grams of the mixed soil were added to each of four 250 ml Erlenmeyer flasks containing 100 ml of either NM, MS, NMA or MSA (all described in section 2.2). The flasks were shaken vigorously for 10 minutes and large particles were removed by straining the soil-media mixtures through cheesecloth. The four 250 ml Erlenmeyer flasks containing the slightly clarified mixtures were incubated at 30°C in an orbital shaker set at 100 rpm. After 2 weeks 1 ml samples from each of these cultures were used to inoculate 100 ml of fresh media also in 250 ml Erlenmeyer flasks. These were incubated at 30°C with shaking at 100 rpm for a further two weeks. Four more enrichment cycles, each lasting two weeks, were carried out but with three adjustments made to the above procedure. Firstly, instead of using 1 ml samples from the preceding cultures to seed fresh media 10 μl samples were used. Secondly, aeration during enrichments was improved by both lowering the culture volume in the flasks from 100 ml to 50 ml and increasing the rate of rotary shaking from 100 rpm to 150 rpm. Lastly, the progress of growth in the enrichment cultures were monitored on a daily basis by measuring their absorbances at 600 nm. This spectrophotometric determination of growth required that uninoculated control flasks also containing 50 ml of the four different enrichment media be incubated together with the enrichment cultures. The absorbances of these cultures was monitored together with those of the enrichments in order to monitor fluctuations in background absorbances in the enrichment cultures which occurred independently of the microbial growth in these cultures.

4.2.4 Screening for Acetochlor Degradation

Between 8 and 10 days after the initiation of enrichment cycles three through five, bacteria surviving the enrichment process were tested for their ability to degrade acetochlor. During cycles three and four 80 bacterial isolates from the four enrichment cultures were selected at random. In cycle five 40 bacterial strains which yielded red colonies after ten days of growth at 30°C on EMBA were selected from the NM and NMA enrichments.

Bacteria from the MS and MSA enrichments which produced red colonies (or at least colonies with red centres) after 10 days of growth at 30°C on MSA-TTC indicator plates, were also selected. The 40 isolates selected from each cycle consisted of 16 from each of the acetochlor containing enrichment cultures and 4 from each of the cultures containing no acetochlor. Pure cultures were obtained of the 120 selected bacterial isolates. The isolates were maintained on either NMA agar if they originated from the NM or NMA enrichment cultures or MSA agar if they originated from the MS or MSA enrichment cultures. All isolates were individually tested for their capacity to either dechlorinate acetochlor and/or utilise it as their sole source of carbon and energy. A differential colony colour reaction between a bacterium's growth on EMB and its growth on EMBA after 10 days at 30°C was taken to imply that it was possibly capable of dechlorinating acetochlor (Loos, 1975). Growth after 10 to 30 days at 30°C in the dark on MSA agar but not on MS agar was used as an indication as to whether the bacteria were capable of utilising acetochlor as their sole carbon and energy source. All tests were carried out in triplicate on plates sealed with masking tape to prevent their drying out. The isolates which gave positive responses in these tests were screened for their potential ability to utilise agar as a carbon source. This was accomplished by comparing their growth after 3 days at 30°C on MS with and without galactose (the monosaccharide subunit of agar) at a concentration of 0.2 $g.l^{-1}$. Using the qualitative bioassay (section 2.5.1) all of the selected isolates lacking the capacity to utilise galactose as a carbon source were tested twice for their ability to noticeably detoxify acetochlor at an approximate concentration of 0.15 $g.l^{-1}$. In the bioassay all isolates, irrespective of whether they had been isolated from the NMA or MSA enrichment cultures, were grown for 18 days at 30°C in MSA.

4.2.5 Quantification of the Acetochlor Detoxifying ability of isolate A108

The quantitative acetochlor bioassay (section 2.5.3) was used to estimate the amount of acetochlor which a selected bacterium, designated A108, was capable of detoxifying over a 28 day period. Herbicide detoxification was tested in both MS medium and NM.1 (described in section 2.2) supplemented with acetochlor at an approximate concentration of 0.06 $g.l^{-1}$. Samples were analysed for acetochlor after 7, 14, 21 and 28 days incubation. Using the scaled down version of the quantitative acetochlor bioassay (section

2.5.3) two more estimates were obtained in both MS and NM.1 of the amount of the herbicide A108 could detoxify in 21 days.

4.2.6 Characterisation and Identification of the Acetochlor Degrading Isolate

This was carried out using both standard microbiological techniques and an automated bacterial identification analyser (ATB Identification; Bio Mérieux).

Initially isolate A108 was Gram stained in order to both characterise its morphology and broadly classify it in relation to the bacterial groups presented in Bergey's Manual of Systematic Bacteriology (9th ed.). The ability of A108 to grow under anaerobic conditions was tested. It was streaked onto three NM plates each of which were subsequently overlaid with 10 ml of sterile mineral oil. The plates were sealed with masking tape and incubated at 30°C for three weeks. The plates were regularly examined for signs of growth over this period.

The size, morphology and flagellation of the isolate were determined with the use of transmission electron microscopy. 1 ml NM in a 5 ml test tube was inoculated with a single A108 colony from a two day old MSA agar plate and incubated overnight at 30°C without shaking. A loop-full of growth was transferred onto a formvar coated transmission electron microscope (TEM) grid. Excess liquid was removed with filter paper and one drop of a 2 % solution of phosphotungstic acid was applied to the surface of the grid. Within 20 seconds the drop of phosphotungstic acid was removed using filter paper. The grid containing the stained cells was left to air dry for approximately five minutes and then viewed in a Joel 100 CX TEM at 80 KV.

Analysis of isolate A108 by an automated bacterial identification analyser was carried out by technicians at Umgeni Water in Pietermaritzberg. To determine which of the possible A108 identities indicated by the bacterial identification analyser was the best fit, the culture was subsequently examined for pigment production, growth at 42°C and liquification of gelatin.

4.3 Results

4.3.1 Enrichment for Acetochlor Degrading Microbes

The measurement of growth in the enrichment cultures was only initiated during the third enrichment cycle because, in enrichment cycles one and two, turbidity resulting from suspended soil particles interfered with absorbance measurements to the extent that no significant growth was discernable in any of the cultures. Lack of any signs of growth may also have been due to poor aeration during these cycles because of the large volumes of liquid present in flasks (100 ml in 250 ml flasks) and the slow rate at which the cultures were agitated (100 rpm). During enrichment cycles 3 through 6 absorbance readings obtained for the enrichment cultures over the two week periods for which they were monitored, required adjustment in order to eliminate the portion of the absorbance which could not be attributed to cell growth. This was of particular importance for the determination of growth in cultures containing acetochlor since it was noted that over the first three days of culturing a significant amount of the absorbance measured was attributable to the herbicide. The drop in absorbance in uninoculated controls containing acetochlor indicated that this absorbance was possibly due to herbicide molecules which had initially failed to go into solution but which subsequently dissolved slowly due to constant mixing at 150 rpm. Adjustment of measured absorbances of each of the inoculated cultures was achieved by subtracting from them the absorbance of the corresponding uninoculated control. The absorbance values thus obtained were plotted against the time after inoculation at which they were taken (Figs. 4.1.1 and 4.1.2). These growth curves demonstrated that over the entire observation period (with the exception of day 2 during enrichment cycle 6) the NM enrichment medium supported the largest cell population. The NMA enrichment medium generally supported the second highest cell population, followed in turn by the MSA and MS enrichment media.

Of all the enrichment media used only MS yielded no recognisable growth curve (Fig 4.1.2). Comparisons of the growth curves obtained for the enrichment cultures exposed to acetochlor between cycles 3 through 6 reveal some weak trends in the growth of the microbial communities comprising these cultures. The growth curves of the enrichment

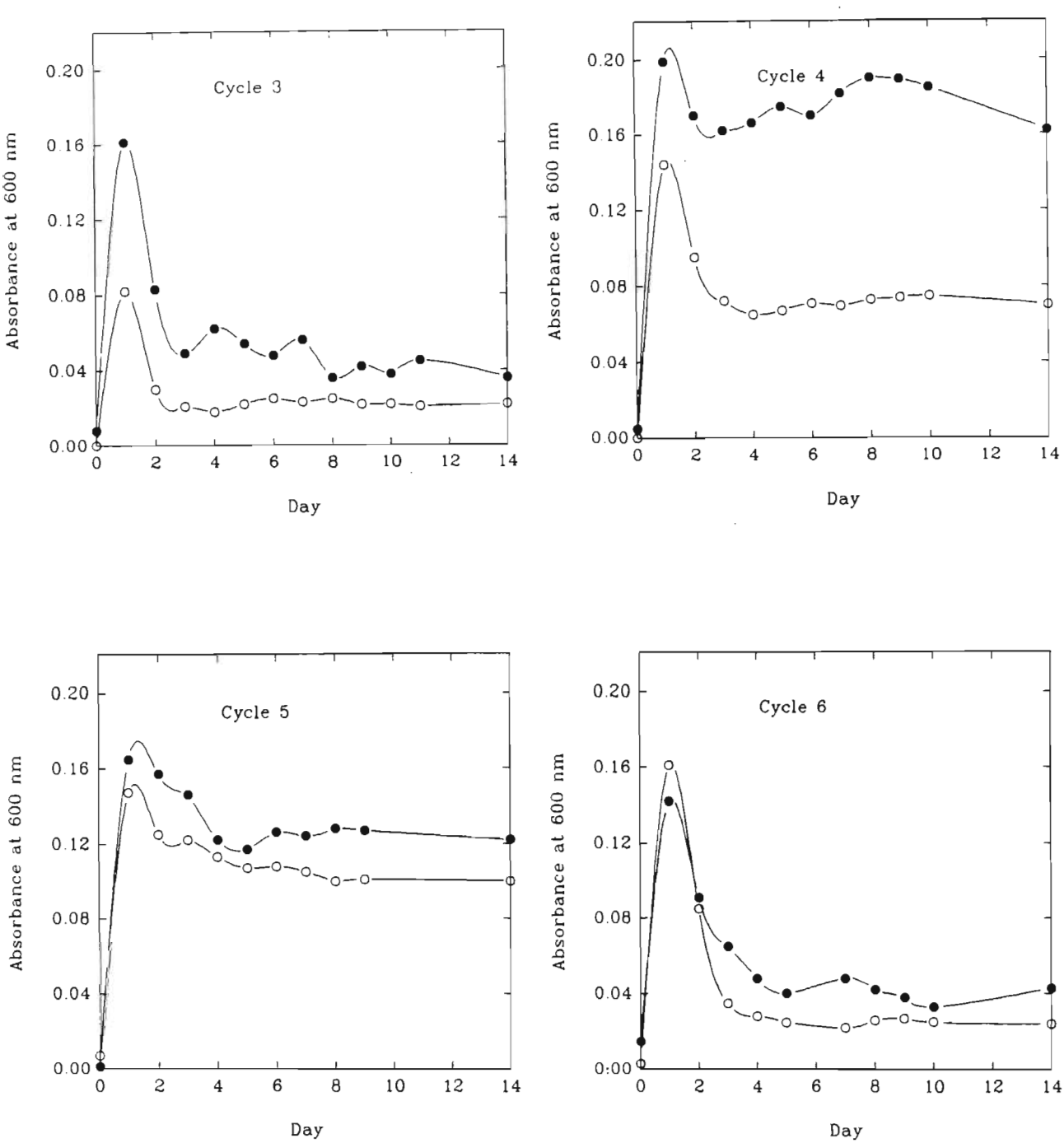

FIG. 4.1.1. The spectrophotometric determination of the growth of soil microbes in the NM and NMA cultures during enrichment cycles 3, 4, 5 and 6 over the 14 days following inoculation of the cultures. (●) NM enrichment culture. (○) NMA enrichment culture.

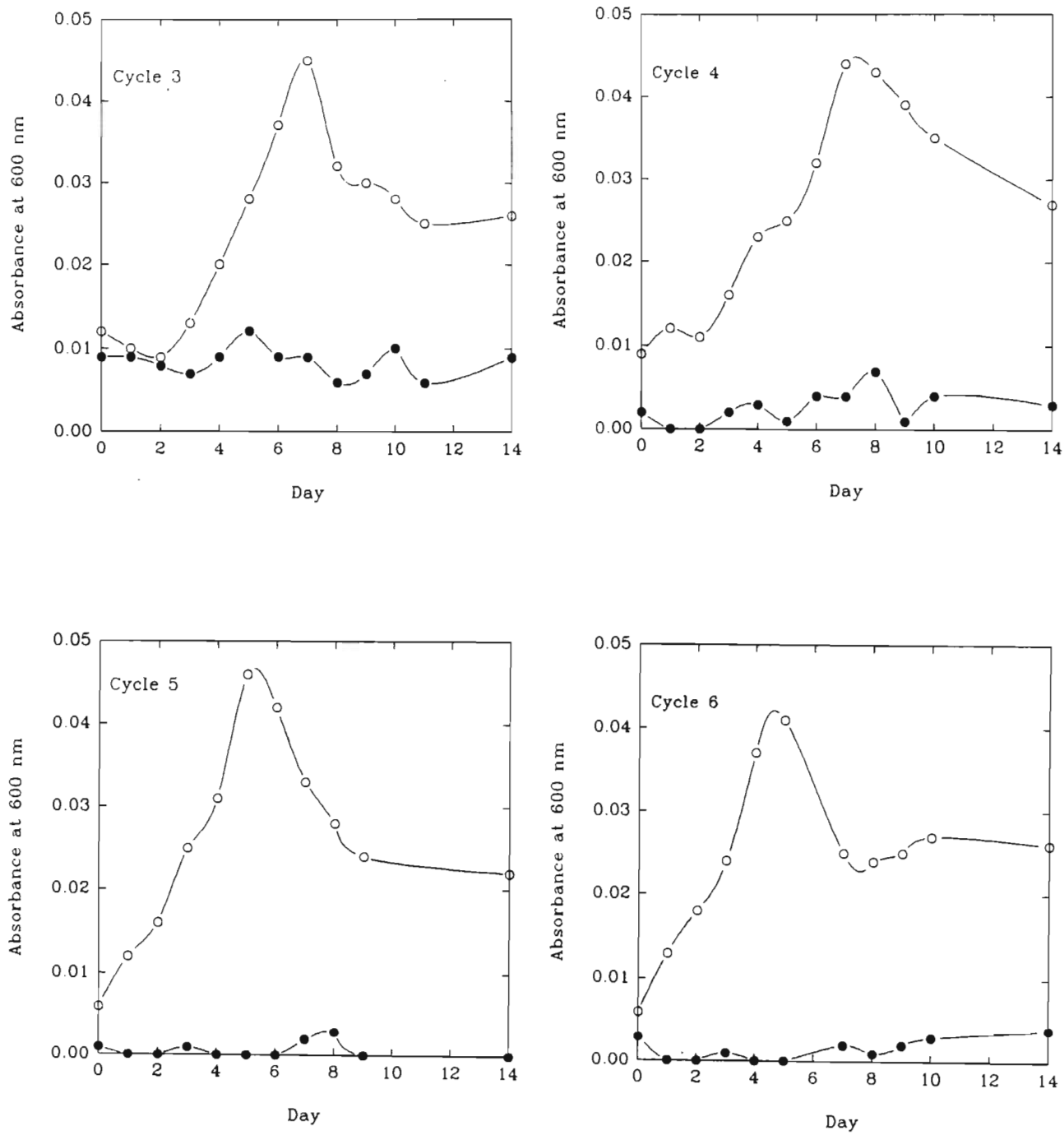

FIG. 4.1.2. The spectrophotometric determination of the growth of soil microbes in the MS and MSA cultures during enrichment cycles 3, 4, 5 and 6 over the 14 days following inoculation of the cultures. (●) MS enrichment culture. (○) MSA enrichment culture.

cultures (Fig. 4.1.1) demonstrate that, over the four cycles of enrichment, the peak absorbance of the cultures at 600 nm (always observable after 1 day of growth) increased steadily from 0.082 during cycle 3 to 0.161 during cycle 6. In the MSA enrichment cultures there was no noticeable increase in the maximum absorption values (OD_{A600}) attained in cycles 3 to 6 (Fig 4.1.2). However, the time taken to reach these maximum absorbances was reduced from 7 days in cycles 3 and 4 to 5 days in cycles 5 and 6 (Fig 4.1.2). This was most marked between cycles 4 and 5, and was probably related to a shortening of the lag period in these cultures due to adaptation.

4.3.2 Screening for Acetochlor Degradation

In the third and fourth enrichment cycles bacterial and fungal strains surviving enrichment were selected at random. Alternatively, in enrichment cycle five, only bacteria which yielded a positive colorimetric reaction on either the EMBA or MSA-TTC indicator media were chosen for further study. Colonies reacting positively on EMBA took on a red appearance while those reacting negatively either remained white or took on a blue appearance (Fig. 4.2.1). On MS-TTC colonies reacting negatively were white, while those reacting positively were either red or had a red spot in their centres.

Table 4.1 shows the results of tests carried out on the 120 isolates obtained from the different enrichment cultures during enrichment cycles three, four and five, to determine whether they could either dechlorinate acetochlor or utilise it as their sole carbon and energy source. Although tests were also carried out on isolates obtained during enrichment cycle six the results were inconclusive and are therefore not included. Five isolates, all of which were selected during the fifth enrichment cycle, produced a different colour reaction when grown on EMBA than when they were grown on EMB. Although all five of the isolates tested were capable of growth on both MSA and MS agar, A108 and A104 appeared to grow slightly better on MSA agar than on MS agar. Neither of these isolates, however, showed any observable growth in liquid MSA medium. Also, neither repeated transfers of A104 and A108 on MS agar, nor starvation in liquid MS medium for up to one month, resulted in any noticeable decrease in the growth of these isolates on MS agar in the absence of any added carbon or energy sources.

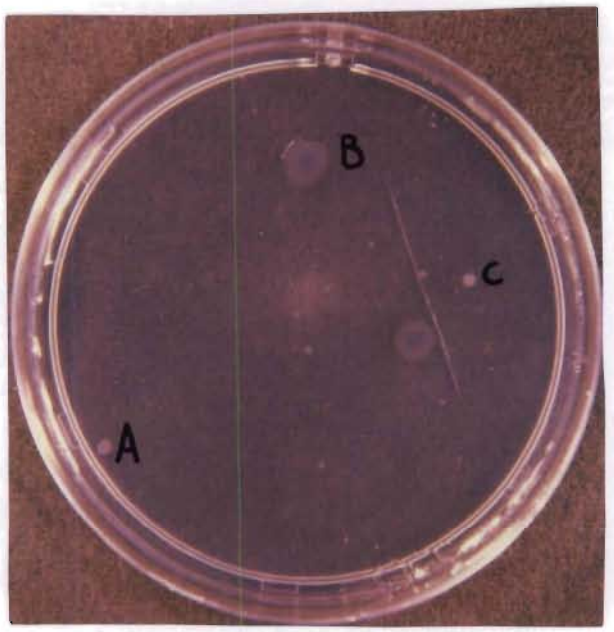

FIG. 4.2. Different colony colours which occurring on EMBA during screening of individuals surviving 5 cycles of enrichment in NM and NMA. Red colony colour (A) indicated the production of acid in the colony and possibly the dechlorination of acetochlor. In the Blue (B) and White colonies (C) no acid production was occurring and hence it was unlikely that dechlorination of acetochlor was taking place.

TABLE 4.1. The results of tests carried out on the 120 isolates obtained during enrichment cycles three, four and five to determine whether they could either dechlorinate acetochlor or utilise it as their sole carbon and energy source.

Strain	Origin of Strain		Colour of Colonies on:		Presence of Growth on:	
	Enrichment Medium	Enrichment Cycle	EMB	EMBA	MS Agar	MSA Agar
A1	NMA	3	White	White	-[a]	-
A2	NMA	3	---[b]	---	-	-
A3	NMA	3	Red	Red	-	-
A4	NMA	3	-	-	-	-
A5	NMA	3	-	-	+-[c]	-
A6[d]	NMA	3	---	---	+	+
A7	NMA	3	Purple	Red	-	-
A8	NMA	3	-	-	-	-
A9	NMA	3	Blue	Blue	-	-
A10	NMA	3	-	-	-	-
A11[d]	NMA	3	---	---	+-	+-
A12	NMA	3	-	Red	+[e]	-
A13	NMA	3	-	-	-	-
A14	NMA	3	White	White	-	-
A15	NMA	3	White	-	-	-
A16	NMA	3	-	-	-	-
A17	MSA	3	Red	Red	+	-
A18	MSA	3	Red	Red	+	-
A19	MSA	3	Purple	Blue	+	-
A20	MSA	3	White	White	+	-
A21	MSA	3	Pink	Pink/White	-	-
A22	MSA	3	White	White	+	-
A23	MSA	3	---	---	+	-
A24	MSA	3	Pink	Pink	+	+
A25	MSA	3	Pink	Pink	+-	-
A26	MSA	3	Red	Red	+	-
A27	MSA	3	Red	Red	+	-
A28	MSA	3	-	-	-	-
A29	MSA	3	Red	Red	+	-
A30	MSA	3	Red/Purple[f]	Purple[f]	+	-
A31	MSA	3	Red	Red	+	-
A32	MSA	3	Red	Red	+	+
A33	NM	3	White	White	-	-
A34	NM	3	Blue	-	-	-
A35	NM	3	-	-	-	-
A36	NM	3	-	-	-	-
A37	MS	3	***[g]	***	+	-
A38	MS	3	***	***	-	-
A39	MS	3	***	***	+	-
A40	MS	3	***	***	+-	-
A41	NMA	4	-	-	***	***
A42	NMA	4	-	-	***	***
A43	NMA	4	Pink	Red	***	***
A44	NMA	4	Red	Red	***	***
A45[d]	NMA	4	---	---	***	***
A46	NMA	4	-	-	***	***
A47	NMA	4	Blue	Blue	***	***
A48	NMA	4	White	White	***	***
A49	NMA	4	White	White	***	***

TABLE 4.1. (Cont.)

Strain	Origin of Strain		Colour of Colonies on:		Presence of Growth on:	
	Enrichment Medium	Enrichment Cycle	EMB	EMBA	MS Agar	MSA Agar
A50	NMA	4	-	-	***	***
A51	NMA	4	-	-	***	***
A52	NMA	4	-	-	***	***
A53	NMA	4	Red	Red	***	***
A54	NMA	4	Purple/Blue	Blue	***	***
A55	NMA	4	White	White	***	***
A56	NMA	4	-	-	***	***
A57	MSA	4	White	White	+-	+-
A58	MSA	4	Blue	Blue	-	-
A59	MSA	4	Red	Red	+-	+-
A60	MSA	4	Red	Red	+	+-
A61	MSA	4	White	White	+	+-
A62	MSA	4	Red	Red	+	+-
A63	MSA	4	White	White	+	-
A64	MSA	4	—	—	+	+-
A65	MSA	4	White	White	-	-
A66	MSA	4	Purpe/Blue	Purple	+	-
A67	MSA	4	Red	Red	+	-
A69	MSA	4	White	White	-	-
A70	MSA	4	White	White	+-	-
A71	MSA	4	Red	Red	+	-
A72	MSA	4	Red	Red	-	-
A73	NM	4	-	-	***	***
A74	NM	4	Red	-	***	***
A75	NM	4	-	-	***	***
A76	NM	4	-	-	***	***
A77	MS	4	***	***	+	+-
A78	MS	4	***	***	+-	+-
A79	MS	4	***	***	+	-
A80	MS	4	***	***	+	-
A81	NMA	5	Red	Red	-	-
A82	NMA	5	Blue	Purple	+	+
A83	NMA	5	Red	Red	-	-
A84	NMA	5	Pink	Pink	-	-
A85	NMA	5	Red	Red	+-	+-
A86	NMA	5	Blue	Purple	-	-
A87	NMA	5	Red	Red	-	-
A88	NMA	5	Red	Red	-	-
A89	NMA	5	Blue	Purple[f]	+	+
A90	NMA	5	Red	Red	-	-
A91	NMA	5	Red	Red	-	-
A92	NMA	5	Red	Red	-	-
A93	NMA	5	Red	Pink	-	-
A94	NMA	5	Red	Red	+-	-
A95	NMA	5	-	-	-	-
A96	NMA	5	Red	Red	-	-
A97	MSA	5	Red	Red	+	+-
A98	MSA	5	Purple	Purple	+	+
A99	MSA	5	Red	Red	+	+
A100	MSA	5	Red	Red	+	-
A101	MSA	5	Purple	Purple	+	+
A102	MSA	5	White	White	+	+
A103	MSA	5	Red	Red	+	+
A104	MSA	5	Purple/Blue	Red	+	+
A105	MSA	5	White	White	+-	-

TABLE 4.1. (Cont.)

Strain	Origin of Strain		Colour of Colonies on:		Presence of Growth on:	
	Enrichment Medium	Enrichment Cycle	EMB	EMBA	MS Agar	MSA Agar
A106	MSA	5	Blue	Blue	+	+
A107	MSA	5	Red	Red	+	+
A108	MSA	5	Blue	Red	+	+
A109	MSA	5	White	White	+	+
A110	MSA	5	Red	Red	-	-
A111	MSA	5	Purple	Purple	+	+
A112	MSA	5	Red	Red	+	+
A113	NM	5	Red	-	***	***
A114	NM	5	Red	-	***	***
A115	NM	5	Red	Red	***	***
A116	NM	5	Red	Red	***	***
A117	MS	5	***	***	+	+
A118	MS	5	***	***	+	-
A119	MS	5	***	***	+	+
A120	MS	5	***	***	+	+

[a] No growth observable.
[b] Normal colony colour interfered with colour determination.
[c] Only slight growth observable.
[d] Fungus.
[e] Obvious growth observable.
[f] Green metallic sheen observed.
[g] Not Tested.

Screening of the isolates on MS agar supplemented with 0.2 g of galactose per litre indicated that growth of A82 and A89 on MSA and their strong colour reactions on EMB were possibly due solely to their ability to efficiently utilise agar as their sole source of carbon and energy.

Use of the qualitative acetochlor detoxification bioassay showed that of the 3 isolates, A86, A104 and A108, only A108 was able to detoxify a detectable quantity of the herbicide in MSA medium over the time-scale tested (Table 4.2). Prior inoculation of media containing no acetochlor with test microorganisms did not obviously effect the growth of the bioassay fungus.

TABLE 4.2. Ability of selected soil bacteria to significantly detoxify acetochlor at a concentration of 0.15 $g.l^{-1}$

Strain	No Acetochlor (Day 8)			Acetochlor (Day 16)		
	Mean Colony Diameter (mm)	Z-value[a]	Probability[b]	Mean Colony Diameter (mm)	Z-value	Probability
Control	10.148 10.146	-- --	-- --	2.513 2.511	-- --	-- --
A86	10.217 -[c]	0.1858 -	0.8528 -	2.625 2.409	0.9992 -0.4253	0.3177 0.6706
A104	10.276 -	0.4646 -	0.6422 -	2.263 2.275	-2.0667 -1.2380	0.0388 0.2157
A108	10.190 -	0.2211 -	0.8250 -	4.711 4.381	5.2835 5.4525	1.3×10^{-7} 4.1×10^{-8}

[a] The Z-values obtained when comparing the set of fungal colony diameter measurements on test and control plates using the Mann-Whitney u-test. The Z-value is equivalent to the U-value described by Neave and Worthington (1988).

[b] The probability that colony diameters on control plates are not different to those observed on control plates.

[c] No data collected.

4.3.3 Quantification of the Acetochlor Detoxifying Ability of Isolate A108

The provision of low concentrations of glucose (0.02 $g.l^{-1}$) and yeast extract (0.005 $g.l^{-1}$) was sufficient to significantly improve the rate at which A108 was able to degrade acetochlor (Figs 4.3.1 and 4.3.2). While approximately 53 % of the added acetochlor had been detoxified after 21 days in NM.1 medium, only approximately 20 % of the herbicide was detoxified over the same time period in MS. While there was no noticeable increase in the turbidity of MS medium inoculated with A108 over the 28 day test period, inoculated NM.1 medium both with and without added acetochlor, showed significant increase in culture turbidity three days after inoculation.

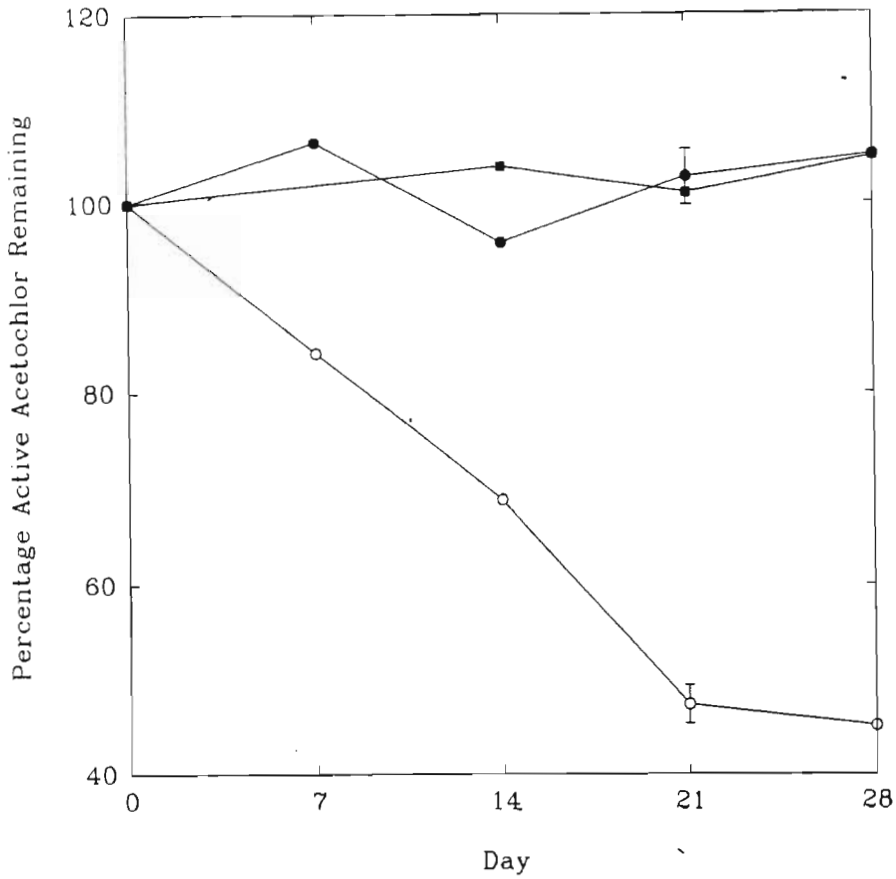

FIG 4.3.1. Degradation of acetochlor by A108 over a 28 day period in NM.1. (○) percentage of added acetochlor remaining in the inoculated medium at different times. (●) percentage of added acetochlor remaining in the uninoculated medium. (■) spectrophotometrically determined percentage of added acetochlor remaining in the uninoculated medium. Error bars at day 21 represent the standard deviation of three independent bioassay measurements of the percentage of active acetochlor remaining at that time.

4.3.4 Characterisation and Identification of the Acetochlor Degrading Isolate

Gram staining showed A108 to be a short Gram negative rod. The very slight growth observed on NM agar under mineral oil after three weeks indicated it was probably aerobic or possibly a weak facultative anaerobic. Electron microscopic examination of the isolate showed that it was approximately 0.45-0.6 μm wide and 0.75-1.0 μm in length with polar flagellation (Fig. 4.4). The ATB Identification analyser gave a 98 % probability that A108 was a member of the genus *Pseudomonas*. While the identification analyser gave a 92 % probability that A108 was either *Pseudomonas putida*, *Pseudomonas mendocina* or *Pseudomonas fluorescens*, it also gave a probability of 6 % that it could be *Pseudomonas*

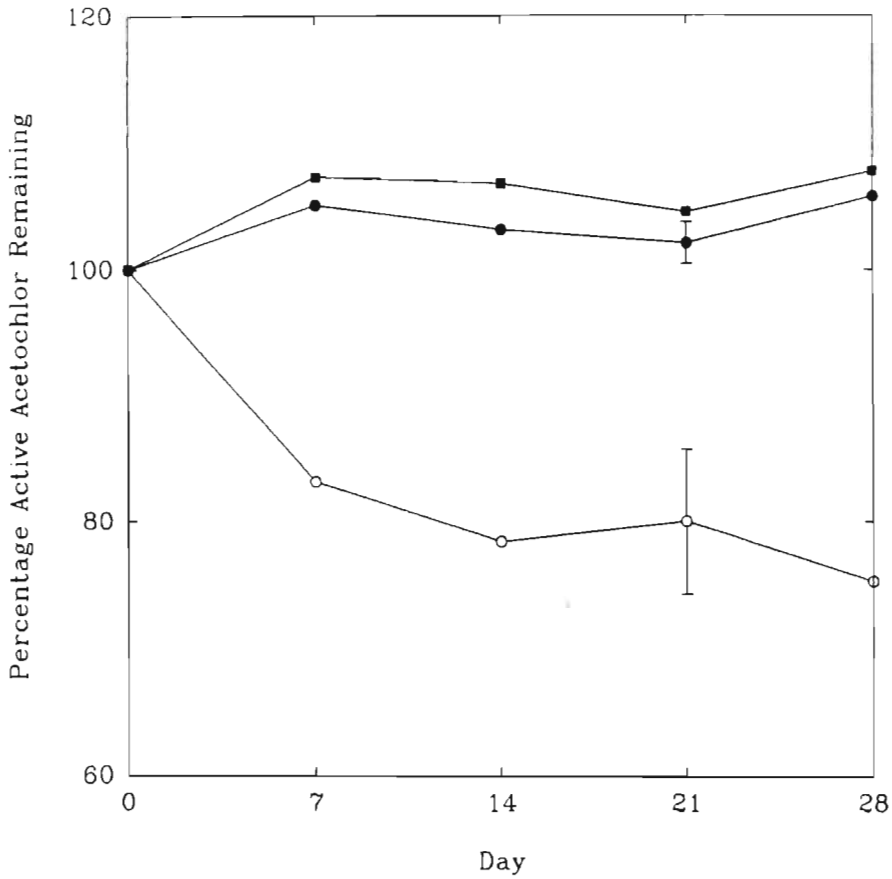

FIG 4.3.2. Degradation of acetochlor by A108 over a 28 day period in MS. (○) percentage of added acetochlor remaining in the inoculated medium at different times. (●) percentage of added acetochlor remaining in the uninoculated medium. (■) spectrophotometrically determined percentage of active acetochlor remaining in the uninoculated medium. Error bars at day 21 represent the standard deviation of three independent bioassay measurements of the percentage of active acetochlor remaining at that time.

aeruginosa. A108's size and flagellation (Fig 4.4), its production of a fluorescent pigment, its ability to slightly liquify gelatin and its inability to grow at 42°C indicated that of all these possible identities *Ps. fluorescens* was the most likely. A light microscopic comparison of Gram stained A108 and a stock culture of *Ps. fluorescens* revealed that the isolate was morphologically indistinguishable from *Ps. fluorescens*.

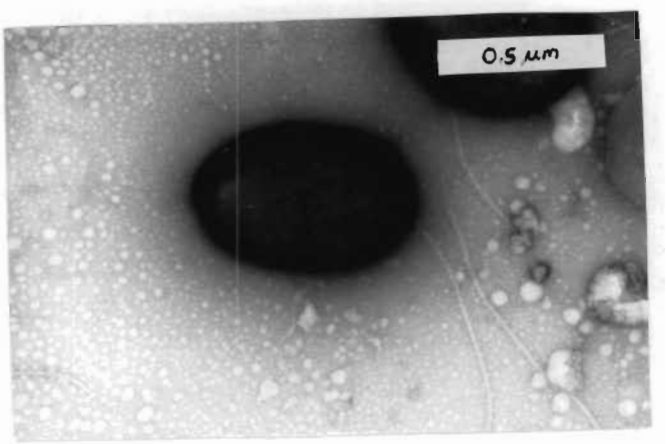

FIG 4.4. Transmission electron micrograph of isolate A108 magnified 33 000 X.

4.4. Discussion

Two procedures were employed in the enrichment of acetochlor transforming microbes. While the first involved the *in vivo* selection of acetochlor resistant microbes by exposing native soils to high dosages of the herbicide, the second procedure involved the *in vitro* selection, in liquid culture of microbes capable of transforming acetochlor. Following the advice of Bartha (1990), the soils used as sources of microbial strains were taken from a variety of sites in order to obtain a high diversity of microorganisms at the onset of enrichment. Ideally, soils should have been sampled from lands with a history of exposure to acetochlor. The chloroacetanilide degrading microbes isolated to date have mostly originated in soils to which these herbicides had been recently and repeatedly applied over a number of years (Krause *et al.*, 1985; Saxena *et al.*, 1987; Novick *et al.*, 1986; Villarreal *et al.*, 1991). It was hoped that repeated treatments of the bulked soil samples with a high concentration of acetochlor (a total addition of approximately 0.275 mg

acetochlor per gram of soil over a 14 week period) would simulate, at least partly, the selection pressure for herbicide degradation which is thought to occur in agricultural soils often exposed to chloroacetanilides (Bailey and Coffey, 1986). Bailey and Coffey (1986) carried out a similar deliberate *in vivo* enrichment for microbial degraders of metalaxyl, a fungicide which is chemically related to acetochlor. These researchers were able to demonstrate that the rate of metalaxyl degradation in a soil with no previous exposure to the fungicide could be enhanced over a 12 week period to match that of a soil which had been subjected to 5 years of treatment with the fungicide. This enhancement in degradation rate was achieved with a total fungicide dosage (0.5 mg per gram of soil) comparable to the herbicide dosage employed in the *in vivo* enrichment carried out in the present study.

In the *in vitro* enrichment experiments two different approaches were employed to obtain microbes capable of transforming acetochlor by using enzymes functional in either detoxification of the herbicide or its use as a carbon and energy source. Whereas the former approach aimed at aimed at obtaining microbes capable of growth in a nutrient medium containing high acetochlor concentrations, the latter aimed at enriching for a microbial community capable of proliferation in the absence of any organic carbon besides acetochlor.

Although six cycles of each enrichment were carried out, it is probable that only the last four were effective in the selection of microbes capable of transforming acetochlor. The reason for this being that in the first two enrichment cycles a significant proportion of the nutrients present in the enrichment media originated in the inocula from which the enrichment cultures were initiated (1 g of soil in the first enrichment cycle and 1 ml of a 10 $g.l^{-1}$ soil suspension in the second enrichment cycle). These inocula not only provided soil particles to which herbicide molecules could become adsorbed but also supplied the cells in the MSA enrichments with carbon and energy sources other than acetochlor. It is probable, therefore, that neither selection for acetochlor resistant strains (in the NMA cultures) nor enrichment for acetochlor utilising populations (in the MSA cultures) were very effective during the first two cycles of enrichment.

During enrichment cycles 3 through 6 a greater proliferation of growth was observed in

the NM than in the NMA cultures. It is probable that the NMA enrichment supported less growth than the NM enrichment because of the inhibitory effects of the acetochlor present in NMA. The marked increased in optical density of the NMA enrichment cultures between the third and sixth enrichment cycles was possibly due to the development of increased tolerance to acetochlor among certain of the microbial strains comprising these cultures. Because growth in the NMA enrichment culture during enrichment cycle 5 was only marginally less prolific than that in the NM enrichment culture it was evident that at least some microbial cells completely resistant to the 0.18 $g.l^{-1}$ acetochlor in NMA had been successfully selected for.

Whereas slight growth was consistently observed in the MSA enrichment cultures between cycles 3 and 6, no visible growth occurred in any of the MS cultures. This indicated that the growth observed in MSA possibly occurred as a result of acetochlor being utilised as a carbon and energy source. It is possible that acetochlor utilisation, if it did occur in the MSA enrichment cultures, took place as a result of synergism. A stable mixed culture able to utilise the chlorinated herbicide, mecoprop, as sole carbon and energy source has been obtained using an enrichment procedure similar to that carried out in the present study (Lappin *et al.*, 1985). The mixed culture contained five bacterial species and the synergistic utilisation of the herbicide was indicated by the fact that none of the microbes were capable of obtaining carbon or energy from mecoprop when grown in pure culture. It is likely, therefore, that observable growth in the MSA enrichment cultures indicated the successful selection of at least one microbial strain which was able to transform acetochlor to a form which could be further degraded by other microbes present in the enrichment culture.

The EMB-EMBA and MSA-TTC colorimetric assays used in the selection of potential acetochlor degraders, appeared to enhance the effectiveness of the isolation procedure. Whereas none of the 80 randomly chosen isolates from enrichment cycles 3 and 4 showed any signs of being able to transform acetochlor, 5 of the 40 isolates selected using these colorimetric assays during cycle 5 gave some indication that they were potential acetochlor degraders. Of these five isolates (which all produced red colonies on EMBA and blue or purple colonies colours on EMB), conclusive evidence for acetochlor detoxification was only obtained for isolate A108.

The EMB based assay was developed by Loos (1975) specifically for the detection of chlorinated pesticide degradation by microorganisms and was used to great effect by Pemberton *et al.* (1979) during the isolation of microbes capable of degrading the herbicide, 2,4-D. The principal upon which the indicator functions is the detection of HCl released during the microbial degradation of chlorinated molecules. If this HCl lowers the pH of the herbicide containing medium in the vicinity of the colonies to such an extent that the eosin dye in the indicator medium becomes mobilised, the colonies should take on a red appearance (Loos, 1975). In the present study the EMB based indicator medium was used both in the initial selection of microbes from the fifth NMA and NM enrichment cycle and in the subsequent screening of all selected isolates (irrespective of the enrichment cultures from which they originated) for an ability to dechlorinate acetochlor. While none of the 20 isolates selected from the NM and NMA enrichment cultures because of their red colony colour on EMBA were found to detoxify acetochlor, screening of microbes from the MSA enrichment cultures on EMB and EMBA revealed one isolate, A108, which, using the qualitative bioassay, was ultimately shown to detoxify acetochlor. Of the four other isolates displaying differential colony colouration on EMB and EMBA, A82 and A89 were not tested for their ability to detoxify acetochlor, while A86 and A104 could not be shown to significantly detoxify the herbicide. One possibly important difference between the colour reactions of A108 and the other isolates tested on both EMBA and EMB was that A108 was the only one with clearly red and blue colonies on EMBA and EMB respectively. Unlike A108, the other four isolates (A82, A86, A89 and A104) selected as potentially active in acetochlor dechlorination gave rise to purple and not clearly blue colonies on EMB. It is possible that the purple colouration of these colonies was misinterpreted as being almost blue. These probably false positive reactions as well as the green metallic sheen observed with colonies of A89 on EMBA, possibly occurred as a result of the assay not being optimised for the detection of acetochlor dechlorination. The assay could not be optimised because no acetochlor dechlorinating bacterium was available for use as a positive HCl generating control in an optimisation procedure. Therefore, the yeast extract and herbicide concentrations used for the assay in the present study were based on those optimised by Loos (1975) for the detection of microbial 2,4-D dechlorination.

The MSA-TTC indicator plate technique was developed by Bochner and Savageau (1977)

as a means of detecting bacterial utilisation of diverse organic substrates as carbon and energy sources. The usefulness of TTC as an indicator dye derives from the fact that in its oxidised state it is colourless and water soluble while in its reduced state it is insoluble in water and has a deep red colour. The principal upon which the TTC containing indicator medium is based is that microbial strains obtaining energy from an organic nutrient via oxidative phosphorylation will simultaneously reduce any intracellular TTC present since TTC has the capacity to displace oxygen as a terminal electron acceptor in oxidative phosphorylation. If either the concentration of the organic carbon source tested is below a certain threshold (which is specific for every organic molecule) or if cells are unable to efficiently utilise the carbon source, then colonies appearing on the indicator plates remain white (Bochner and Savageau, 1977). In the present study use of the MSA-TTC plates during enrichment cycle 5 to select potential acetochlor utilisers from the MSA and MS enrichment cultures, ultimately yielded one isolate capable of demonstrably detoxifying the herbicide. However, use of the indicator to detect utilisation of acetochlor was unsuccessful. One possible reason for this was that the concentration of acetochlor in MSA-TTC was over five times lower (0.018 %) than the minimum test substrate concentration of 0.1 % recommended for the assay by Bochner and Savageau (1977). Because of acetochlor's limited solubility (0.233 $g.l^{-1}$) it was, however, not possible to increase its concentration to the 0.1 % threshold concentration recommended by these researchers. It was accepted, therefore, that the red colouration of colonies selected from the indicator plates may have been due to the ability of the cells to effectively utilise other nutrients available in the indicator medium.

No irrefutable evidence was obtained that any of the microbial strains investigated were capable of utilising acetochlor as a sole carbon and energy source. Although it appeared as though isolates A108 and A104 were capable of better growth on MS agar in the presence of acetochlor than in its absence, the fact that both were able to survive on MS agar without any added carbon and energy sources cast doubt on the assumption that they could utilise the herbicide. Many non-agarolytic microbes are known to be capable of growth on mineral salts media by virtue of their ability to utilise impurities in agar and the atmosphere. The growth of these microbes is recognised as a common problem in studies attempting to demonstrate the utilisation of a carbon source through the occurrence of growth in its presence and lack of growth in its absence (Bartha, 1991). Since neither

A108 nor A104 could utilise galactose (the monosaccharide subunit of agar) as a carbon source and because highly pure water and purified agar were used to make up the MS agar medium, it is likely that the nutrients permitting the growth of these microbes on MS agar originated either from the atmosphere or as contaminants of the mineral salts used to make up the medium. The presence of a qualitative difference in the growth of these bacteria on MS and MSA could be explained by the fact that the technical grade acetochlor used in this study was only 90.5 % pure. It was thus possible that the 0.095 mg of inert ingredients added to MSA with every milligram of acetochlor permitted improved growth. If a combination of contaminants in the atmosphere, the herbicide and the mineral salts used were the cause of cell proliferation on solid MS and MSA media, these same contaminants could possibly have supported observable cell growth in liquid MS or MSA. It was found, however, that even after long incubation periods, no discernable growth of A108 or A104 occurred in either liquid MS or liquid MSA.

Reports on the failure of isolation procedures to yield microbes capable of utilising the chloroacetanilides metolachlor (Bailey and Coffey, 1986; Saxena *et al.*, 1987; Bollag, 1990), alachlor (Novick *et al.*, 1986) and propachlor (Novick and Alexander, 1985; Novick *et al.*, 1986) as sole sources of either carbon or nitrogen are fairly common. It appears that acetochlor, like these other chloroacetanilides, may be highly resistant to complete microbial degradation. This is because acetochlor and these related molecules contain tertiary amine moieties and alkyl groups at positions 2 and 6 on their aniline rings. By protecting the bonds to the nitrogen atoms of chloroacetanilides from enzymatic attack, these structural features probably make these herbicides very poor nutrient sources (Saxena *et al.*, 1987).

While three of the forty isolates chosen for further study during enrichment cycle 5 met the selection criteria used for identifying potential acetochlor detoxifiers, only one of these (A108) could be shown to significantly detoxify the herbicide. This, however, might not accurately reflect the number of acetochlor degraders present in the enrichment cultures. Bailey and Coffey (1986) isolated microbes from soils treated with metalaxyl (a fungicide chemically related to the chloroacetanilides) with an ability to degrade ^{14}C-labelled metolachlor (a chloroacetanilide herbicide) using TLC for the detection of ^{14}C-labelled degradation products. Without using any *in vitro* enrichment for herbicide degraders,

these researchers found that of the 25 isolates examined, 14 (56 %) were capable of carrying out metabolic transformation of the herbicide. Thus, it is possible that a far higher proportion than just 2.5 % (1 out of 40) of the microbes selected from enrichment cycle 5 were capable of transforming acetochlor.

There are two possible reasons why the isolation procedures used during the present study were apparently less effective than those used by Bailey and Coffey (1986). The first is that the vast majority of the microbes capable of metabolising acetochlor would not have elicited any positive response in the EMBA-EMB phenotypic assay. This was as a result of the test being specifically aimed at the identification of strains capable of dechlorinating acetochlor. It is likely that, as is the case with the other chloroacetanilides, the vast majority of possible acetochlor transformations do not involve dechlorination (Figs. 1.5.1, 1.5.2, 1.5.3, 1.5.4).

The second possible reason was that detection of acetochlor detoxification using the qualitative bioassay was dependent on test microbes being capable of transforming the herbicide in MS medium. In a number of instances it has been found that the successful transformation of chloroacetanilides by known degraders of these herbicides, is strongly correlated with the availability of utilisable carbon sources (Bailey and Coffey, 1986; Krause *et al.*, 1985; Novick and Alexander, 1985; Smith and Phillips, 1975). Krause *et al.* (1985), in experiments involving the determination of a soil actinomycete's ability to transform metolachlor, demonstrated how essential nutrient availability may be in permitting the detection of a microbe's ability to degrade a chloroacetanilide herbicide. While in 24 days the actinomycete was capable of completely transforming over 50 % of the metolachlor (at a concentration of 0.05 $g.l^{-1}$) in a growth medium containing 4.0 $g.l^{-1}$ sucrose, it was only able to transform 6 % of the herbicide over the same time period in a medium containing 0.5 $g.l^{-1}$ sucrose. Adding yeast extract to the test medium at a concentration of 4.0 $g.l^{-1}$ further stimulated the actinomycete's degradative capacity permitting it to transform 100 % of the metolachlor present in less than 16 days.

Therefore the finding that the rate at which acetochlor was degraded by A108 was accelerated in the presence of 0.02 $g.l^{-1}$ glucose and 0.005 $g.l^{-1}$ yeast extract supported the observations made by other researchers examining the microbial degradation of

chloroacetanilide herbicides. Increased degradation of chloroacetanilide herbicides in the presence of carbon sources other than the herbicides has been cited as being merely a function of these nutrients supporting the growth of a greater number of herbicide degrading cells (Krause *et al.*, 1985; Novick and Alexander, 1985). In this regard differences in the turbidity of MS and NM.1 media inoculated with isolate A108 indicated that the additional nutrients in NM.1 did support a greater number of A108 cells than did the nutrients in MS.

In the present study the motivating force behind the isolation of an acetochlor degrading microbe was to obtain a gene which would be of use in the genetic engineering of acetochlor resistance in crops. Because dechlorination both detoxifies chloroacetanilides (Hamm, 1972) and is typically the first step in the degradation pathways of these herbicides (Figs 1.5.1, 1.5.2 and 1.5.3), it was realised that a gene encoding acetochlor dechlorination would be ideal for use as an acetochlor resistance gene in genetically engineered crops. Therefore, the isolation procedures used were specifically aimed at obtaining a single microbial strain active in dechlorinating acetochlor. Isolate A108 both elicited a strong response in the EMB-EMBA phenotypic assay and significantly detoxified acetochlor according to both the qualitative and quantitative bioassays. Despite the fact that the degradation products arising from the metabolism of acetochlor by isolate A108 could not be identified due to limited facilities, the results obtained indicated that there was a strong possibility that A108 was capable of metabolically dechlorinating acetochlor.

5 CLONING AND PARTIAL SEQUENCING OF A GENE ASSOCIATED WITH ACETOCHLOR DETOXIFICATION

5.1 Introduction

Successes have been reported in the cloning of microbial genes encoding the detoxification of the herbicides glufosinate (Murakami *et al.*, 1986; Wohlleben *et al.*, 1988), bromoxynil (Stalker and McBride, 1987), 2,4-D (Streber *et al.*, 1987), cyanamide (Maier-Greiner *et al.*, 1991), dalapon (Thomas *et al.*, 1992b), chlorsulfuron (Omer *et al.* 1990), melamine (Eaton and Karns, 1991), paraquat (Salleh and Pemberton, 1993) and phenmedipham (Pohlenz *et al.*, 1992). While the motivation behind the isolation of these genes has been to obtain a better understanding of how microbes are capable of metabolising chemicals foreign to their environments, it has also been the acquisition of genes with the potential for use in engineering herbicide resistant plants. In this regard the glufosinate, bromoxynil, 2,4-D, cyanamide and dalapon degradative genes have been noted to increase levels of herbicide resistance in transgenic plants in which they have been expressed (Buchanan-Wollaston *et al.*, 1992; DeBlock *et al.*, 1987; Maier-Greiner *et al.*, 1991; Stalker *et al.*, 1988; Streber and Willmitzer, 1989).

Although a number of microbes capable of metabolically transforming one or more of the chloroacetanilides have been discovered (Bailey and Coffey, 1986; Krause *et al.*, 1985; Liu *et al.*, 1989; Novick and Alexander, 1985; Novick *et al.*, 1986; Saxena *et al.*, 1987; Tiedje and Hagedorn, 1975; Villarreal *et al.*, 1991), none of the genes encoding the degradation of any of these herbicides have been cloned from these organisms. A possible reason for this is that the isolation and examination of chloroacetanilide degraders has been carried out almost exclusively to facilitate studies on the mechanisms of chloroacetanilide degradation in the environment (Bailey and Coffey *et al.*, 1986; Krause *et al.*, 1985; Novick and Alexander, 1985; Novick *et al.*, 1986; Saxena *et al.*, 1987; Tiedje and Hagedorn, 1975; Villarreal, 1991). The potential for certain of the microbes isolated during the course of this research to be used in the clean-up of chloroacetanilide contaminated soil and water has also been investigated (Gizzarelli *et al.*, 1993; Liu *et al.*, 1990).

Despite the fact that the chloroacetanilides are among the most frequently used agricultural chemicals in the world today, there have been no reported attempts to produce transgenic chloroacetanilide resistant plants. While genes encoding glutathione transferases responsible for the detoxification of chloroacetanilide herbicides in plants have been cloned (Kutchan and Hochberger, 1992; Shah *et al.*, 1986b; Timmerman, 1989) and successfully expressed in bacteria and *Saccharomyces cerevisiae* (Kutchan and Hochberger, 1992) the successful use of these genes in the engineering of herbicide resistance in crops has yet to be reported. As an alternative to using degradative genes from plants, it should be possible to engineer chloroacetanilide resistance in crops by employing microbial genes encoding the detoxification of these herbicides. In particular genes such as those encoding the dechlorination of chloracetanilides would be ideal for use in genetically engineering resistance to these herbicides in crops. The reason being that dechlorination reactions both detoxify chloroacetanilides (Hamm *et al.*, 1974) and are characteristically a first step in microbial mediated degradation pathways of these herbicides (Figs 1.5.1, 1.5.2 and 1.5.3).

The cloning of a gene encoding acetochlor detoxification, from soil isolate A108 (resembling *Pseudomonas fluorescens*), is described here. The gene was isolated by virtue of its ability to provide the *E. coli* cells in which it was expressed with elevated levels of acetochlor resistance. Partial sequencing of the gene revealed it to be highly homologous with the gene encoding the dihydrolipoyl transacetylase component of the pyruvate dehydrogenase complexes of *Azotobacter vinlandii*, *E. coli*, and *Alcaligenes eutrophus*. Theories are proposed regarding the role this gene may play in the detoxification of acetochlor in recombinant *E. coli* cells.

5.2 Materials and Methods

5.2.1 Enzymes and Chemicals

RNAase A, lysozyme proteinase K, agarase, klenow, T4 DNA ligase, exonuclease III, S1 nuclease and all restriction enzymes where obtained from Boehringer Mannheim and where used according to the manufacturers' instructions. T7 promoter specific primers were either obtained from Boehringer Mannheim or synthesised using a Pharmacea DNA

synthesiser at the University of Natal, Durban medical school. dNTP's were obtained from Boehringer Mannheim. Redistilled nucleic acid grade phenol was obtained from Bethesda Research Laboratories. Both agarose and low melting point agarose (LMP agarose) were obtained from Bethesda Research Laboratories. Acrylamide and N-N'-methylenebisacrylamide were obtained from BDH. Molecular Weight Marker II (MWMII) (Hind III digested phage λ DNA), Molecular Weight Marker III (MWMIII) (Hind III and EcoRI digested phage λ DNA) and PFGE Marker II (*Saccharomyces cerevisiae* YPH 755 chromosomes) were all obtained from Boehringer Mannheim. Wild type phage λ DNA was obtained from Amersham International.

DNAase free RNAase A was prepared using the technique of either Sambrook *et al.* (1989) (used in the isolation of A108 genomic DNA and the preparation of A108 DNA for inversion field agarose gel electrophoresis) or Feliciello and Chinali (1993) (used during the isolation of plasmid DNA from *E. coli*). The method of Sambrook *et al.* (1989) was as follows: RNAase A dissolved at a concentration of 50 $mg.ml^{-1}$ in 10 mM Tris-Cl (pH7.5) and 15 mM NaCl was heated to 100°C for 15 minutes and allowed to cool slowly to room temperature. The method of Feliciello and Chinali (1993) was as follows: 15 mg RNAase A was dissolved in 9 ml sterile distilled water. After the addition of a 1 ml of 0.2 M HCl, 2 ml aliquots of the solution were distributed into sterile 5 ml disposable plastic test-tubes and incubated for five minutes in boiling water. Tubes were chilled on ice and the RNAase A solution was incrementally neutralised by adding both 0.2 ml of 0.2 M Tris-HCl (pH 7.6) and 0.1 ml of an 80 mM solution of NaOH every 2 minutes over a 10 minute period. Following the addition of every aliquot of the Tris-HCl and NaOH solutions tubes were inverted several times to mix their contents. The final RNAase A solution contained 10 $mg.ml^{-1}$ RNAase A, 15 mM NaCl and 15 mM Tris-HCl. The DNAase free RNAase A solutions produced by both methods were stored in 20 μl aliquots at -20°C.

The pH of phenol used in this study was raised to 7.8 following the method described by Sambrook *et al.* (1989).

The TaqTrack® Sequencing Systems kit from Promega was used for DNA sequencing.

5.2.2 Media and Growth Conditions

SOB and SOC media used in the transformation of *E. coli* were prepared as described by Hanahan (1983). SOB contained the following per litre of distilled water: 20 g Bacto Tryptone; 5 g yeast extract, 0.584 g NaCl, 0.186 g KCl; 2.033 g $MgCl_2.6H_2O$; 2.5 g $MgSO_4.7H_2O$. SOC comprised SOB together with 3.6 g Glucose per litre. SOD was made up in the same manner as SOB but without any magnesium salts and with tetracycline at a concentration of 10 $mg.l^{-1}$. Yeast extract-tryptone (YT) broth contained the following per litre of distilled water: 16 g bacto-tryptone; 10 g yeast extract; 5 g NaCl.

5.2.3 Agarose Gel Electrophoresis

Generally, unless DNA was to be isolated from gels, 1 % agarose gels were used with 0.5 X TBE (45 mM Tris-borate, 1 mM EDTA, pH 8) employed as the running buffer. If DNA embedded in agarose was to be cut out of a gel for later use, 1 % agarose and 0.5 X TBE were replaced by 1.1 % LMP agarose and TAE (40 mM Tris-acetate, 1 mM EDTA, pH 8) respectively. Power for running agarose gels was supplied by Hoefer Scientific Instruments PS500X or PS250 power-packs. Horizontal submarine mini-gel (Minnie the Gel-Cicle™ from Hoefer Scientific Instruments), midi-gel (Electro-4® from Hybaid) and custom built maxi-gel units were used. 20 μl volumes of DNA solutions (usually containing approximately 0.25 μg DNA) mixed with 2 μl of a loading buffer solution [2.5 % bromophenol blue, 40% (w/v) sucrose in distilled water], were used to load gel wells. Unless otherwise stated agarose gel electrophoresis was carried out under the following conditions: Room temperature using gels 6 mm thick at constant current and 4 $V.cm^{-1}$. The electrophoresis times varied. Following electrophoresis of a gel, nucleic acids which were not to be isolated from the gel were stained by immersion of the gel in a 50 $\mu g.ml^{-1}$ ethidium bromide (EtBr) solution for 10 minutes. Stained nucleic acids were visualised under long wavelength ultraviolet (UV) light.

The set up and use of an improvised inversion field agarose gel electrophoresis apparatus is described later in the text.

5.2.4 Analysis of Isolated DNA for Determining its Concentration and Purity

The concentration of a solution of isolated DNA in either TE buffer or distilled water was estimated by electrophoresing different concentrations of the solution beside a 0.25 μg quantity of bacteriophage λ DNA on an agarose gel for 30 minutes. Following staining with EtBr and visualisation under long wavelength UV the approximate amount of DNA in various lanes could be determined by comparing the brightness of stained DNA bands with that of the known quantity of λ DNA.

The purity of DNA solutions was determined by spectrophotometry. The ratio of the absorbance of a DNA solution between 260 nm (OD_{A260}) and 280 nm (OD_{A280}) was used as a general measure of DNA purity. DNA solutions which were found to have OD_{A260}/OD_{A280} values either larger than 2.0 or lower than 1.6 where not used. Solutions containing DNA isolated using organic solvents was tested for solvent contamination using a Beckman DU®640 spectrophotometer to scan the absorbance of the solution between 220 and 320 nm. An absorption peak at 270 nm was taken to be indicative of solvent contamination. DNA solutions contaminated with organic solvents were discarded.

5.2.5 Screening *E. coli* Strains for Resistance to Acetochlor

5.2.5.1 Sensitivity of XL1Blue and JM109 to acetochlor

A series of formulation acetochlor concentrations (0 $g.l^{-1}$, 4 $g.l^{-1}$, 8 $g.l^{-1}$, 16 $g.l^{-1}$ and 32 $g.l^{-1}$) in 5 ml of MS media (mineral salts medium described in section 2.2) was prepared in 25 ml Erlenmeyer flasks. The flasks were autoclaved after preparation at 121°C for 15 minutes. Ten single isolated colonies of XL1Blue or JM109 grown on NA (nutrient agar described in section 2.2) overnight were transferred, using a sterile inoculating loop, into 1 ml sterile MS medium. The MS medium and cells were mixed thoroughly by vortexing. The number of cfu's in the mixture was determined by dilution plating. Between 3.7 X 10^6 and 4.0 X 10^7 cfu's in 100 μl MS medium were transferred to each of the five flasks comprising the acetochlor concentration series. The flasks were reciprocally shaken at approximately 150 strokes per minute at 30°C for 7 days. At exactly 24 hour intervals the

number of cfus.ml^{-1} in each of the flasks was determined by dilution plating. The procedure was repeated 4 times for both JM109 and XL1Blue.

5.2.5.2 Selection of acetochlor resistant JM109 and XL1Blue strains

Between 3.5 X 10^5 and 5 X 10^6 JM109 or XL1Blue cells in MS medium (determined by dilution plating) were used to inoculate 5 ml MS medium containing formulation acetochlor at a concentration of either 32 g.l^{-1} (if selecting for acetochlor resistant JM109 cells) or 16 g.l^{-1} (if selecting for acetochlor resistant XL1Blue cells) in a 25 ml Erlenmeyer flask. Flasks were incubated at 30°C and shaken reciprocally at approximately 150 strokes per minute. Every 24 hours the number of cfus.ml^{-1} remaining in each flask was estimated using dilution plating. This was continued either until less than 10 cfus.ml^{-1} remained or until the number of cfus.ml^{-1} remaining increased or became constant. A single isolated colony of either one of the last ten surviving cfus.ml^{-1} or one of the cfus obtained from a stable or growing population of resistant cells, was selected and streaked onto the appropriate maintenance medium in order to obtain a pure culture.

5.2.5.3 Testing the resistance of recombinant *E. coli* XL1Blue strains to acetochlor

Six single isolated colonies of recombinant XL1Blue strains which had been grown on NA overnight were transferred, using a sterile inoculating loop, into 2 ml sterile MS medium. The MS medium and cells were mixed thoroughly. Between 10^6 and 10^7 recombinant XL1Blue cells in 100 μl were used to inoculate 5 ml MS medium containing either 16 or 32 g.l^{-1} formulation acetochlor in a 25 ml Erlenmeyer flask. The flask was incubated at 30°C and shaken reciprocally at approximately 150 strokes per minute. At exactly 24 hour intervals an estimate of the number of cfus.ml^{-1} remaining was obtained using dilution plating. This was continued for 4 days or until there was less than one viable cfu.ml^{-1} remaining in a flask. The procedure was repeated between 3 and 12 times. As a control XL1blue containing non-recombinant pUC18 was used treated in precisely the same manner test XL1Blue strains containing selected recombinant plasmids.

5.2.6 Use of Inversion Field Agarose Gel Electrophoresis (IFAGE) to Analyse the Genome of A108

The preparation of unsheared A108 genomic DNA was carried out using an adaptation of the method described by Grothues *et al.* (1990). A single A108 colony was used to inoculate 10 ml NB (nutrient broth described in section 2.2) containing acetochlor at a concentration of 0.4 $g.l^{-1}$. The culture was grown at 30°C with constant rotary shaking at 150 rpm until it reached an OD_{A600} of 0.9. Samples of 0.5 ml were transferred to sterile 1.5 ml microfuge tubes. The tubes were centrifuged at 11000 g for 30 seconds, the supernatant was discarded and the pelleted cells were washed twice with 1.5 ml SE (75 mM NaCl, 25 mM EDTA, pH 7.5) before resuspending them in a final volume of 0.5 ml SE. The tubes were warmed to 37°C in a water-bath and 0.5 ml of melted 1.5 % LMP agarose (cooled to 37°C) was added to each tube and mixed with the suspended cells. The contents of 5 of the tubes were poured into a sterile petri dish. The 5 ml of liquid in the petri dish was spread over the base of the dish to obtain a cell-SE-LMP agarose layer approximately 1 mm thick. The spread mixture was left to solidify for 30 minutes on a level surface at 4°C. As many approximately 1 cm^2 agarose blocks as possible were cut from the solidified LMP agar-SE-cell layer. These blocks were all cut in half (to obtain rectangular blocks approximately 1 cm X 0.5 cm) and individually transferred to sterile 1.5 ml microfuge tubes. The blocks were immersed in 0.2 ml ES [0.5 $mg.ml^{-1}$ proteinase K, 1% (w/v) N-lauroylsarcosine, 0.5 M EDTA, pH 9.5] and incubated at 56°C for 15-18 hours. ES was pipetted out of the microfuge tubes and the blocks were rinsed twice in 1 ml TE1 (10 mM tris, 10 mM EDTA, pH 7.5). The blocks were equilibrated for 8 hours in 1 ml TE1 (with one change of the TE1 after 4 hours). The agarose blocks were cut in half again (to obtain blocks 0.5 cm^2) and stored at 4°C for up to 3 weeks before being used.

Digestion of the unsheared genomic DNA within agarose blocks was carried out using the restriction enzymes BamHI, SmaI, KpnI, SacI, HaeII, PvuII, EcoRI, SfiI, HindIII, SwaI, PstI and NotI. Prior to the addition of restriction enzymes the two agarose blocks in each tube were equilibrated for 18 to 24 hours in 0.2 ml of the appropriate Boehringer Mannheim SuRE Cut restriction enzyme buffers. In all cases bovine serum albumin was

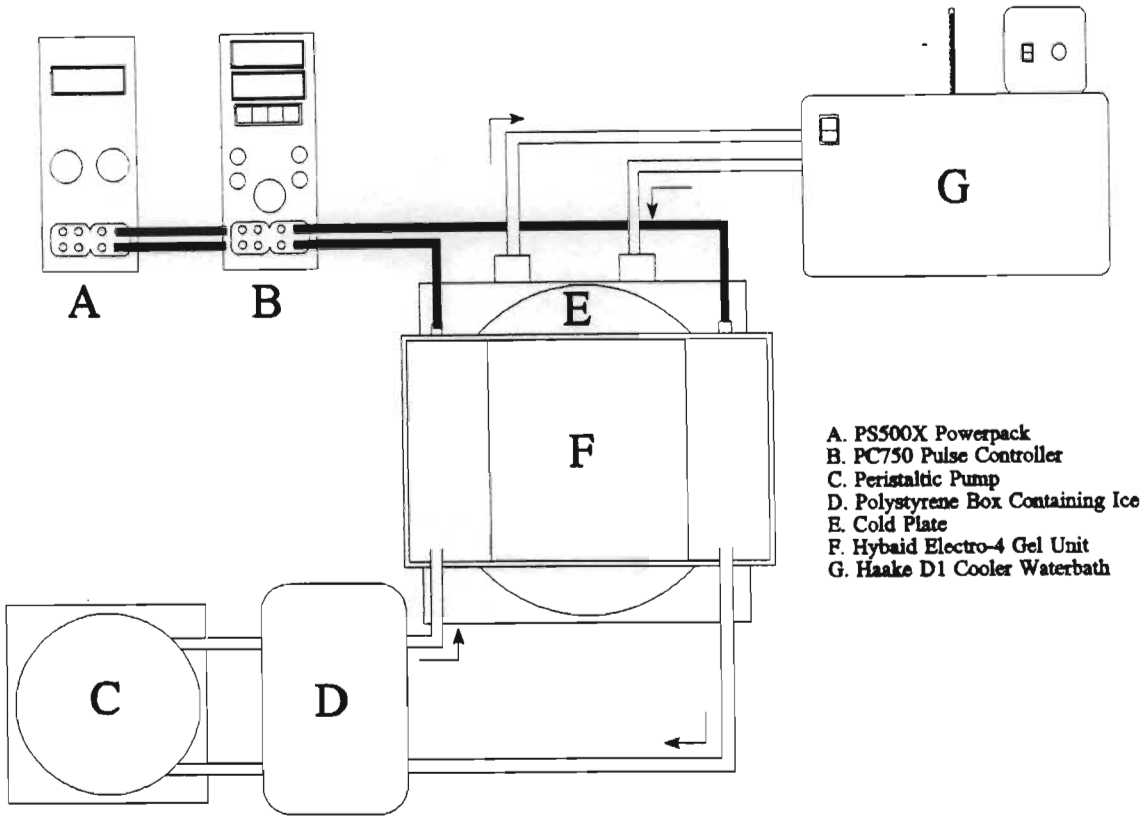

FIG. 5.1. Schematic representation of an improvised IFAGE apparatus. Arrows indicate the direction of water/buffer flow. Two mechanisms were employed to maintain the temperature of the electrophoresis buffer at 15°C: (1) Running Buffer in the gel unit (F) was circulated by a peristaltic pump (C) through crushed ice maintained in an ice bucket (D); (2) Cooled water was circulated between a cooler water bath (G) and a cold plate (E) upon which the gel unit (F) was placed. The diagram is not to scale.

added to the restriction enzyme reaction mixtures to give a final concentration of 0.2 $mg.ml^{-1}$. The equilibration buffer was changed once after 4 hours. 10 units of the appropriate restriction enzymes were added and incubated at the required temperature for 15 hours. The reaction was terminated by the addition of 1 ml TE1 buffer. Blocks were stored at 4°C until use.

Electrophoresis of the DNA within the agarose blocks was carried out using an improvised IFAGE apparatus (Fig. 5.1). One percent agarose gels 8.3 cm wide, 12.3 cm long and

0.6 cm thick were made using 0.5 X TBE. Wells with the capacity to contain previously prepared agarose blocks were 1.5 mm long and 6 mm wide. Agarose blocks (including ones containing the PFGE II marker) were trimmed to a size which permitted their insertion into the wells of prepared gels. Blocks were inserted into wells and fixed in position with 1% agarose. The following electrophoresis conditions were found to permit the separation of 17 out of the 18 bands in the PFGE II yeast chromosome marker: 66 V; 21 s initial forward pulse; 7 s initial reverse pulse; ramp factor of 2; 68 hour electrophoresis period; constant 16°C electrophoresis buffer temperature. Following IFAGE bands were visualised by staining gels with ethidium bromide.

5.2.7 Isolation of Total Genomic DNA From A108

A single A108 colony grown on MSA agar was used to inoculate 25 ml NB (in a 100ml Erlenmeyer flask) containing the herbicide at a concentration of 0.2 $g.l^{-1}$. The culture was grown at 30°C overnight with shaking at 150 rpm. Media and cells were transferred to a 50 ml polypropylene centrifuge tube and cells were pelleted at 4°C by centrifugation at 8000 rpm in a Beckman JA20 rotor. The supernatant was discarded and the cells were resuspended in 20 ml STE (0.01 M Tris, 0.001 M EDTA, 0.1 M NaCl, pH 7.5). The cells were washed a further two times with 20 ml STE before resuspending them in a 4.5 ml volume of the buffer. Twelve 375 μl aliquots were pipetted into 1.5 ml microfuge tubes. Ten μl of a 45 $mg.ml^{-1}$ lysozyme solution and 10 μl of a 40 $mg.ml^{-1}$ DNAase free RNAase A solution were added to each tube. The tubes were incubated for 25 minutes at 37°C. To each tube was added 375 μl STE containing 0.075 mg proteinase K. The tubes were incubated at 50°C for one hour. An equal volume (750 μl) of a phenol-chloroform mixture [comprising equilibrated phenol (pH 7.9), chloroform and isoamyl in a ratio of 25:24:1 respectively] was added to each tube. The two phases in each tube were gently mixed. To separate precipitated proteins and the aqueous and organic phases the tubes were centrifuged at 11000 g for 2 minutes. Due to the high viscosity of the aqueous phase, the organic and protein layers formed during centrifugation were removed from tubes before transferring the aqueous phase to a fresh tube. Phenol-chloroform extractions were repeated until no band of precipitated protein could be observed between the aqueous and organic phases following centrifugation. Following the final phenol-chloroform

extraction three extractions with water saturated ether were carried out. Ether dissolved in the aqueous phase after the last extraction was removed by incubating the tubes with their lids open at 50°C for 3 hours. DNA was precipitated overnight at - 20°C with a one tenth volume of 3M sodium acetate and a three quarter volume of isopropanol. Precipitated DNA was pelleted by centrifugation at 11000 g for 10 minutes. The supernatants were discarded and DNA pellets were washed with 70 % ethanol without dislodging them from the microfuge tube walls. As much of the 70% ethanol as possible was removed from the tubes before leaving the pellets to dry in a partial vacuum. The DNA pellets were each resuspended in 21 μl TE2 (0.01 M Tris-HCl, 0.001 M EDTA, pH 8). Samples of 1 μl from each tube were diluted 300 times in TE2 and examined for purity using spectrophotometry (section 5.2.4). Pure DNA solutions were bulked and the concentration of the bulked sample was determined (section 5.2.4).

5.2.8 Plasmid DNA Isolations

Three plasmid isolation procedures were employed at various stages and for various reasons during the present study. For the isolation from *E. coli* of pure pUC18, pBluescript and recombinant plasmid DNA, the modified alkaline lysis method of Feliciello and Chinali (1993) was employed. For the rapid isolation of moderately pure plasmid DNA a simplified alkaline lysis method was employed. One or more large plasmids from the soil isolate, A108, were isolated using enzymatic lysis of cells, phenol-chloroform extraction of proteins and separation of closed circular DNA from linear and nicked circular DNA on a caesium chloride density gradient.

5.2.8.1 Isolation of pure plasmid DNA from *E. coli*

The method employed in the isolation of pure plasmid DNA was that developed by Feliciello and Chinali (1993). A single plasmid containing *E. coli* colony was used to inoculate 25 ml LB-amp (described in section 2.2) in a 100 ml Erlenmeyer flask. The flask was shaken at 37°C overnight at 150 rpm. Media and cells were decanted into a 50 ml centrifuge tube and cells were pelleted by centrifugation at 10000 rpm in a Beckman JA20 rotor for 10 minutes at 4°C. The supernatant was discarded and cells were

resuspended in 2 ml ice cold STE. Samples of 1 ml were transferred to two 1.5 ml microfuge tubes. The tubes were centrifuged at 11000 g in a microfuge for 1 minute. Supernatants were discarded and the pellets resuspended in 250 μl ice cold GTE (0.05 M glucose, 0.01 M Tris-HCl, 0.001 M EDTA). 500 μl of a lysis solution (LS) (0.2 M NaOH, 1% SDS) was added to the tubes, the contents of which were then thoroughly but gently mixed until they cleared. Following storage of the tubes on ice for 5 minutes, 750 μl of an ice-cold neutralisation solution (NS) (4 M potassium acetate, 2 M acetate) was added to each tube. The contents of tubes were thoroughly but gently mixed until particles of the white precipitate that formed were no larger than approximately 1 mm^3. The tubes were then placed on ice for 10 minutes before centrifugation them at 11000 g for 2 minutes. The supernatant was carefully removed from each tube in two 700 μl aliquots which were decanted into two separate 1.5 ml microfuge tubes. To each of the tubes was added 500 μl isopropanol. Following centrifugation of the tubes for 10 minutes at 11000 g, supernatants were discarded. Tubes replaced in the rotor in their original orientations and they were respun at 11000 g for 5 seconds. Supernatants remaining at the bases of the tubes were removed using a micropipette fitted with a small bore sterile disposable tip. The pellets were resuspended in 250 μl TE2 (0.01 M Tris-Hcl, 0.001 M EDTA, pH8) containing 10 $\mu g.ml^{-1}$ DNAase free RNAase A and incubated at room temperature for 15 minutes. To each tube was added 300 μl of an 88% isopropanol-0.2 M potassium acetate solution. Tubes were then centrifuged at 11000 g for 5 minutes. Immediately following centrifugation supernatants were poured away. Tubes were briefly recentrifuged and residual supernatants were removed as described above. To dissolve the pure pelleted plasmid DNA, 20 μl TE2 was added to each tube. The purity of the DNA in each of the tubes was established (section 5.2.4). The pure DNA solutions were mixed with one another and the approximate concentration of the bulked solution was determined (section 5.2.4).

5.2.8.2 Rapid isolation of plasmids from *E. coli*

A simplified alkaline lysis method similar to that described by Feliciello and Chinali (1993) was used for rapid plasmid isolations. A single plasmid containing *E. coli* colony was used to inoculate 5 ml of LB-amp media in a 25 or 50 ml Erlenmeyer flask. The

flask was shaken at 150 rpm for between 5 and 15 hours at 37°C. Media and cells were poured from the flask into a 1.5 ml microfuge tube. Cells were pelleted by centrifugation at 11000 g for 1 minute. The supernatant was discarded and cells were resuspended in 500 μl ice cold STE. The tube were recentrifuged at 11000 g for 1 minute. The supernatant was discarded and the pellet was resuspended in 125 μl ice cold STE. Immediately after adding 250 μl of LS and thoroughly but gently mixing it with the contents of the tube, 375 μl of ice-cold NS was added to the tube. The contents of the tube were thoroughly but gently mixed and then placed on ice for between 5 and 10 minutes before centrifuging it at 11000 g for 2 minutes. The supernatant was carefully removed and decanted into a fresh tube. A half to equal volume of isopropanol was added and mixed thoroughly but gently with the supernatant in the fresh tube which was then centrifuged for 5 minutes at 11000 g. The supernatant was discarded and the pellet resuspended in 60 μl TE2. Immediately prior to loading a 10 μl sample of the DNA for electrophoresis, RNA contaminating the DNA solution was digested during a fifteen minute treatment at room temperature with DNAase free RNAase A. The RNAase A was added in a 1 μl volume to obtain at a final concentration of 10 $\mu g.ml^{-1}$.

5.2.8.3 Isolation of plasmid DNA from A108

A single A108 colony grown on MSA agar was used to inoculate 500 ml NB (in a 2 litre Erlenmeyer flask) containing the herbicide at a concentration of 0.2 $g.l^{-1}$. The culture was grown at 30°C overnight with shaking at 150 rpm. Media and cells were transferred to eight 50 ml polypropylene centrifuge tubes and cells were pelleted at 8000 rpm for 10 minutes at 4°C in a Beckman JA20 rotor. Supernatant was discarded and the cells in each tube were resuspended in 10 ml STE and bulked in two 50 ml polypropylene tubes (each containing 40 ml). Cells were pelleted at 4°C by centrifugation at 8000 rpm for 10 minutes in a Beckman JA20 rotor. Supernatants were discarded and the cells in each tube were resuspended in 5 ml STE. The cell suspensions were bulked in one tube. After adding 10 mg lysozyme to the tube it was incubated at 37°C for 30 minutes. Ten ml STE containing 1.5 mg proteinase K was added and the tube and it was incubated at 50°C for one hour. Ten ml STE containing 0.25 g CTAB was added to the tube. The mixture was incubated for 3 hours at 60°C with slight agitation in a reciprocal shaking water-bath. Two

phases, one a non-viscous aqueous phase and the other an apparently semi-solid cluster of cell debris, formed in the tube. The aqueous phase was transferred to a fresh sterile 50 ml polypropylene centrifuge tube. An equal volume of a phenol-chloroform mixture [containing equilibrated phenol (pH7.9),chloroform and isoamyl in the ratio 25:24:1 respectively] was added and the polar and non-polar phases in the tube were gently mixed. The tube was centrifuged at 11000 g for 2 minutes at 4°C in a Beckman JA20 rotor. The aqueous phase was transferred to a fresh tube with care being taken not to transfer any of the precipitated material in the process. Another two phenol-chloroform extractions were carried out. Removal of phenol and chloroform dissolved in the aqueous phase was carried out using ether extractions in the same manner as described for the isolation of total A108 genomic DNA (section 5.2.7). DNA was precipitated overnight at -20°C with a 0.1 volume of 3M sodium acetate and a 0.75 volume of isopropanol. Precipitated DNA was pelleted at 11000 g for 10 minutes at 4°C in a Beckman JA20 rotor. The supernatant was discarded and the DNA pellet was washed without dislodging it from the centrifuge tube wall with 70% ethanol. As much of the 70% ethanol as possible was removed from the tube before drying the pellet under vacuum. The DNA pellets were resuspended in 10 ml TE3 (0.01 M Tris, 0.001 M EDTA, pH 7.4).

Closed circular plasmid DNA was separated from linear DNA, nicked circular DNA, and RNA on a caesium chloride density gradient according to the method of Ausubel *et al.* (1992). Ten grams of CsCl were added to the Nucleic acid-TE3 mixture in the 50 ml polypropylene centrifuge tube. CsCl was dissolved by slow inversion of the centrifuge tube. A 1.6 ml volume of a 10 $mg.ml^{-1}$ ethidium bromide solution was added to and mixed with the contents of the tube by gentle inversion. The density of the mixture within the centrifuge tube was adjusted to 1.55 $g.ml^{-1}$ with the addition of distilled water. Triton X-100 was added to the tube to attain a final concentration of 1 % (v/v). This was to facilitate movement of RNA to the bottom of the CsCl gradient during ultracentrifugation. The contents of the tube were pipetted into a disposable 13.5 ml Quick-Seal® ultracentrifuge tube (from Beckman). The ultracentrifuge tube was balanced against another identical centrifuge tube containing a CsCl solution of the same density. The two tubes were sealed using a Beckman heat sealing device and then placed opposite one another in a Beckman NVT650 rotor. Spacers and plugs were put over the tubes and the

plugs were torqued to 13.6 Nm. The tubes were spun in a Beckman L80 ultracentrifuge at 65000 rpm for 4 hours at 20°C. The tube containing the plasmid DNA was removed from the ultracentrifuge and the positions of DNA and RNA bands on the CsCl gradient were visualised under long wavelength UV light.

5.2.9 Isolation of Single Stranded DNA for DNA sequencing

Single stranded DNA (ssDNA) for sequencing was isolated following instructions presented in the Bluescript Cloning Manual from Stratagene (1991). XL1Blue containing recombinant pBluescript SK+ was grown overnight at 37°C in 5 ml YT-tet-amp broth in a 25 ml Erlenmeyer flask with shaking at 150 rpm. From the overnight culture 50 μl of cells and media were transferred to 12 ml of fresh YT-tet-amp broth in a 50 ml Erlenmeyer flask. The fresh culture was grown to an OD_{A600} of 0.3. To the culture was added 10 μl of an R408 phage stock with a titre of 8 X 10^{10} pfu.ml^{-1}. The culture was incubated at 37°C with shaking at 150 rpm for a further 8 hours. The infected culture was transferred to a sterile 50 ml polypropylene centrifuge tube which was placed in a 65°C water-bath for 15 minutes. Cells and cell debris were harvested by centrifugation at 4°C in a Beckman JA20 rotor at 12000 rpm for 10 minutes. With care being taken not to contaminate it with the pelleted material, 1.2 ml aliquots of the supernatant was transferred to a 1.5 ml microfuge tubes. To each tube was added 300 μl of a 3.5 M sodium acetate-20 % (w/v) PEG solution. The contents of the tubes were thoroughly mixed and incubated at room temperature for 15 minutes. The precipitates which formed were pelleted by centrifugation at 11000 g for 10 minutes. The supernatants were discarded and the pellets were resuspended in 300 μl TE2 (0.01 M Tris-HCl, 0.001 M EDTA, pH 8). The mixture was extracted twice with 300 μl of a phenol-chloroform mixture [containing equilibrated phenol (pH7.9), chloroform and isoamyl in the ratio 25:24:1 respectively]. Following extractions with phenol-chloroform the aqueous phases were extracted twice with equal volumes of chloroform. An equal volume of a 7.5 M ammonium acetate solution and a double volume of ice cold ethanol was added to each tube. The tubes were stored for 15 minutes at -20°C before pelleting the ssDNA by centrifugation at 11000 g for 10 minutes. Following removal of supernatants DNA pellets were washed with 100 μl 70 % ethanol. Without removing pellets, ethanol was carefully pipetted from tubes. Traces of ethanol

were removed from tubes under a partial vacuum. Pellets were resuspended in 30 μl TE1 (0.01 M Tris, 0.01 M EDTA, pH 7.5) and approximate DNA concentrations and purities were determined (section 5.2.4).

5.2.10 Transformation of *E. coli*

Two different transformation techniques were employed during the course of the present study. While one of the procedures yielded a high frequency of transformation and was employed during the construction of a complete genomic library of A108 in pUC18, the other was a more rapid procedure employed during subcloning of an acetochlor resistance gene in pBluescript SK+ and pUC18.

5.2.10.1 High frequency of transformation

The method employed was that described by Inoue *et al.* (1990). Ten *E. coli* JM109 or XL1Blue colonies which had been grown overnight at 37°C on either LA (JM109) or LA-tet (XL1Blue) were transferred with a sterile inoculating loop to 250 ml of either SOB (JM109) or SOD (XL1Blue) in a 2 l Erlenmeyer flask. The flask was incubated at 18°C and shaken at 200 rpm until the culture reached an OD_{A600} of 0.6. The 250 ml culture was aseptically transferred to a sterile 500 ml Erlenmeyer flask which had been precooled on ice. The flask was kept on ice for 10 minutes before transferring four 40 ml aliquots to sterile 50 ml polypropylene centrifuge tubes. The tubes were centrifuged in a Beckman JA20 rotor at 3000 rpm for 10 minutes at 4°C. Each of the cell pellets thus obtained was resuspended in 12.8 ml ice cold TB (10 mM Pipes; 55 mM $MnCl_2$; 15 mM $CaCl^2$; 250 mM KCl; pH adjusted to 6.7 with KOH). The four cell suspensions were added to a single tube and left on ice for 10 minutes. Cells were repelleted in a Beckman JA20 rotor at 3000 rpm for 10 minutes at 4°C following which they were resuspended in 12.8 ml of ice cold TB. To the tubes was added 0.9 ml DMSO with gentle mixing to give a final concentration of 7 %. After incubating on ice for 10 minutes the cell suspension was aliquoted in 0.2 ml volumes into 1.5 ml microfuge tubes. The tubes were immersed and stored in liquid nitrogen for between 30 seconds and five days. Tubes were thawed at room temperature before placing them on ice for between five and sixty minutes. Plasmid

DNA in a 20 μl volume was added to each tube and the tubes were left on ice for between 30 minutes and 15 hours. The tubes were heat shocked at 42°C for exactly 30 seconds before returning them to storage on ice. Following the addition of 0.8 ml SOC to each tube, the tubes were incubated without agitation at 37°C for one hour. Cells from each tube were plated in two 490 μl and two 10 μl aliquots onto dry LA-amp (JM109) or LA-amp-tet (XL1Blue) plates. Cells were spread across the surface of plates until all liquid on the plates was absorbed. Two controls were run with each transformation experiment carried out. One of the controls consisted of a tube of competent cells which were treated in exactly the same manner as described above with the exception that no plasmid DNA was added to it. If colonies occurred on the LA-amp or LA-amp-tet plates inoculated with cells from this tube then the transformation experiment was abandoned. The second control involved the addition of 1 ng of pUC18 DNA (from a stock of pUC18 DNA of known concentration from Clontech) to the tubes instead of test DNA. This was used in order to obtain an estimate the efficiency of each transformation experiment.

5.2.10.2 Rapid transformation

The method employed was a modification of that described by Sambrook *et al.* (1989). The *E. coli* strains JM109 and XL1Blue were grown overnight in 25 ml of either NB (JM109) or NB-tet (XL1Blue) at 37°C with reciprocal shaking at approximately 150 strokes per minute. A 100 μl sample of the culture was used to inoculate 25 ml of either NB (JM109) or NB-tet (XL1Blue) which was then shaken at 200 rpm at 37°C until it reached an OD_{A600} of 0.6. Two 1 ml samples of the culture were aseptically transferred to two sterile 1.5 ml microfuge tubes. Cells were pelleted by centrifugation of the tubes at 11000 g for 30 seconds. Supernatants were discarded and the cells resuspended in 400 μl ice cold $MgCl_2$. The cells were again pelleted by centrifugation of the tubes for 1 min at 11000 g. Supernatants were discarded and pelleted cells were resuspended in 200 μl ice cold 0.1 M $CaCl_2$. The tubes were kept on ice for 30 minutes. Between 1 and 0.1 μg of plasmid DNA in 20 μl TE2 was added to one of the tubes. The tubes were left on ice for a further 30 minutes. The cells in the two tubes were heat shocked at 42°C for 30 seconds. Following the addition of 1 ml prewarmed NB (37°C) to each of the tubes they were incubated at 37°C for 1 hour. Samples of 500 μl, 50 μl and 5 μl from the tube to

which DNA was added were spread onto either pre-dried NA-amp (in transformation employing JM109) or pre-dried NA-amp-tet (in transformations employing XL1Blue) plates. From the tube to which no plasmid DNA was added a 500 μl sample was plated onto NA-amp. Plates were incubated overnight at 37°C. If no growth occurred on the NA-amp plate inoculated with untransformed cells then a single isolated colony from one of plates inoculated with cells exposed to the plasmid was picked and aseptically streaked onto a fresh NA-amp plate. If untransformed cells grew on the ampicillin containing plate the procedure was abandoned and restarted.

5.2.11 Construction of a Complete A108 Genomic Library

Two separate genomic libraries of A108 were constructed in pUC18 using the *E. coli* host strains XL1Blue and JM109.

5.2.11.1 Size fractionation of Sau3A digested A108 genomic DNA

Using the method described by Ausubel *et al.* (1992) a combination of restriction enzyme units and digestion periods yielding Sau3A restriction fragments between 1 and 4-Kb was determined. Approximately 7 μg pure A108 genomic DNA (OD_{A260}/OD_{A280} = 1.86) in 27 μl of 1X Boehringer Mannheim restriction buffer A was prewarmed to 37°C for ten minutes in a sterile 1.5 ml microfuge tube. Before adding Sau3A 3 μl of the DNA-restriction buffer mixture was withdrawn from the tube into a second fresh tube containing 17 μl of sterile distilled water. The tube was incubated at 65°C for 10 minutes before placing in an ice bath. 10, 1 or 0.2 units (all in a 1 μl volume) of Sau3A were added to and mixed with the contents of the first tube being maintained at 37°C. Six 3 μl samples were withdrawn from the tube at 20 minute intervals. Each of the samples was added to 17 μl of sterile distilled water in a sterile microfuge tube and incubated for 10 minutes at 65°C before being placed in an ice bath. The sets of Sau3A restriction fragments thus obtained were separated by agarose gel electrophoresis and examined for the presence of restriction fragments between 1 and 4-Kb in size.

Approximately 7 μg pure A108 genomic DNA (OD_{A260}/OD_{A280} = 1.86) in 27 μl 1X Boehringer Mannheim restriction buffer A was prewarmed to 37°C for ten minutes in a sterile 1.5 ml microfuge tube. To the DNA-restriction buffer solution was added 0.2 units Sau3A (in a 1 μl volume) diluted in 1X restriction buffer A. The DNA-restriction buffer-restriction enzyme solution was gently mixed. After 50 minutes digestion was stopped by incubating the tube at 65°C for ten minutes before transferring it to an ice bath. The digested DNA was divided into an 8 μl sample and a 20 μl sample. The 8 μl sample was made up to 20 μl with 1X restriction buffer A represented a diluted reference digest. The concentrated digest and the reference digest were loaded beside one another into the wells of a 1.1 % LMP agarose gel. Electrophoresis was carried out in either 0.5 X TBE (for the size fractionation of fragments used to construct an A108 library in JM109) or TAE (for the construction of a library in XL1Blue). Before staining of the DNA with EtBr the entire lane containing the concentrated Sau3A digested DNA (save a 1 mm strip immediately bordering the lane containing the reference DNA) was cut out of the gel. Following staining of the rest of the gel with ethidium bromide and visualisation of DNA under UV, it was determined whether the expected proportion of restriction fragments were between 1 and 4-Kb. The portion of the unstained lane containing fragments between 8 and 1-Kb (as indicated by the reference lane) was cut on a clean, sterile surface into 10 approximately 1 mm thick slices with a clean sterile scalpel blade. The top of the excised lane (the end containing the largest fragments) was cut first with subsequent slices being made progressively further down the lane. Immediately as the agarose slices were made they were transferred to marked, sterile microfuge tubes. The tubes containing the gel slices were stored at -20°C for up to 3 months before being used. While the agarose slices used to construct an incomplete A108 genomic library in JM109 contained partially Sau3A digested A108 DNA fragments between 1 and 3-Kb, agarose slices containing fragments between 3 and 5-Kb were used in the construction of a complete library in XL1Blue.

5.2.11.2 Insertion of A108 genomic DNA fragments into pUC18

Approximately 3 μg purified pUC18 DNA (OD_{A260}/OD_{A280} = 1.67) in 9.5 μl 1X Boehringer Mannheim restriction buffer H was digested to completion with 5 units SalI (in a 0.5 μl volume) for 3 hours. Linearised pUC18 was prepared for ligation with A108

DNA by partial filling in its ends. The partial end fill reaction mixture contained the following in a 20 μl reaction volume: 10 μl SalI digested pUC18-restriction buffer-restriction enzyme mixture; 1 μl of a solution containing both dTTP and dCTP, each at a concentration of 1 mM; 2.0 μl 10 X T4 DNA ligase buffer (from Boehringer Mannheim); 1.5 μl of a 2 unit.μl^{-1} solution of Klenow; 5.5 μl sterile distilled water. The mixture was incubated at room temperature for 20 minutes before incubating it at 75°C for 15 minutes. The partially end filled pUC18 DNA was stored at -20°C until used.

Size fractionated A108 Sau3A restriction fragments embedded in LMP agarose blocks were prepared for ligation with pUC18 by carrying out a similar partial end filling reaction. The agarose block containing the A108 DNA was completely melted at 65°C following which it was cooled to 37°C. The volume of the melted agarose thus obtained was between 40 and 60 μl. The partial end-fill reaction mixture contained the following in a 70 μl reaction volume: Between 40 and 60 μl Sau3A digested A108 DNA-melted LMP agarose mixture; 3.5 μl of a solution containing dATP and dGTP, each at a concentration of 1 mM; 7 μl of 10 X T4 DNA ligase buffer (from Boehringer Mannheim); 3.5 μl of a 2 unit.μl^{-1} solution of klenow; sterile distilled water to make the reaction volume up to 70 μl. The reaction mixture was incubated at 37°C for 1 hour before incubating it at 75°C for 10 minutes. The Sau3A and partially end filled A108 DNA fragments were stored in the partial end-fill reaction mixture at -20°C until they were used. During storage the LMP agarose reset and the mixture had to be heated to 65°C to melt the agarose in it so that the DNA in the mixture could be efficiently ligated with pUC18. The melted agarose-DNA mixture was cooled to 37°C before adding and mixing into it 1 μl (0.15 μg) of the linearised pUC18 DNA (with its ends partially filled in) and 5 Weiss units of T4 DNA ligase. As a control a ligation reaction was set up containing the following in a 70 μl reaction volume: 1 μl (0.15 μg) linearised and partially end-filled pUC18 DNA; 7 μl 10 X ligation buffer (from Boehringer Mannheim; 5 Weiss units of T4 DNA ligase; 60 μl molten LMP agarose (containing no DNA) cooled to 37°C. Ligations were left to proceed at 16°C for between 18 and 24 hours. The ligation mixtures were heated to 65°C until the LMP agarose in them melted. They were cooled to 45°C before adding 2.8 μl 25 X agarase buffer and 1 unit of agarase to each. The mixtures were incubated at 45°C for 2 hours following which they were stored at -20°C.

To approximate the proportion of recombinant and non-recombinant plasmids present in the test and control ligation mixtures 10 μl of each was used to transform *E. coli* JM109 using the rapid transformation procedure (section 5.2.10.2). The number of ampicillin resistant transformants containing plasmids from the control ligation was noted in order to obtain an approximation of the success of the pUC18 end-filling reaction. Using the rapid plasmid isolation procedure (section 5.2.8.2) plasmids were isolated from five of the transformants and examined for the presence of inserts using agarose gel electrophoresis. If none of the five transformants thus examined contained recombinant plasmid DNA then the ligation mixture was discarded.

For the construction of an A108 genomic DNA library 20 μl aliquots of ligation mixtures containing plasmid populations consisting of at least 20 % recombinants, were used to transform the *E. coli* strains, JM109 and XL1Blue, using the high frequency of transformation procedure (section 5.2.10.1). After 24 hours of growth the number of successfully transformed bacteria were carefully counted and the approximate number of these transformants containing recombinant plasmids was calculated. Plates containing transformants from a single transformation experiment were flooded with 2ml LB. Cells were suspended in the LB using a hockey-stick spreader. From each plate 1 ml of the transformant-LB mixture was withdrawn and bulked in a sterile plastic disposable test tube. The bulked transformant sample was decanted in 200 μl aliquots into sterile 1.5 ml microfuge tubes each containing 100 μl glycerol. The contents of the tubes were thoroughly mixed before being immersed in liquid nitrogen for 1 minute. Without allowing any time for them to thaw the tubes were transferred to a -80°C freezer for storage. A sample from the collection of transformant cells was diluted 100X with sterile MS medium and 100 μl of the diluted cells suspension (containing between 3.5 X 10^5 and 5 X 10^6 cfus) was screened immediately for the presence of acetochlor resistant recombinants (section 5.2.5.2).

5.2.12 Screening of Libraries for Herbicide Resistant Recombinants

The plasmids of apparently acetochlor resistant transformants selected from the A108 genomic libraries were isolated using the pure plasmid isolation procedure (section 5.2.8.1)

and examined for the presence of insert DNA. Non-recombinant plasmids were discarded. Plasmids containing insert DNA were used to transform *E. coli* XL1Blue using the rapid transformation procedure (section 5.2.10.2). Successfully transformed cells were screened between 3 and 15 times for the possession of elevated acetochlor resistance (section 5.2.10.2). A transformed XL1Blue strain containing a recombinant plasmid, designated pR5, which provided the strain with elevated acetochlor resistance was tested for an ability to detoxify acetochlor using the scaled down version of the quantitative bioassay (section 2.5.3). This assay was carried out in triplicate with both untransformed XL1Blue and uninoculated media as controls. An approximation was obtained of the proportion of acetochlor that the recombinant *E. coli* cells were able to detoxify over a 21 day period in NM.1.

5.2.13 Restriction Mapping of pR5, a Recombinant Plasmid Carrying a Gene Associated With Acetochlor Detoxification

A recombinant plasmid, pR5, which appeared to contain a gene encoding the detoxification of acetochlor was restriction mapped according to the method described by Ausubel *et al.* (1992). The restriction enzymes used were EcoRI, PstI, PvuII, SmaI, SphI, HindIII, SacI, XhoI, AvaI, ClaI, XbaI and SalI. Approximately 250 ng of plasmid DNA was digested for 3 hours with 10 units of these enzymes in 25 μl of the appropriate restriction buffers. Those enzymes which were found to cut the plasmid within the insert region were selected for use in restriction analysis of the insert fragment. Double digests were carried out with enzymes cutting more than once in the insert region of the plasmid. For double digests approximately 0.25 μg pR5 DNA was digested with 5 units (in a 0.5 μl volume) of each enzyme in 25 μl of an appropriate 1X Boehringer Mannheim SuRE Cut buffer (A buffer in which, according to Boehringer Mannheim, both enzymes would function at greater than 75% capacity). Restriction fragment sizes were determined relative to MWMII and MWMIII size standards from Boehringer Mannheim using the UVP 500 Imagestore and Gel Documentation systems.

5.2.14 Localisation of a Gene Associated With Acetochlor Detoxification on pR5

Using the restriction map of pR5 three deletions in the insert region of this plasmid were constructed. The resulting plasmids pR51, pR52 and pR53 contained the insert fragment of pR5 minus the 1460 bp EcoRI fragment, the 1265 and 640 bp PstI fragments and the 1450 bp SalI fragment respectively. pR5 was digested to completion with either EcoRI, PstI or SalI. The reaction mixtures contained the following: Approximately 0.5 μg pR5 DNA; 3 μl of the appropriate 10X restriction enzyme buffer; 10 units of the appropriate restriction enzyme; sterile distilled water to give a reaction volume of 30 μl. Following digestion 10 and 20 μl samples of each digestion mixture were loaded onto 1.1 % LMP agarose gels and fragments were separated by electrophoresis using TAE as a running buffer. After removing the lanes containing DNA from the 20 μl samples, the gels was stained with ethidium bromide and visualised under long wavelength UV light. The distance from the wells of the bands containing the 4440 bp (for the EcoRI digest), 4010 bp (for the PstI digest) and the 4450 bp (for the SalI digest) fragments were measured. The corresponding section of the unstained lanes containing DNA from the 20 μl samples were cut from the gels and transferred to sterile microfuge tubes. Care was taken to cut the bands from gels using a flame sterilised scalpel blade on a clean sterile surface. The removed agarose blocks were melted at 65°C. A 7 μl sample was withdrawn from each melted block and placed in a fresh sterile microfuge tube. To each of the tubes was added 10 μl sterile distilled water which had been warmed to 37°C. The tubes were placed in a 37°C water-bath. To each of the tubes was added 2 μl 10X T4 DNA ligase buffer and 1 unit of T4 DNA ligase (in 1 μl). The contents of the tubes were gently but thoroughly mixed before incubating them overnight at 15°C. The tubes were heated to 65°C until the LMP agarose in them melted. They were cooled to 45°C before adding 2.8 μl of 25 X agarase buffer and 1 unit of agarase to each. Following incubation for 1 hour the contents of each tube were used to transform *E. coli* XL1Blue using the rapid transformation procedure (section 5.2.10.2). The plasmids contained by two ampicillin resistant transformants from each transformation experiment were isolated using the pure plasmid isolation procedure (section 5.2.8.1). The success of the construction procedures were assessed by digesting each plasmid with the specific restriction enzyme used during its

construction from pR5. Transformants containing the desired plasmid constructs were screened for resistance to acetochlor (section 5.2.5.3).

A fourth plasmid, pR511, was constructed by deleting the 650-Kb PstI fragment of pR51 using the techniques described above. Transformants containing this plasmid were also screened for resistance to acetochlor (section 5.2.5.3).

5.2.15 Construction of a Deletion Series

The insert DNA in pR51 was cloned into pBluescript SK+. Approximately 1 μg of pR51 and pBluescript SK+ were digested for 3 hours with HindIII and EcoRI. The restriction fragments were separated by electrophoresis on a 1.1 % LMP agarose gel using TAE as a running buffer. Following staining of the gel with ethidium bromide two bands, one containing linearised pBluescript and the other containing the insert DNA fragment from pR51, were removed from the gel using a flame sterilised scalpel blade and transferred to a sterile microfuge tube. The agarose blocks containing the linearised pBluescript and insert DNA were first melted at 65°C for 10 minutes and then cooled to and maintained at 37°C. pBluescript and insert DNA were ligated in the following reaction mixture: 16 μl melted LMP agarose containing pR5 insert DNA; 1 μl melted LMP agarose containing pBluescript; 2 μl 10 X T4 DNA ligation buffer; 1 unit T4 DNA ligase (in a 1 μl volume). Before the agarose solidified the ligation mixture was gently but thoroughly mixed. Ligation was left to occur overnight at 16°C. The ligation mixture was heated to 65°C until the LMP agarose in it melted. It was cooled to 45°C before adding 3 μl sterile distilled water, 1 μl 25 X agarase buffer and 1 unit of agarase. The mixture was incubated at 45°C for 2 hours following which it was used to transform *E. coli* JM109 (section 5.2.10.2). The plasmid DNA from 3 ampicillin resistant transformant colonies was isolated (section 5.2.8.1), digested to completion with PstI, and separated on an agarose gel. The PstI banding pattern was used to confirm whether the insert DNA from pR51 had been successfully inserted into pBluescript in the expected orientation. The resulting recombinant plasmid was designated pR7.

A nested deletion series was produced in pR7 using the method described by Ausubel *et al.* (1992). Approximately 7 μg of pure pR7 DNA (OD_{A260}/OD_{A280} = 1.76) was isolated from JM109 (section 5.2.8.1). Care was taken during the isolation procedure to avoid excessive agitation of DNA suspensions in order to ensure that a large proportion of the plasmid DNA obtained was in the closed circular state. The plasmid was linearised by double digestion for 3.5 hours with KpnI (giving rise to a 3′, 4 base pair overhang) and HindIII (giving rise to a 5′, 4 base pair overhang). HindIII cut the plasmid 36 base pairs from the KpnI site between the KpnI site and the insert region. The digestion mixtures contained the following in a 50 μl volume: 5 μl 10 X Boehringer Mannheim's SuRE Cut restriction buffer A; 10 units of both KpnI and HindIII (each in a 1 μl volume); approximately 7 μg plasmid DNA in 42 μl distilled water. Following digestion for 3.5 hours at 37°C the restriction enzymes were precipitated by extracting the digestion mixtures with 50 μl of a phenol-chloroform mixture [containing equilibrated phenol (pH 7.9), chloroform and isoamyl in the ratio 25:24:1 respectively]. The aqueous phase was transferred to a fresh tube. Following the addition of 5 μl 3M sodium acetate and 100 μl ethanol the linearised plasmid DNA was precipitated overnight at -20°C. The plasmid DNA was pelleted by centrifugation at 11000 g for five minutes. The pelleted DNA was washed with 70 % ice cold ethanol and dried on a laminar flow bench. The dried pellet was resuspended in 74 μl exonuclease III buffer (15 mM Tris.Cl, 0.66 mM $MgCl_2$, pH 8.0). Approximately 3.75 μg linearised plasmid DNA in 37 μl exonuclease III buffer was incubated for 2 minutes at 37°C before adding 150 units of exonuclease III. Continuing the 37°C incubation, twelve 3 μl aliquots were removed at 1 minute intervals. Each removed sample was immediately placed at -20°C for five minutes before adding 3 μl water and heating to 70°C for 10 minutes to inactivate the exonuclease III. Samples were kept on ice until they were mixed with 15 μl of S1 nuclease buffer (400 mM NaCl, 16 mM sodium acetate, 1.6 mM $ZnSO_4$, 8% glycerol, pH 4.6) and 4 μl (4 units) of S1 nuclease. Digestion with S1 nuclease was left to occur for 20 minutes at room temperature. S1 nuclease digestion was stopped by the addition of 5 μl of S1 nuclease stop buffer (0.8 M Tris-HCl, 20 mM EDTA, 80 mM $MgCl_2$, pH 8.0). Using TAE as a running buffer an 8 μl aliquot of each sample was electrophoresed on a 1.1 % LMP agarose gel. Ethidium bromide was included in the running buffer at a concentration of 0.5 $mg.l^{-1}$. Following electrophoretic separation agarose blocks containing desirably sized

linearised exonuclease III digested DNA were cut from the gel using a flame sterilised scalpel blade and transferred to marked sterile microfuge tubes. The agarose in the tubes was melted by heating the tubes to 65°C for 15 minutes. Twenty microlitre samples of the melted agarose blocks were transferred to fresh sterile tubes and cooled to 37°C. To each tube was added 2.5 μl 10 X T4 DNA ligase, 1 μl of mixture of dATP, dGTP, dTTP and dCTP (each at a concentration of 1 mM), and 2 units Klenow (in a 1 μl volume). The mixtures was incubated at 37°C for 1 hour before heating to 75°C for 10 minutes. Following cooling to 37°C, 1 unit of T4 DNA ligase (in a 1 μl volume) was added to and mixed with the contents of each tube. Ligations were left to proceed at 16°C overnight. The ligation mixtures were heated to 65°C until the LMP agarose in them melted. They were cooled to 45°C before adding 1 μl of 25 X agarase buffer and 1 unit of agarase to each. The mixtures were incubated at 45°C for 2 hours following which 10 μl of each was used to transform *E. coli* XL1Blue (section 5.2.10.2). Four colonies were picked from each plate inoculated with cells transformed with DNA from the different ligation mixtures. The plasmids of these different transformants were isolated (section 5.2.8.2) and examined to determine their size relative to non-recombinant pBluescript. From all the plasmids examined a series of 8 clones, designated pR71, pR72, pR73, pR74, pR75, pR76 and pR77, was selected with deletions sequentially spanning the entire 1.7-Kb insert region of pR7. The clones differed in size from one another by at least 100 base pairs.

5.2.16 Partial Sequencing of a Gene Associated With Acetochlor Detoxification

Sequencing was carried out with both single stranded DNA (ssDNA) and denatured double stranded DNA (dsDNA) templates using an adaptation of the dideoxy method of DNA sequencing developed by Sanger *et al.* (1977) which is described in the TaqTrack• Sequencing Systems manual from Promega (1993). Single stranded recombinant pBluescript SK+ DNA (ssDNA) templates were isolated in the manner described in section 5.2.9. Double stranded recombinant pUC18 and pBluescript SK+ DNA isolated by the pure plasmid isolation procedure (section 5.2.8.1) was prepared for sequencing using alkaline denaturation. Approximately 8 μg of pure closed circular plasmid DNA (OD_{A260}/OD_{A280} between 1.65 and 1.84) in 18 μl sterile distilled water was transferred to a microfuge tube. To the tube was added 2 μl of a solution containing 2M NaOH and 2

mM EDTA. The mixture was incubated for five minutes at room temperature. The reaction mixture was neutralised with the addition of 8 μl of a 5 M ammonium acetate solution (pH 7.5). Denatured DNA was precipitated with the addition of 112 μl of 100% ethanol followed by centrifugation for ten minutes at 11000 g. The pellet of denatured DNA was washed with 1 ml 70% ethanol and dried on a laminar flow bench. The dried pellet was resuspended in 18 μl sterile distilled water.

Primers used during sequencing were either the forward and reverse M13/pUC universal primers (for sequencing with recombinant pUC18) or the T7 promoter specific primer (for sequencing with recombinant pBluescript). ssDNA and denatured dsDNA templates and primer were annealed in a sterile 1.5 ml microfuge tube containing a reaction mixture comprising the following: Either 2 μg ssDNA or 4 μg denatured dsDNA template; 13 ng primer; 5.0 μl 5X *Taq* DNA polymerase buffer; 2.0 μl Extension/Labelling Mix; sterile distilled water to bring the reaction volume up to 25 μl. The reaction mixture was incubated at 37°C for 10 minutes. 0.5 μl of [α-^{35}S]dATP (1,000 Ci.mM^{-1} or approximately 10 μCi.μl^{-1}) was added to the annealed primer/template mixture. Five units of Sequencing Grade *Taq* DNA Polymerase (in a 1 μl volume) was added and thoroughly but gently mixed with the contents of the tube. The mixture was incubated at 37°C for between 2 and 5 minutes. During incubation of the extension/labelling reaction 1 μl of d/ddATP Mix, d/ddCTP Mix, d/ddGTP Mix and d/ddTTP Mix were added to four separate labled sterile 1.5 ml microfuge tubes. Upon completion of the extension/labelling reaction, 6 μl aliquots of the reaction mixture were pipetted into each of the four d/ddNTP containing microfuge tubes and the contents of the tubes were mixed by gently pipetting them up and down. The four tubes were incubated at 70°C for 15 minutes. Following incubation 4 μl of Stop Solution was added to and gently mixed with the contents of each tube. If not used immediately reaction mixtures were stored overnight at -20°C. Immediately prior to loading on a sequencing gel the reaction mixtures were heated to 70°C for 5 minutes. Sequencing gels were 6% polyacrylamide and were run using 1 X TBE as a running buffer at a constant power of 130W. Both the Poker Face™ SE1500 sequencing gel apparatus and the PS2500 power-pack used for running sequencing gels were from Hoefer Scientific Instruments.

Analysis of sequencing data was carried out using the DNA Strider™ 1.2 software package. Nucleotide and amino acid sequence comparisons were made with sequences stored in the GenBank data base using BLAST (Basic Local Alignment Search Tool; Altschul *et al.*, 1990).

5.3 Results

5.3.1 Screening *E. coli* Strains for Resistance to Acetochlor

In order to detect the expression of a foreign acetochlor detoxifying gene in *E. coli* cells containing recombinant plasmids, a method was required for screening mixed populations of transformed cells for increased acetochlor resistance. However, a combination of acetochlor's limited solubility in water (0.223 $g.l^{-1}$ at room temperature; Worthing and Hance, 1991) and its relative non-toxicity to the *E. coli* strains used, created problems when attempting to formulate a method for detecting herbicide resistant *E. coli* cells. It was found that technical grade acetochlor at a concentration of 0.3 $g.l^{-1}$ in MS medium, NM, NA, NB and M9 was non-toxic to the *E. coli* strains JM109 and XL1Blue. Even with constant agitation technical grade acetochlor added to media at concentrations higher than 0.3 $g.l^{-1}$ would neither fully dissolve nor form a stable emulsion. Acetochlor in formulation (Harness®) was found to form a relatively stable emulsion when added to liquid and solid media at concentrations up to 32 $g.l^{-1}$. Formulation acetochlor was, therefore, used instead of technical grade acetochlor in the screening of transformants for elevated acetochlor resistance. While none of the acetochlor concentrations tested (up to 32 $g.l^{-1}$) prevented growth of *E. coli* on NM, NA or M9 agar, survival of *E. coli* cells in liquid MS medium was influenced by the herbicide at concentrations as low as 4 $g.l^{-1}$. A controlled means of using acetochlor to kill *E. coli* cells in MS medium was therefore developed for use in both the selection of individual herbicide resistant cells from heterogenous transformant populations and the detection of herbicide resistance conferred by certain selected recombinant plasmids.

JM109 was found to be significantly more resistant to acetochlor than was XL1Blue (Figs 5.2.1 and 5.2.2). While acetochlor at a concentration of 32 $g.l^{-1}$ in MS medium was

required to kill between 7 X 10^5 and 8 X 10^6 JM109 cells in under seven days, a concentration of 16 g.l^{-1} in MS medium killed a comparable number of XL1Blue cells over the same time period. Therefore, when selecting for acetochlor resistant transformants from the A108 genomic libraries constructed in JM109 and XL1Blue, acetochlor concentrations used were 32 and 16 g.l^{-1} respectively. Because of the higher sensitivity of XL1Blue to acetochlor, plasmids isolated from selected herbicide resistant transformants were retransformed into this strain in order to test whether they contained an acetochlor resistance gene.

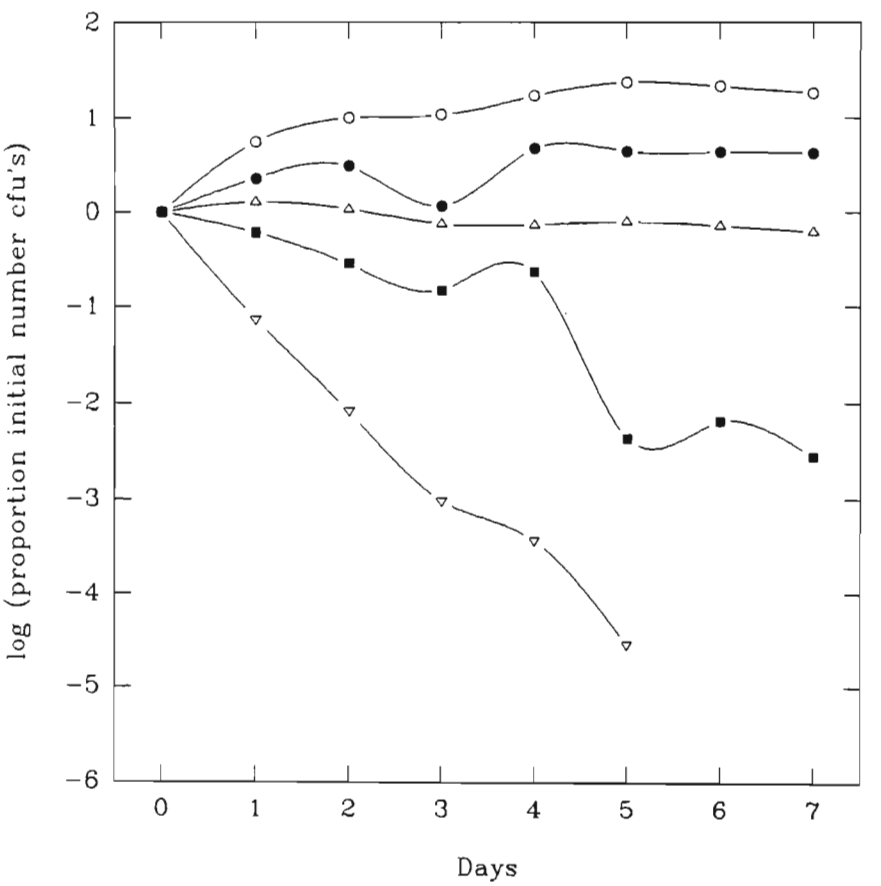

FIG 5.2.1. The effect of different formulation acetochlor concentrations on the survival of *E. coli* JM109 in MS medium. (○) No acetochlor; (●) 4 g.l^{-1}; (△) 8 g.l^{-1}; (■) 16 g.l^{-1}; (▽) 32 g.l^{-1}.

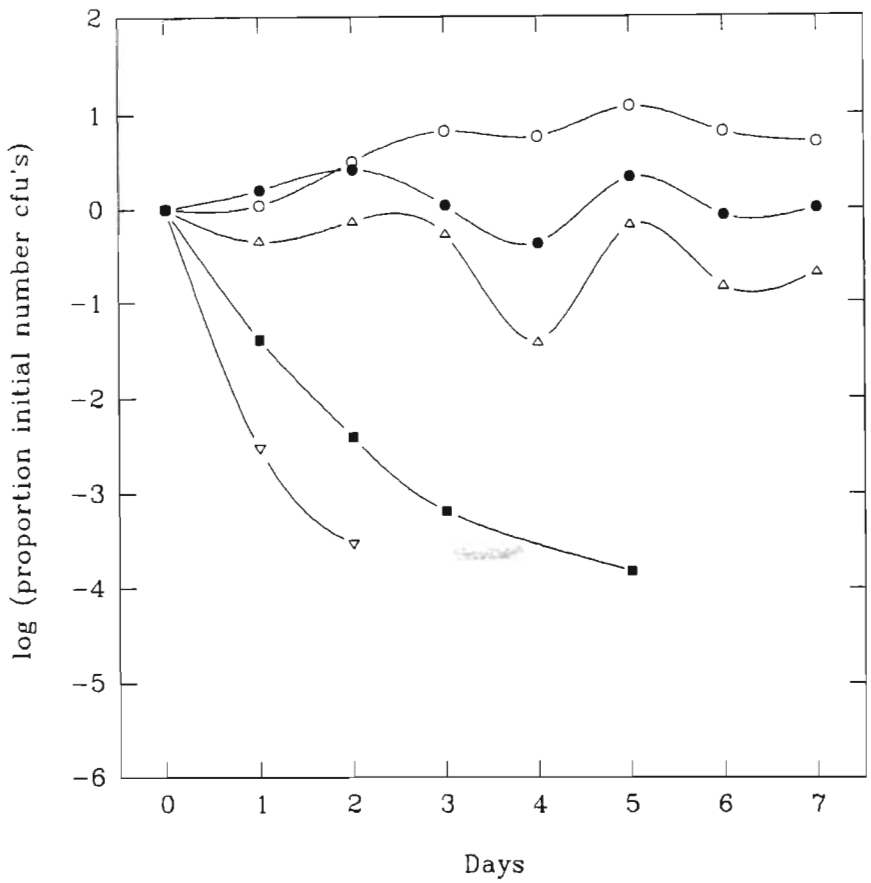

FIG 5.2.2. The effect of different formulation acetochlor concentrations on the survival of *E. coli* XL1Blue in MS medium. (○) No acetochlor; (●) 4 $g.l^{-1}$; (△) 8 $g.l^{-1}$; (■) 16 $g.l^{-1}$; (▽) 32 $g.l^{-1}$.

5.3.2 Use of IFAGE to analyse the genome of A108

A set of IFAGE parameters was developed which permitted the distinct electrophoretic separation of all but the largest of the 18 yeast chromosome and chromosome fragments comprising the PFGE Marker II. The largest fragment in the PFGE Marker II (2200-Kb) could not be made to migrate from gel wells during IFAGE.

IFAGE revealed the presence of what appeared to be one or more extrachromosomal DNA elements in A108 (Fig 5.3). The presence of one or more plasmids in A108 was verified using caesium chloride gradient centrifugation of isolated A108 genomic DNA. According to Sambrook *et al.* (1989) the banding pattern observed upon visualisation of the gradient under long wavelength UV light (Fig. 5.4) did indicate the presence of what was probably closed circular plasmid DNA.

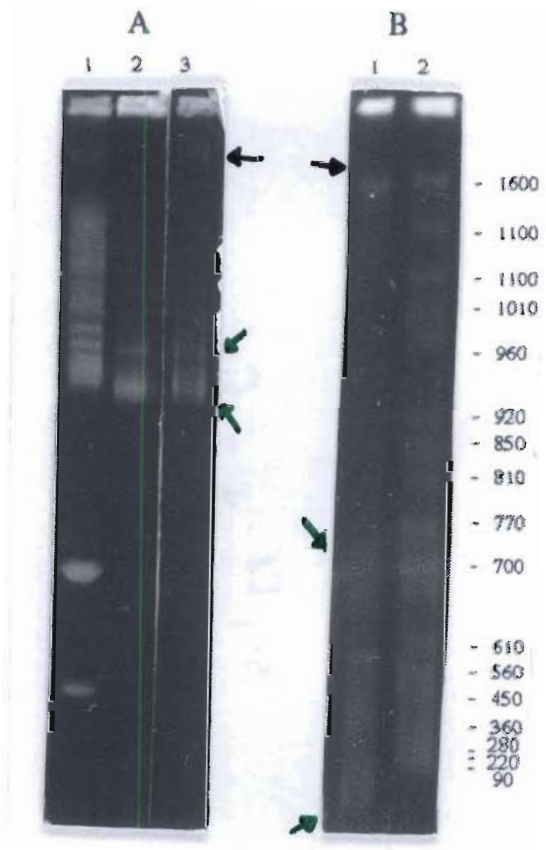

FIG 5.3. Use of IFAGE to analyse the genome of A108. Gel A: lane 1, *S. cerevisiae* YPH 755 chromosomes and chromosome fragments (PFGE marker II); lane 2, undigested A108 genomic DNA; lane 3, KpnI digested A108 genomic DNA. Electrophoresis conditions were as follows: 30 s initial forward pulse; 10 s initial reverse pulse; ramp factor of 0.3; 28 hour electrophoresis period. Gel B: Lane 1, KpnI digested A108 DNA; lane 2, *S. cerevisiae* YPH 755 chromosomes and chromosome fragments (PFGE marker II). Electrophoresis conditions: 21 s initial forward pulse; 7 s initial reverse pulse; ramp factor of 2; 68 hour electrophoresis period. Black arrows indicate the single observable 1.45-Mb restriction fragment obtained following digestion of A108 DNA with KpnI. The bands produced by the one or more large plasmids possessed by A108 are occupy the areas between the green arrows.

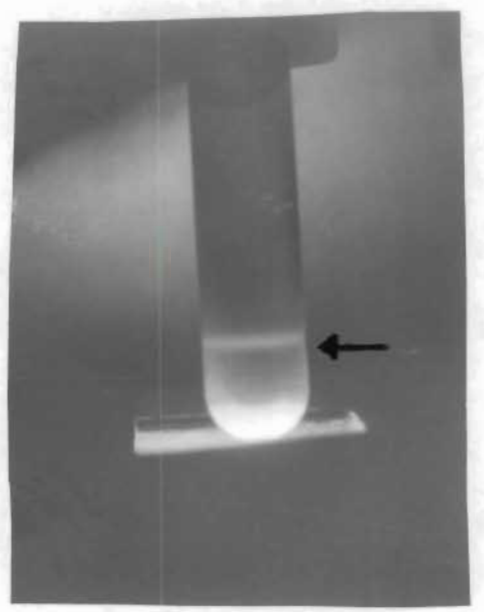

FIG 5.4. The separation of A108 genomic DNA on a caesium chloride density gradient. The arrow indicates closed circular plasmid DNA. Two other bands which are not visible in the photograph were observed above the band containing the supercoiled DNA.

IFAGE of high molecular weight A108 genomic DNA digested with different restriction enzymes revealed that of all the enzymes tested only digestion with KpnI yielded high molecular weight [in the megabase (Mb) size range] restriction fragments. Digestion with KpnI produced at least one high molecular weight restriction fragment approximately 1.45-Mb in size (Fig 5.3). Because the migration and separation of DNA fragments larger than 2-Mb could not be achieved, it could not be determined whether KpnI digestion of A108 DNA produced any larger restriction fragments than the one observed. It was therefore not possible to obtain an estimate of the genome size of A108 using IFAGE.

5.3.3 Construction of a complete A108 Genomic DNA Library

Digestion of 7 μg A108 DNA with 0.2 units of Sau3A for 50 minutes was found to produced a high proportion of restriction fragments between 2 and 5-Kb in size (Fig. 5.5). It was observed that when TAE was used instead of 0.5 X TBE as a running buffer during electrophoretic size fractionation of partially Sau3A digested A108 DNA, the number of recombinant transformants yielded by the cloning procedure increased approximately ten-fold. While electrophoresis using 0.5 X TBE as a running buffer was used to size fractionate the A108 fragments during construction of an A108 genomic library in JM109, TAE was used as the running buffer to size fractionate fragments when constructing a similar library in XL1Blue. This procedural difference meant that while 4 repeats of the cloning process were required to obtain 4340 JM109 transformants, a single repeat of the process yielded 13600 transformed XL1Blue cells.

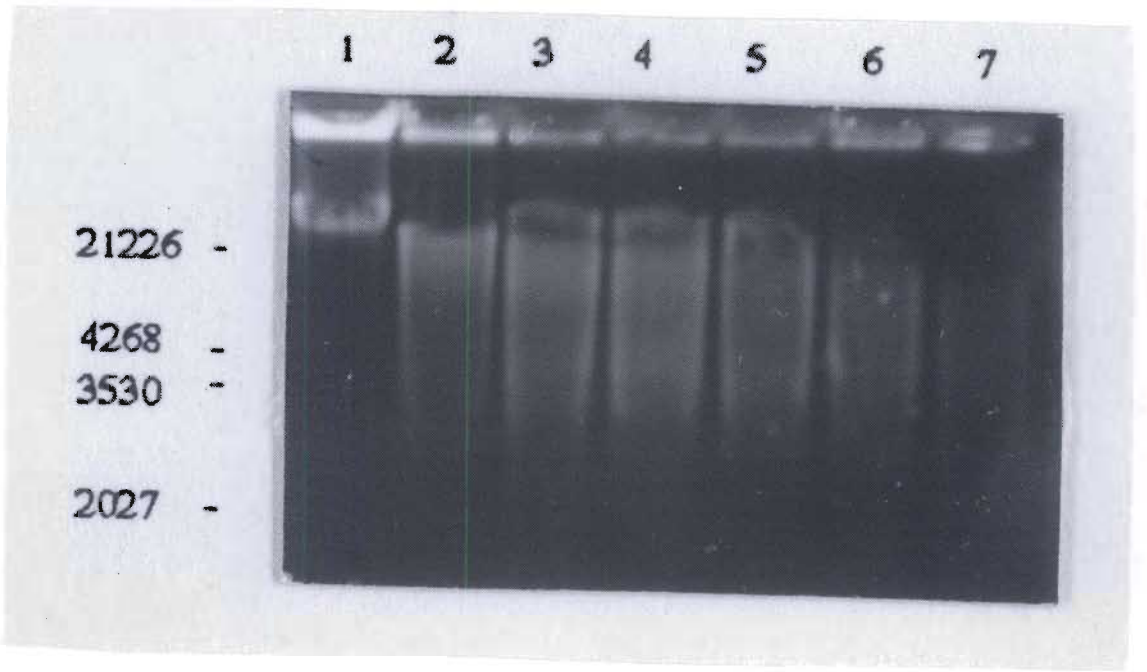

FIG 5.5. Analysis of timed digestion of A108 DNA with Sau3A by agarose gel electrophoresis. Lane 1, undigested A108 genomic DNA; lanes 2-7 A108 DNA digested with 0.2 units of Sau3A for 10, 20, 30, 40, 50 and 60 minutes respectively.

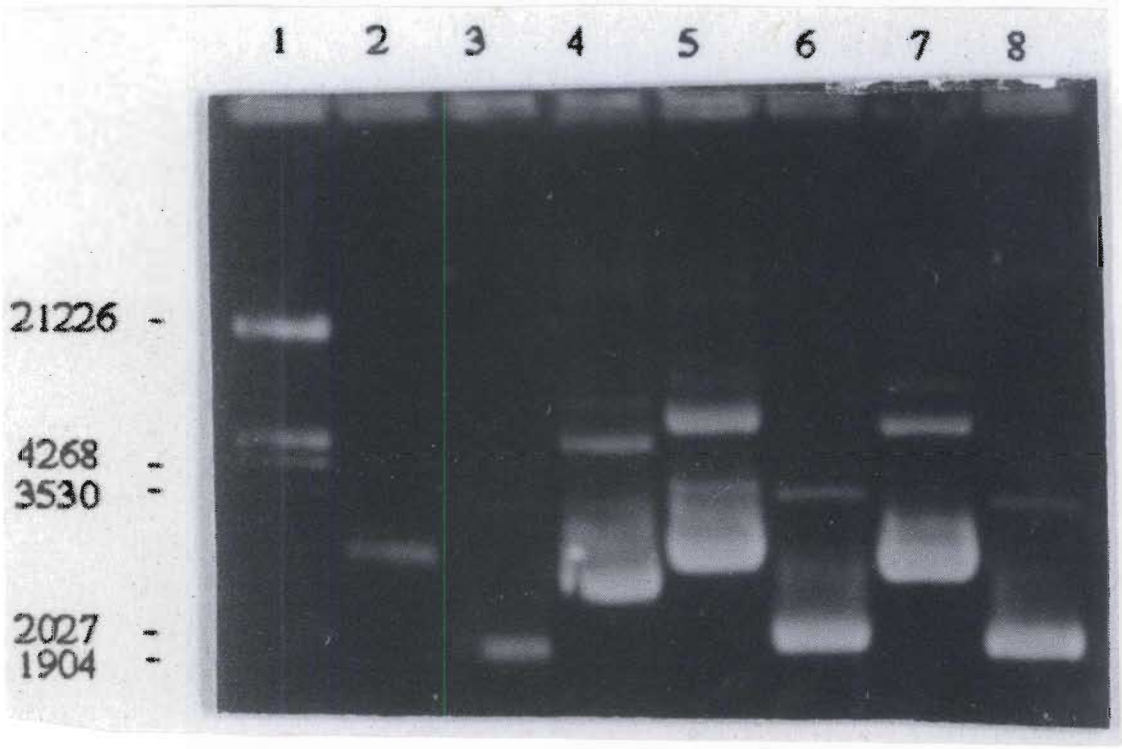

FIG 5.6. An example of how, in the present study the proportion of recombinant transformants in transformant populations was determined by agarose gel electrophoresis. Lane 1, EcoRI-HindIII digested λ DNA fragments (MWMIII); lane 2, SalI digested (linearised) pUC18 DNA containing no insert; lane 3, undigested pUC18 DNA containing no insert; lane 4-8, plasmids isolated (using the rapid plasmid isolation procedure) from randomly picked transformed JM109 cells containing either no inserts (lanes 6 and 8) or inserts 1 to 2-Kb in size (lanes 4, 5 and 7).

Using electrophoresis to examine plasmids isolated from randomly sampled transformants from both libraries (Fig 5.6), it was noted that between 60 and 80 % of successfully transformed JM109 cells and more than 80 % of successfully transformed XL1Blue cells possessed recombinant plasmids.

The probability (P) that any one portion of the genome of A108 would be represented in the two constructed libraries, was calculated. The value of P was dependent on the following variables: the genome size of A108 (G), the average size of A108 genomic DNA

fragments incorporated into recombinant plasmids (I) and the number of different recombinant plasmids present in the library (N) (Ausubel *et al.*, 1992). These variables were used to calculate the completeness of the A108 genomic libraries constructed in JM109 and XL1Blue using the following equation:

$$P = 1 - [1 - (I \div G)]^N \qquad (21)$$

There was a strong possibility that A108 was a *Pseudomonas* species. The genome sizes thus far determined for various members of the Genus *Pseudomonas* have been between 2.2 and 4.16-Mb (Grothues and Tummler, 1987; Park and DeLey, 1967; Pemberton, 1974). The maximum likely value of G for A108 was therefore taken to be 4.16×10^6 base pairs. The minimum likely values of N for the two libraries constructed using JM109 and XL1Blue as host strains were approximately 3218 and 10880 respectively. The values of I for the two libraries were approximately 1500 for the library constructed in JM109 and 3500 for the library constructed in XL1Blue. Using equation (21) the value of P for the library constructed in JM109 was 68.7 %, while for the library constructed in XL1Blue it was greater than 99.9 %.

5.3.4 Screening of Libraries for Herbicide Resistant Recombinants

While four acetochlor resistant transformants were selected from the A108 genomic library constructed in JM109 (one for each of the four repeats of the cloning procedure required to obtain the library), only one was selected from the library in XL1Blue. The plasmids isolated from these transformants were designated pR1, pR2, pR3, pR4 and pR5 (pR5 being the plasmid isolated from the library in XL1Blue). Of these plasmids only pR4 was found to contain no insert. Respectively pR1, pR2, pR3 and pR5 contained inserts approximately 1.2, 1.1, 0.8, and 3.2-Kb in size. All of these plasmids and non-recombinant pUC18 were used to transform XL1Blue. Testing of XL1Blue transformed with these plasmids revealed that only pR5 supplied the *E. coli* strain with elevated resistance to acetochlor (Fig. 5.7).

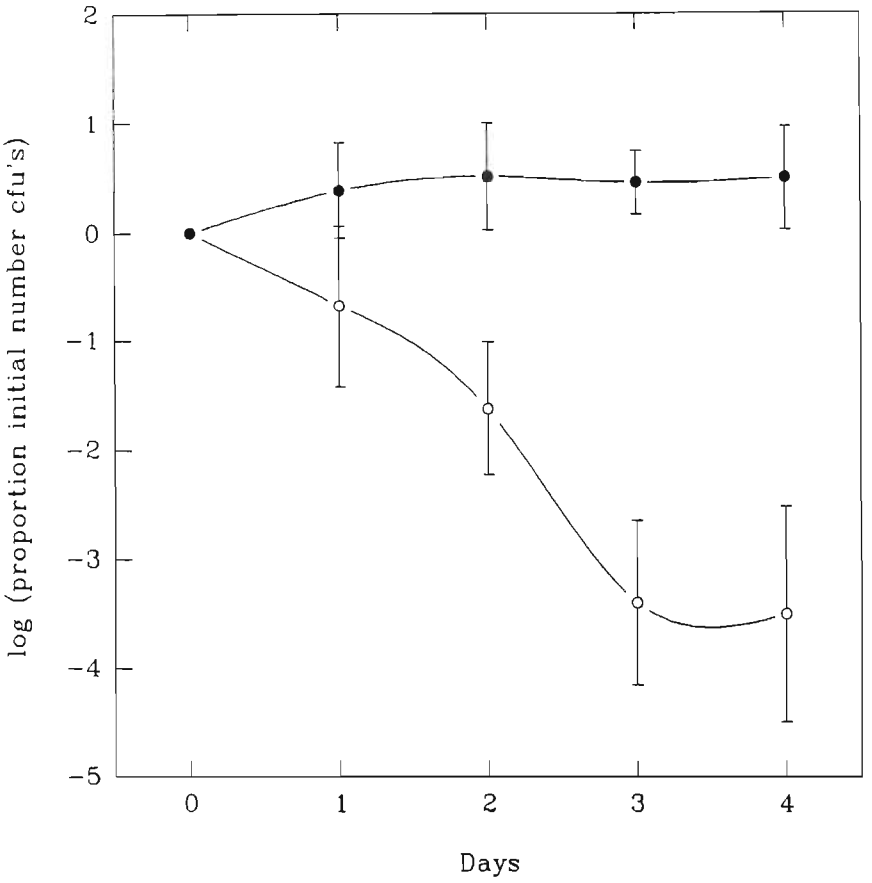

FIG 5.7. Increased resistance to 16 $g.l^{-1}$ acetochlor in MS medium amoung XL1Blue cells containing pR5. (●) XL1Blue containing pR5. (○) XL1Blue containing non-recombinant pUC18. The error bars indicate standard deviations.

Using the scaled down version of the quantitative acetochlor bioassay it was observed that *E. coli* XL1Blue cells containing pR5 were capable of detoxifying approximately 14.8 % of the active acetochlor in NM.1 over a 21 day period. Growth of untransformed cells in NM.1 containing acetochlor had no effect on the active concentration of the herbicide (Fig 5.8). The percentage of acetochlor detoxified in 21 days by XL1Blue containing pR5 was considerably less than that detoxified by A108 under comparable conditions and over the same time period.

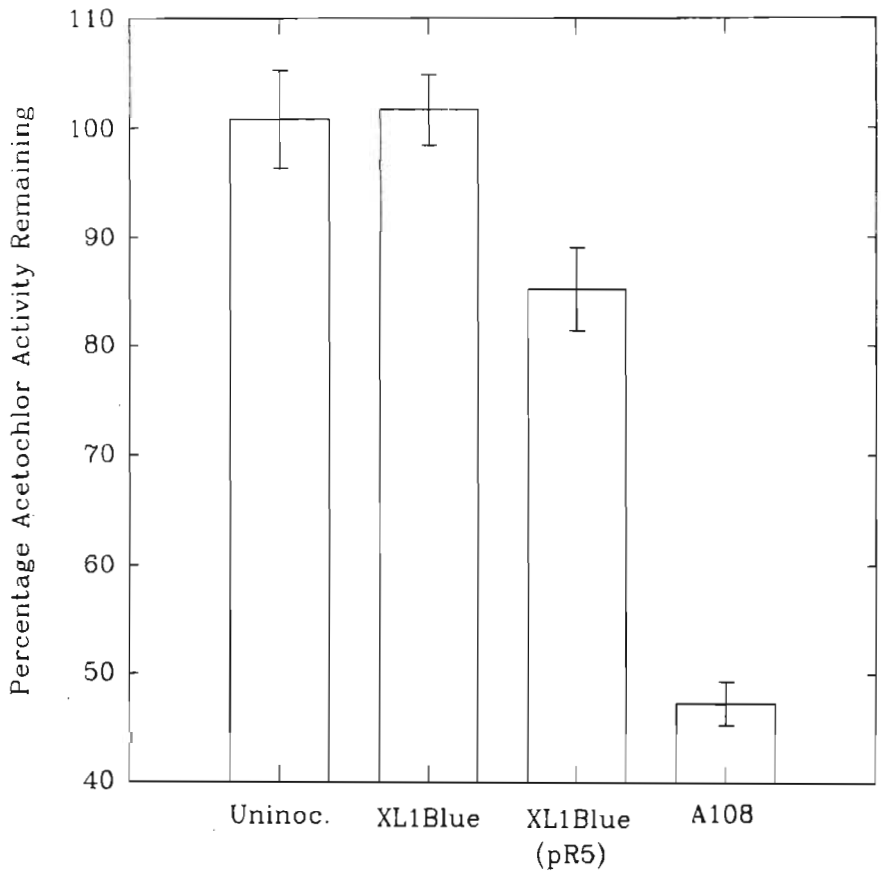

FIG 5.8. Percentage of initial acetochlor concentrations remaining active after 21 days in both uninoculated media and media used to support the growth of soil isolate A108, untransformed XL1Blue or XL1Blue containing pR5. Error bars represent standard deviations.

5.3.5 Restriction Mapping of pR5, a Recombinant Plasmid Carrying a Gene Associated With Acetochlor Detoxification

Of the restriction enzymes tested EcoRI, PvuII, PstI, KpnI, SalI and SphI were found to cleave within the approximately 3.2-Kb of A108 DNA present in pR5. Restriction fragment sizes arising through both single and double digestion of pR5 with these enzymes (Fig. 5.9.1) are given in Table 5.1. Using this data a restriction map of pR5 was produced (Fig. 5.9.2).

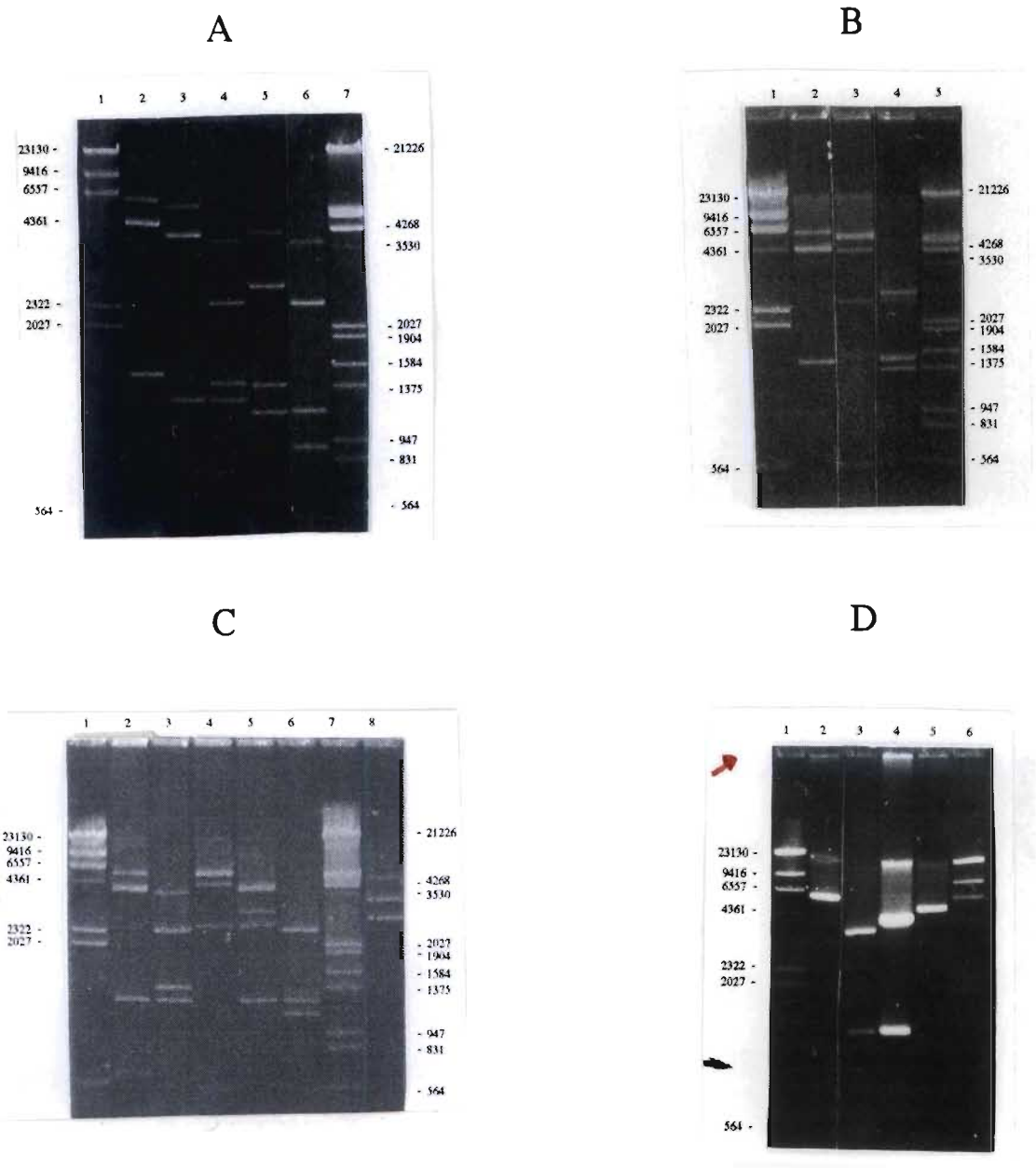

FIG. 5.9.1. Restriction analysis of pR5 with EcoRI, PvuII, PstI, KpnI, AvaI, BamHI, SphI and SalI. Gel A: lane 1, HindIII digested λ DNA (MWMII); lane 2, EcoRI digested pR5; lane 3, PstI digested pR5; lane 4, PvuII digested pR5; lane 5 EcoRI-PstI digested pR5; lane 6, PstI-PvuII digested pR5; lane 7, HindIII-EcoRI digested λ DNA (MWMIII). Gel B: lane 1, HindIII digested λ DNA; lane 2, EcoRI digested pR5; lane 3, SphI digested pR5; lane 4, EcoRI-SphI digested pR5; lane 5, HindIII-EcoRI digested λ DNA. Gel C: lane 1, HindIII digested λ DNA; lane 2, PstI digested pR5; lane 3, PvuII digested pR5; lane 4, SphI digested pR5; lane 5 SphI-PstI digested pR5; lane 6, SphI-PvuII digested pR5; lane 7, HindIII-EcoRI digested λ DNA; lane 8, AvaI digested pR5. Gel D: lane 1, HindIII digested λ DNA; lane 2, BamHI digested pR5; lane 3, SalI-BamHI digested pR5; lane 4, SalI digested pR5; lane 6, KpnI digested pR5; lane 7, HindIII digested λ DNA.

TABLE 5.1. DNA fragment sizes produced following restriction digestion of pR5.

Restriction Enzyme	Fragment Sizes (in bp) Resulting From a Single Digest	Fragment Sizes (in bp) Resulting From Double Digestion With:				
		PvuII	PstI	EcoRI	SphI	SalI
PvuII	2375 1380 1306 464	2375 1380 1306 464	2375 1065 959 464	-[a]	2375 1200 1033 464	-
PstI	4009 1255 636	2375 1065 959 464	4009 1255 636	2658 1351 1144 636	4009 1255 464	-
EcoRI	4438 1462	-	2658 1351 1144 636	4438 1462	2652 1462 1227 559	-
SphI	5341 559	2375 1200 1033 464	4009 1255 464	2652 1462 1227 559	5341 559	-
SalI	4450 1450	-	-	-	-	4450 1450
BamHI	5900	-	-	-	-	4227 1450 510
AvaI	3255 2645	-	-	-	-	-
KpnI	5441 459	-	-	-	-	-
HindIII	5900	-	-	-	-	-
SmaI	5900	-	-	-	-	-
XbaI	5900	-	-	-	-	-
ClaI	Uncut	-	-	-	-	-
XhoI	Uncut	-	-	-	-	-

[a] No Data Collected.

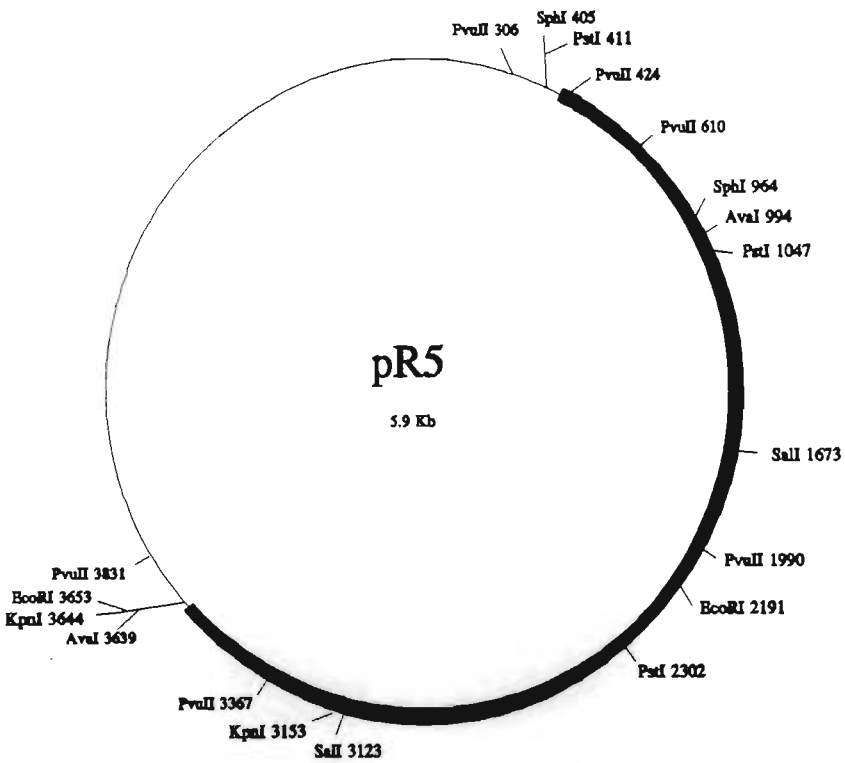

FIG. 5.9.2. Restriction map of pR5 indicating the positions of restriction sites for the restriction enzymes, EcoRI, PstI, PvuII, SphI, SalI, KpnI, and AvaI. The thick line on the map indicates the A108 DNA inserted into the SalI site of pUC18 (thin line).

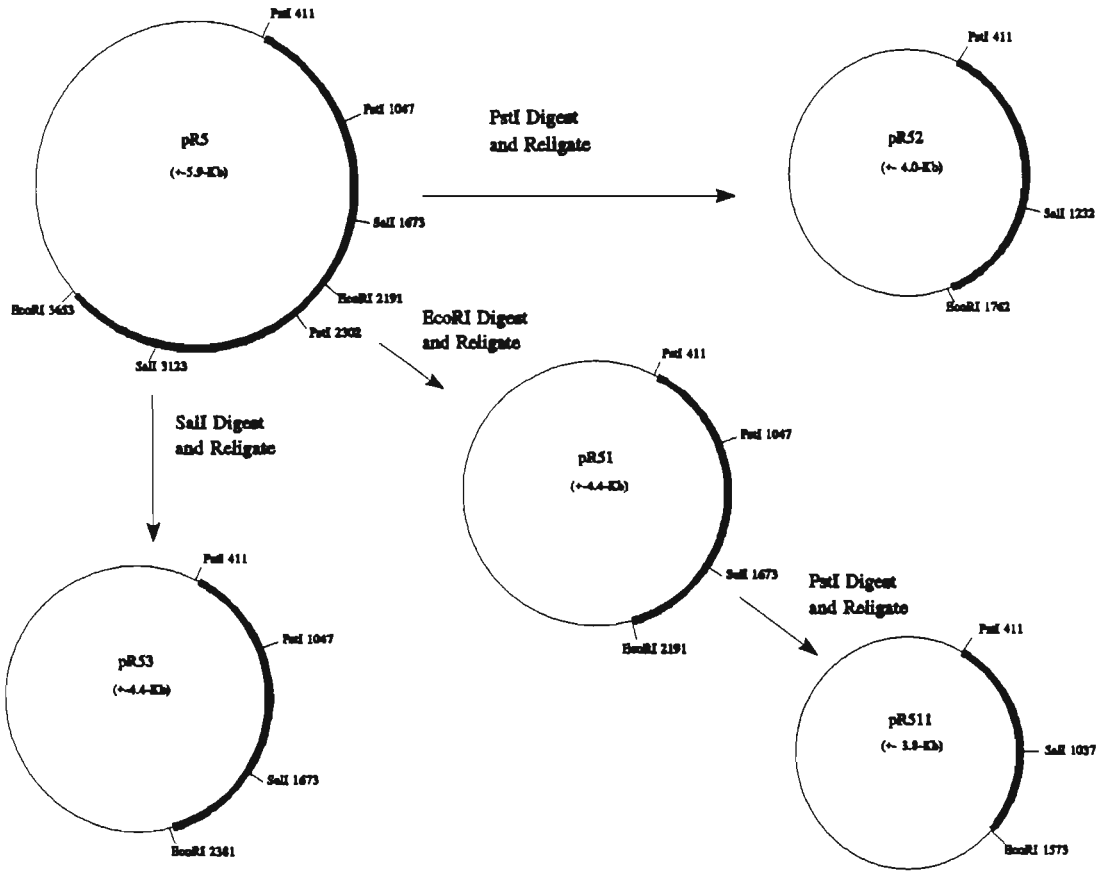

FIG. 5.10. Construction of plasmids pR51, pR52, pR53 and pR511 from pR5.

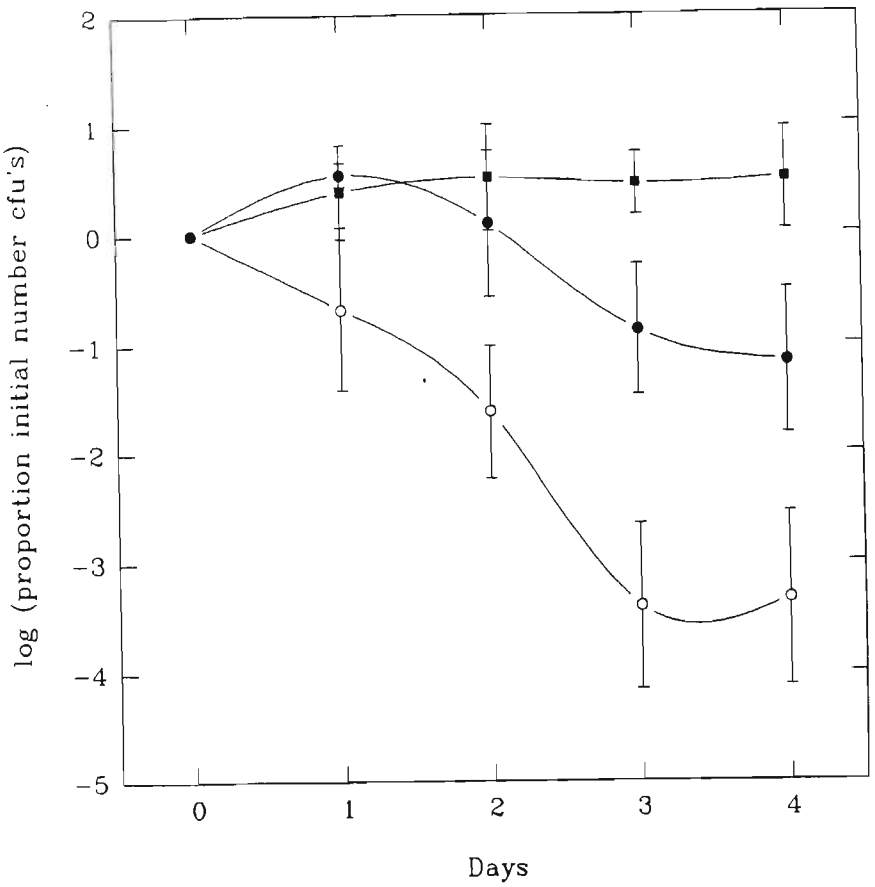

FIG. 5.11.1 Increased resistance to 16 $g.l^{-1}$ of formulation acetochlor amoung XL1Blue cells containing pR52. (●) XL1Blue containing pR52. (○) XL1Blue containing non-recombinant pUC18. (■) XL1Blue containing pR5. Error bars indicate standard deviations.

5.3.6 Localisation of a Gene Encoding Acetochlor Detoxification on pR5

Fig. 5.10 illustrates the production of plasmid constructs in which portions of the insert DNA present in pR5 were deleted. Of these constructs only pR511 was found to provide no elevated acetochlor resistance to the *E. coli* XL1Blue cells into which it was transformed. While XL1Blue cells containing pR52 and pR53 showed more resistance to acetochlor than XL1Blue containing non-recombinant pUC18, the degree of resistance observed appeared to be lower than that of pR5 (Figs. 5.11.1 and 5.11.2). pR51 appeared to provide XL1blue cells with acetochlor resistance in excess of that provided by pR5 (Fig 5.11.3).

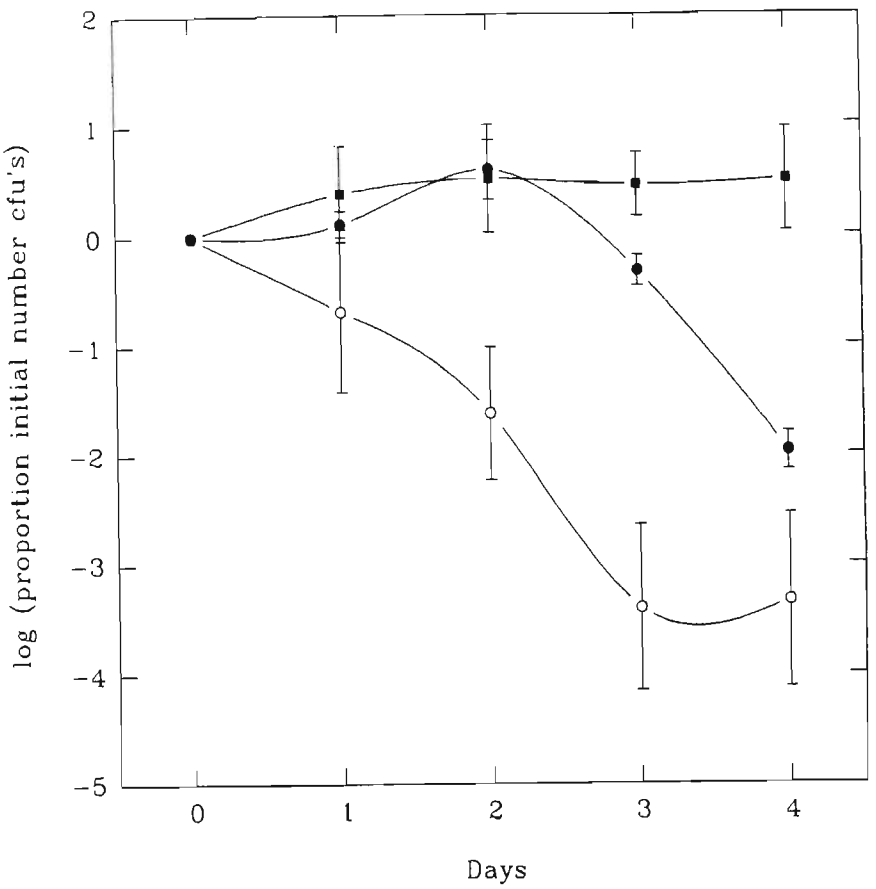

FIG. 5.11.2 Increased resistance to 16 $g.l^{-1}$ formulation acetochlor amoung XL1Blue cells containing pR53. (●) XL1Blue containing pR53. (○) XL1Blue containing non-recombinant pUC18. (■) XL1Blue containing pR5. Error bars indicate standard deviations.

5.3.7 Construction of a Deletion Series and Partial Sequencing of a Gene Associated with Acetochlor Detoxification

A series of nested deletions in the insert region of pR7 (a pBluescript derivative containing the same fragment of A108 DNA as pR51) was constructed in order to facilitate the partial sequencing of the A108 DNA fragment encoding elevated resistance to acetochlor. During construction of the nested deletion series, exonuclease III digested approximately 250 base pairs of pR7 per minute and electrophoresis of the digested DNA revealed that a series of deletions was obtained from the KpnI end of the insert DNA which traversed the entire insert region of pR7 (Fig. 5.12).

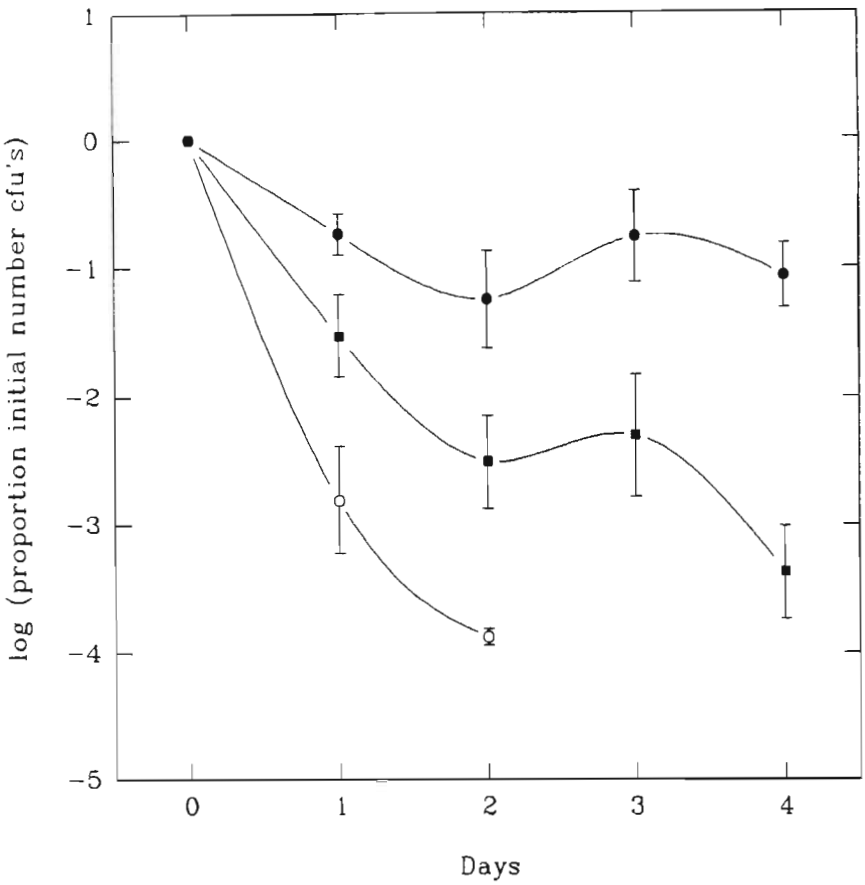

FIG. 5.11.3 Increased resistance to 32 g.l^{-1} formulation acetochlor amoung XL1Blue cells containing pR51. (●) XL1Blue containing pR51. (○) XL1Blue containing non-recombinant pUC18. (■) XL1Blue containing pR5. Error bars indicate standard errors.

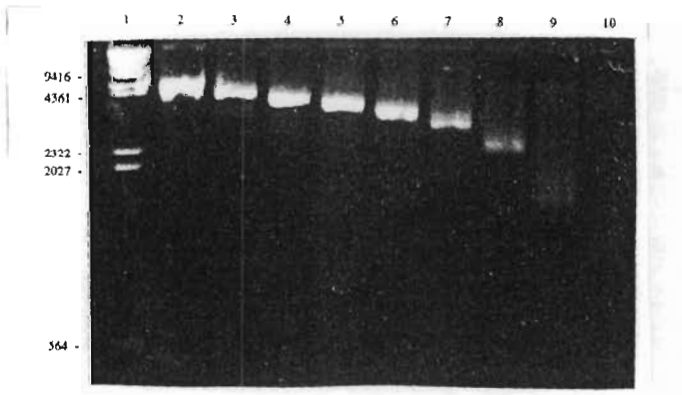

FIG. 5.12. The series of pR7 deletions constructed from the KpnI end of the insert DNA present in this plasmid. Agarose blocks were cut from this LMP agarose gel during the construction of a nested pR7 deletion series. Lane 1 contains HindIII digested λ DNA. Lanes 2-10 contain pR7 digested with exonuclease III for 1, 2, 3, 4, 5, 6, 7, 8 and 9 minutes respectively.

Portions of both strands of the A108 DNA common to pR7 and pR51 were sequenced to aid in obtaining an identity for any gene(s) present on this DNA fragment. From both the forward and reverse pUC18/M13 universal primer binding sites of pR51 and each of the T7 promoter specific primer binding sites of plasmids pR72, pR73, pR76, and pR77, between 150 and 250 base pairs were sequenced using either double or single stranded sequencing techniques (Fig. 5.13). In total the nucleotide sequence of approximately 43 % of the 1.78-Kb fragment of A108 DNA common to pR51 and pR7 was determined (Fig. 5.14). Comparisons of the nucleotide sequences determined in the present study with those stored in the GenBank database using BLAST revealed a single 315 bp region of pR51 and pR7 which contained an open reading frame that displayed sequence homology with portions of the dihydrolipoyltransacetylase genes of *Azotobacter vinelandii* (Hanemaaijer *et al.*, 1988), *E. coli* (Stephens *et al.*, 1983) and *Alcaligenes eutrophus* (Hein and Steinbuchel, 1994) (Table 5.2; Fig 5.14). Additionally the theoretical amino acid sequence encoded by this portion of A108 DNA was slightly homologous with the dihydrolipoyltransacetylase enzymes of *Clostridium magnum* and *Bacillus subtilis* (Table 5.2).

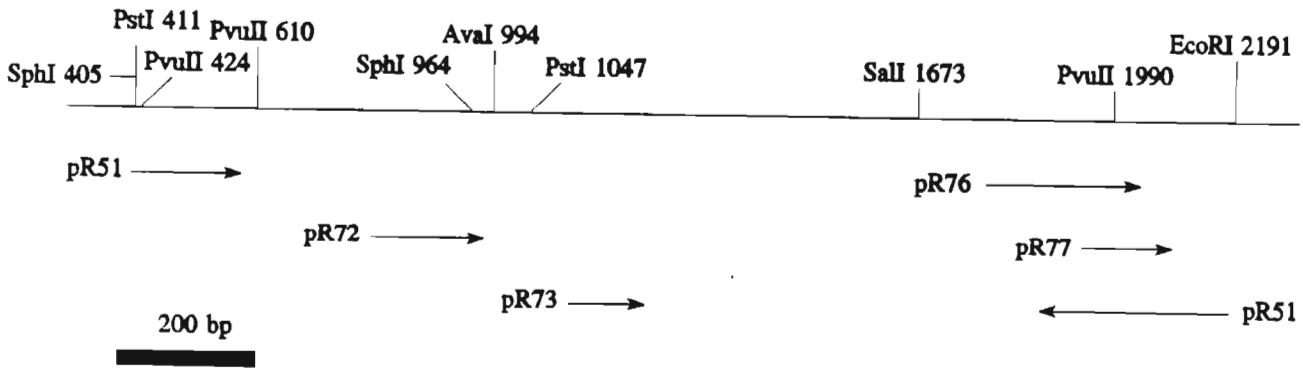

FIG. 5.13. The strategy employed to sequence portions of the 1.78-Kb fragment of A108 DNA common to both pR51 and pR7. The numbering system for the restriction sites presented here are based on the first nucleotide of A108 DNA being situated at position 422. Arrows indicate the direction and extent of DNA sequenced from the pUC/M13 forward and reverse universal primer binding sites of pR51 and T7 promoter specific binding site of pR72, pR73, pR76 and pR77.

```
2240 CCTGCCACCNCCCGCACAGGTCGCATCCTGAAAGAAGACGTAGCAGGT
     ProAlaThrXXXArgThrGlyArgIleLeuLysGluAspValAlaGly

2192 CTGACGATCAAGGCCATGATGCAGAAGGCCAAGGAAGCGCCGGCAGCC
     LeuThrIleLysAlaMetMetGlnLysAlaLysGluAlaProAlaAla

2144 GCCGGTGCGACCGGTGCTGCTGGCATCCCGCCGATCCCTGCCGTGGAC
     AlaGlyAlaThrGlyAlaAlaGlyIleProProIleProAlaValAsp

2096 TTCAGCAAGTTCGGTGAAGTGCAAGAAGTGGCCCTGACCCGCCTGATG
     PheSerLysPheGlyGluValGlnGluValAlaLeuThrArgLeuMet

2048 CAGGTCGGTGGTGCCAACCTGCACCGCAGCTGGCTCAACGTGCCGCAC
     GlnValGlyGlyAlaAsnLeuHisArgSerTrpLeuAsnValProHis

2000 GTGACCCAGTTCGACTCTGCCGACATCACCGAGCTGGAAGAACCTTCC
     ValThrGlnPheAspSerAlaAspIleThrGluLeuGluGluProSer

1952 GCGTTCCGCGTTGTCTGTCAGAAGCCCANNN......NNNCGACGTGC
     AlaPheArgValValCysGlnLysPro

1212 TGGGCGATATCGCACCATGCTGCTGTAAGCCAGCACACTGCCNGCTCC

1164 TTGCGCGA GGGGNAGTAGCNCTTTCGAGCTGNCACCTCGTCAGCCTC

1116 AACCCCGNNN......NNNAGGCCCCTCCCCACAGGCAATCGCAGTCC

 941 ACATTGTGGGAGTAACTGTCTTCTCAATTTCTAAAAGCTGGCCGATCC

 893 TGTGNGAGCGACCTTGTGTCTCNATGGGCCGCAAANCGGCGCNNAAAA

 845 TCTCCACGCCCAAACTGCAGAAGTACAGGCACGAGCGCTCCACGCCGA

 797 TACATCACTGNNN......NNNCACGACCTGGAATCAANNAACAACGC

 569 TGCACCTGGNCCTGCAGNTGAACCANGNANGCAGNAAGAACAANTGGA

 521 GGACCTGGAGNCCCACNGCGGCCAGCAGGANCTTATTCCTGCNCCTCG

 473 NCCGCCATCGCTACAGNCCGGGCAACTCGCTGCTCGAAGCGGCCCAGC

 425 TGA
```

FIG. 5.14. Partial sequence of the A108 DNA common to both pR7 and pR51. The amino acid sequence encoded by the region of DNA with homology to a portion of the dihydrolipoyltransacetylase genes of *Azotobacter vinelandii*, *E. coli* and *Alcaligenes eutrophus* is also given. The nucleotides presented in bold type indicate individual residues which are in positions identical to corresponding residues in the *Azotobacter vinelandii* dihydrolipoyltransacetylase gene. Amino acid residues presented in bold type indicate individual amino acids which were either the same or functionally equivalent to residues in corresponding positions in the *Azotobacter vinelandii* dihydrolipoyltransacetylase enzyme. The numbering system used is based on the first nucleotide of A108 DNA being situated at position 422.

TABLE 5.2. Results of the search (using BLAST) of established DNA and protein sequences stored in the GenBank database for regions of nucleotide and amino acid homology with the A108 DNA cloned in pR51 and pR7.

GenBank Acquisition Number	Species	Gene/Protein Identity	High Score[a]	Smallest Sum Probability [P(N)] [b]	N [c]
Nucleic Acid					
emb:X12455	*Azotobacter vinelandii*	Gene encoding dihydrolipoyltransacetylase	648	1.2×10^{-43}	1
emb:V01498	*E. coli*	*aceA*(encoding dihydrolipoyltransacetylase)	410	1.0×10^{-23}	1
gb:U09865	*Alcaligenes eutrophus*	*pdhB*(encoding dihydrolipoyltransacetylase)	356	3.4×10^{-19}	1
Protein					
sp:P10802	*Azotobacter vinelandii*	Dihydrolipoyltransacetylase	268	2.0×10^{-28}	1
sp:P06959	*E. coli*	Dihydrolipoyltransacetylase	238	1.3×10^{-24}	1
gp:U09865	*Alcaligenes eutrophus*	Dihydrolipoyltransacetylase	198	2.0×10^{-18}	1
gp:L31844	*Clostridium magnum*	Dihydrolipoyltransacetylase	65	0.049	2
gp:M57435	*Bacillus subtilis*	Dihydrolipoyltransacetylase	45	0.1	3

[a] Maximum possible value calculated according to the system described by Altschul *et al.* (1990). Generally high scores above 100 for nucleotide comparisons and above 50 for amino acid comparisons indicate some significant degree of homology between compared sequences.

[b] Probability of achieving the presented high score by chance.

[c] Number of distinct high scoring regions of homology in the sequences being compared.

5.4 Discussion

5.4.1 Use of IFAGE to Analyse the Genome of A108

The initial purpose behind the use of IFAGE was to estimate the genome size of A108 in a manner similar to that used by Grothues and Tummler (1987) to determine the genome size of *Pseudomonas aeruginosa*. This was not possible in the present study, however, since no restriction enzyme could be found which digested the genome of A108 into fragment sizes which could be analysed using IFAGE. Although digestion of intact A108 DNA with KpnI yielded a single megabase sized (1.45-Mb) fragment, it was unlikely that this fragment represented the linearised A108 chromosome. It was expected that because A108 was almost certainly a *Pseudomonas* sp., its chromosome should have been between 2.2 and 4.16-Mb which is a size characteristic of the members of this genus (Grothues and Tummler, 1987; Park and DeLey, 1967; Pemberton, 1974). It was probable, therefore, that KpnI digestion of intact A108 genomic DNA produced either a multitude of small fragments not observable on ethidium bromide stained gels or a second fragment larger

than 2-Mb which could not be resolved with IFAGE employing the Hoefer PC 750 pulse controller (Hoefer Scientific Instruments Catalogue, 1988). It was unlikely that a large number of small unobservable fragments were produced by digestion with KpnI because staining of IFAGE gels with ethidium bromide revealed DNA remaining in the agarose blocks subjected to digestion with this enzyme. It is also not probable, however, that KpnI sites are very rare in the genome of A108 since A108 DNA cloned into pR5 could be cut with this enzyme. It is also possible that the results were the product of reduced KpnI activity which occurred when attempting to digest A108 DNA in LMP agarose.

Because A108 contains one or more large plasmids it is possible that genes encoding acetochlor degradation may not be borne on the chromosome of the isolate. There have been no reports regarding the situation of microbial genes encoding chloroacetanilide degradation on plasmids. While no evidence could be obtained to indicate whether any A108 genes encoding acetochlor detoxification were plasmid bourn, it would be of interest in future studies to determine the location of such genes in the genome of A108.

5.4.2 Cloning, Isolation and Identification of a Gene Associated With Acetochlor Detoxification

It is probable that the different efficiencies of the cloning procedure when using different running buffers during electrophoretic size fractionation of insert DNA, was related to the relative activities of T4 DNA ligase in the presence and absence of borate. This is probably because borate may inhibit the enzyme, T4 DNA ligase, used to ligate vector and insert DNAs in LMP agarose during the cloning procedure (Struhl, 1985).

Because the cloning procedure involved partially filling in the ends of SalI digested pUC18 DNA, the X-gal phenotypic assay for identifying recombinant and non-recombinant transformants could not be used. If the partially filled in ends of pUC18 ligated with one another during the cloning procedure, the frame shift mutations induced by the end filling reactions would have prevented the *lacZ'* gene on the plasmid effectively complementing the *lacZ*ΔM15 on the chromosome of the host *E. coli* strain. A large proportion of transformant colonies containing non-recombinant pUC18 would thus have been WHITE

(*lac*$^-$). A method involving the selection of transformants, the isolation of their plasmids, and the use of electrophoresis for detecting the presence of inserts was therefore adopted in order to indicate the approximate proportion of recombinant individuals in transformant populations.

Together the gene libraries constructed in JM109 and XL1Blue represented a complete A108 genomic library. Although there was a 68.7 % probability that the library constructed in JM109 contained any particular region of DNA in the genome of A108, none of the recombinant plasmids isolated from this library contained A108 DNA which provided *E. coli* cells with acetochlor resistance. While it is possible that this was due to chance, it is also possible that the size of A108 DNA fragments present in the library (a mean of approximately 1.5-Kb) were too small for there to be a high probability of the library containing an intact acetochlor detoxification gene.

In the present study no DNA or antibody probes were available for screening the A108 gene libraries for either acetochlor detoxification genes or acetochlor detoxifying enzymes. It was, therefore, necessary to attempt the isolation of acetochlor detoxification genes by selecting for recombinant *E. coli* cells in which they were expressed. It was believed that the expression of a gene encoding the degradation of acetochlor in *E. coli* cells would provide the cells with increased resistance to the herbicide. The success of this approach to the isolation of a gene encoding acetochlor detoxification was dependent on two factors. Firstly, it was essential that a means of screening populations of *E. coli* cells for elevated acetochlor resistance be available and, Secondly, it was necessary that the native transcription and translation sequences of the desired acetochlor detoxification gene be similar enough to those of *E. coli* to permit its efficient expression in this microbe.

Selection of recombinant *E. coli* cells resistant to acetochlor was complicated by the naturally high degree of acetochlor resistance displayed by the *E. coli* strains used. Only very high concentrations of the herbicide would consistently kill *E. coli* cells and then only if the cells were exposed to the herbicide for prolonged periods. Since it is likely that acetochlor has a multitude of target sites in living cells (LeBaron *et al.*, 1988; Sharp, 1988), it is not probable that *E. coli* would have been resistant to the herbicide by virtue

of a single altered, herbicide insensitive target molecule. Resistance may, however, have been due to any ONE OR MORE of a number of other mechanisms. One mechanism may have been that *E. coli* was capable of detoxifying acetochlor. Use of the bioassay to detect the detoxification of acetochlor by *E. coli* XL1Blue, however, revealed that this strain was incapable of metabolically converting acetochlor to a form less toxic to the bioassay fungus. A second potential resistance mechanism may have been the total absence in *E. coli* of enzymes or structural proteins targeted by the herbicide. However, because the primary site(s) of acetochlor activity are unknown (Sharp, 1988), it could not be determined whether *E. coli* possessed any potentially sensitive acetochlor target sites. A third mechanism of resistance may have been that uptake of the herbicide by *E. coli* was somehow impaired. This may have been the result of either the failure of *E. coli* cells to transport herbicide molecules across their cell membranes or the prevention of efficient contact between acetochlor molecules and cell surfaces due to the limited solubility of the herbicide. The number of acetochlor molecules dissolved in MS media when testing the toxicity of the herbicide to *E. coli*, was almost certainly less than the number of acetochlor molecules added to the MS medium. This was because the concentration of the herbicide required to kill *E. coli* cells exceeded the solubility of the herbicide in water at room temperature (0.223 $g.l^{-1}$; Worthing and Hance, 1991). Thus, although very high concentrations of the herbicide were applied to MS media to kill *E. coli* (either 16 or 32 $g.l^{-1}$ of formulation acetochlor), it is highly likely that the vast majority of herbicide molecules did not enter into solution and were, therefore, not available for uptake by *E. coli* cells.

The successful expression of an acetochlor detoxification gene from A108, a probable Pseudomonad, in *E. coli*, an Enterobacter, was dependent on the gene having promoter, transcription termination and ribosome binding site sequences comparable with analogous sequences in *E. coli*. Attempts to express different *Pseudomonas* genes in *E. coli* under the control of their own promoters have been both successful (Eaton and Karnes, 1991; Fitzgibbon and Braymer, 1990; Goldberg *et al.*, 1992; Kahn and Walia, 1990) and unsuccessful (Kimbara *et al.*, 1989). It has been suggested that the poor expression of certain *Pseudomonas* genes in *E. coli* has been due to these genes having either promoters which are unrecognisable to *E. coli* RNA polymerases (Deretic *et al.*, 1989; Kimbara *et*

al., 1989), or a high frequency of codons which are only rarely used by *E. coli* (Sayers, 1994; personal communication[5]).

Screening recombinant *E. coli* cells for the expression of acetochlor detoxification genes occurred in three stages. Firstly, transformants surviving long term exposure to high acetochlor concentrations were selected from the A108 genomic libraries. Secondly, it was necessary to verify whether the apparently elevated acetochlor resistance observed in the selected transformants was encoded on the recombinant plasmids they contained. Lastly, it was determined whether or not acetochlor resistant recombinant transformants were capable of detoxifying the herbicide. Following the selection of acetochlor resistant individuals from the A108 genomic libraries, it was necessary to verify whether they contained resistance genes from A108 since it was possible that cells could survive exposure to the herbicide either by chance or through mutation of chromosomal genes. Recombinant plasmids isolated from selected acetochlor resistant cells were therefore retransformed into XL1Blue. The transformants thus obtained were assessed to determine whether they were more resistant to acetochlor than XL1Blue cells containing non-recombinant pUC18. It was necessary to screen cells apparently possessing acetochlor resistance genes for their ability to detoxify acetochlor since it was possible that acetochlor resistance may also have been provided by genes that did not encode the metabolic transformation of the herbicide. For example, Fitzgibbon and Braymer (1990) while attempting to isolate a glyphosate degradative gene from *Pseudomonas* sp. PG2982, obtained a gene which provided recombinant *E. coli* cells with elevated glyphosate resistance without encoding transformation of this herbicide. It was also noted that the gene neither encoded any known enzyme target of glyphosate nor did it interfere with uptake of the herbicide in cells possessing it.

The approach employed in the isolation of an A108 gene which when present in *E. coli* XL1Blue cells would enable them to detoxify acetochlor yielded a segment of DNA containing what appeared to be a gene encoding the dihydrolipoyltransacetylase component

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of the pyruvate dehydrogenase enzyme complex. There are a number of possible explanations as to how such a gene may have provided recombinant *E. coli* cells with elevated acetochlor resistance. All of these explanations, however, are based on the assumption that acetochlor is capable of attachment to some component of the pyruvate dehydrogenase complex. If the complex is a primary target site of acetochlor activity then resistance to this herbicide could occur in recombinant cells through either the overproduction of dihydrolipoyltransacetylase or the expression of an acetochlor resistant variety of the enzyme. Although it has been noted that the chloroacetanilides influence both lipid biosynthesis (Yenne and Hatzios, 1989; Weisshaar and Boger, 1991) and the catabolism of pyruvate to CO_2 (Warmund *et al.*, 1985) in higher plants, in no instances has the herbicide been noted to target any particular enzyme in these metabolic pathways. While there is no direct evidence to indicate that the pyruvate dehydrogenase complex is a target of the chloroacetanilide herbicides, the only common reaction step in the synthesis of lipids and the catabolism of pyruvate to CO_2 is the conversion of pyruvate to acetyl-coenzyme A that is catalysed by this enzyme complex (Stryer *et al.*, 1988). Generally, involvement of coenzyme A appears to be a common factor in many metabolic processes inhibited by the chloroacetanilides (Couderchet and Boger, 1993; Molin *et al.*, 1986; Tevini and Steinmuller, 1987; Weisshaar and Boger, 1987; Wilkinson, 1981; Yenne and Hatzios, 1989). A theory proposed by Molin *et al.* (1986) to explain the effect of chloroacetanilides on reactions involving coenzyme A is that these herbicides inhibit some aspect of coenzyme A reduction or recycling.

Additional evidence that the pyruvate dehydrogenase complex may be a target site of chloroacetanilide herbicides comes from studies involving the herbicide EPTC. In plants a primary mode of action of this herbicide appears to be its competitive inhibition of the pyruvate dehydrogenase complex (Wilkinson and Oswald, 1987). EPTC has both physiological (Wilkinson, 1988) and biochemical (Wilkinson, 1985) effects in plants which are similar to those induced by the chloroacetanilides. Also, detoxification of both chloroacetanilide and EPTC molecules in plants is known to occur via conjugation of these molecules with glutathione (Cottingham *et al*, 1993). It is therefore feasible that, like EPTC, the numerous biochemical effects characteristic of chloroacetanilide activity in plants, may occur as a result of pyruvate dehydrogenase inhibition.

A possible biochemical mechanism whereby chloroacetanilides such as acetochlor may function in pyruvate dehydrogenase inhibition is through alkylation of the thiol groups belonging to the lipoamide ($lipS_2$) cofactors associated with the active sites of the dihydrolipoyltransacetylase components of this enzyme complex. Once alkylated these thiol groups would no longer be available for reaction with the hydroxyethyl-thiamine pyrophosphate intermediary metabolite produced by the pyruvate decarboxylase component of the pyruvate dehydrogenase complex (Fig 5.15, A and B). The chloroacetanilides are known alkylating agents and will bind covalently with an attacking nucleophile in a reaction coupled with the displacement of their chlorine atoms (Lindley, 1960). The nucleophilic thiol groups of the $lipS_2$ coenzyme associated with the active site of dihydrolipoyltransacetylase, could potentially react non-enzymatically with acetochlor. It has been observed that *in vitro* the chloroacetanilide, alachlor, reacts non-enzymatically in this manner with the thiol containing compounds, cysteine, glutathione and coenzyme A (Leavitt and Penner, 1979). The high reactivity of the chloroacetanilides with thiol containing compounds has lead to speculation that activity of the chloroacetanilides at multiple target sites may occur through these compounds reacting with the thiol containing amino acid residues of a variety of sulphur rich proteins. While McFarland and Hess (1985; cited by LeBaron *et al.*, 1988) have found chloroacetanilides to bind covalently with a number of unidentified proteins both *in vivo* (intact oat seedlings) and *in vitro*, Marsh *et al.* (1988; cited by LeBaron *et al.*, 1988) found that the chloroacetanilide, alachlor, did not inhibit the activity of any of the sulphur rich enzymes they tested.

A feasible mechanism whereby the expression of dihydrolipoyltransacetylase in recombinant cells may have enabled them to detoxify acetochlor is that the enzyme actually played a role in transforming the herbicide in these cells. The enzyme may have contributed to the detoxification of acetochlor molecules in two ways. Firstly, it is possible that the dihydrolipoyltransacetylase enzyme participated directly in the transformation of acetochlor. By covalently binding with the thiol groups of the $lipS_2$ coenzyme associated with the active site of this enzyme, bound herbicide molecules would become irreversibly inactivated with the loss of their chlorine atoms (Fig 5.15). Secondly, it is possible that dihydrolipoyltransacetylase may have participated indirectly in acetochlor detoxification by enhancing the non-enzymatic binding of acetochlor molecules with

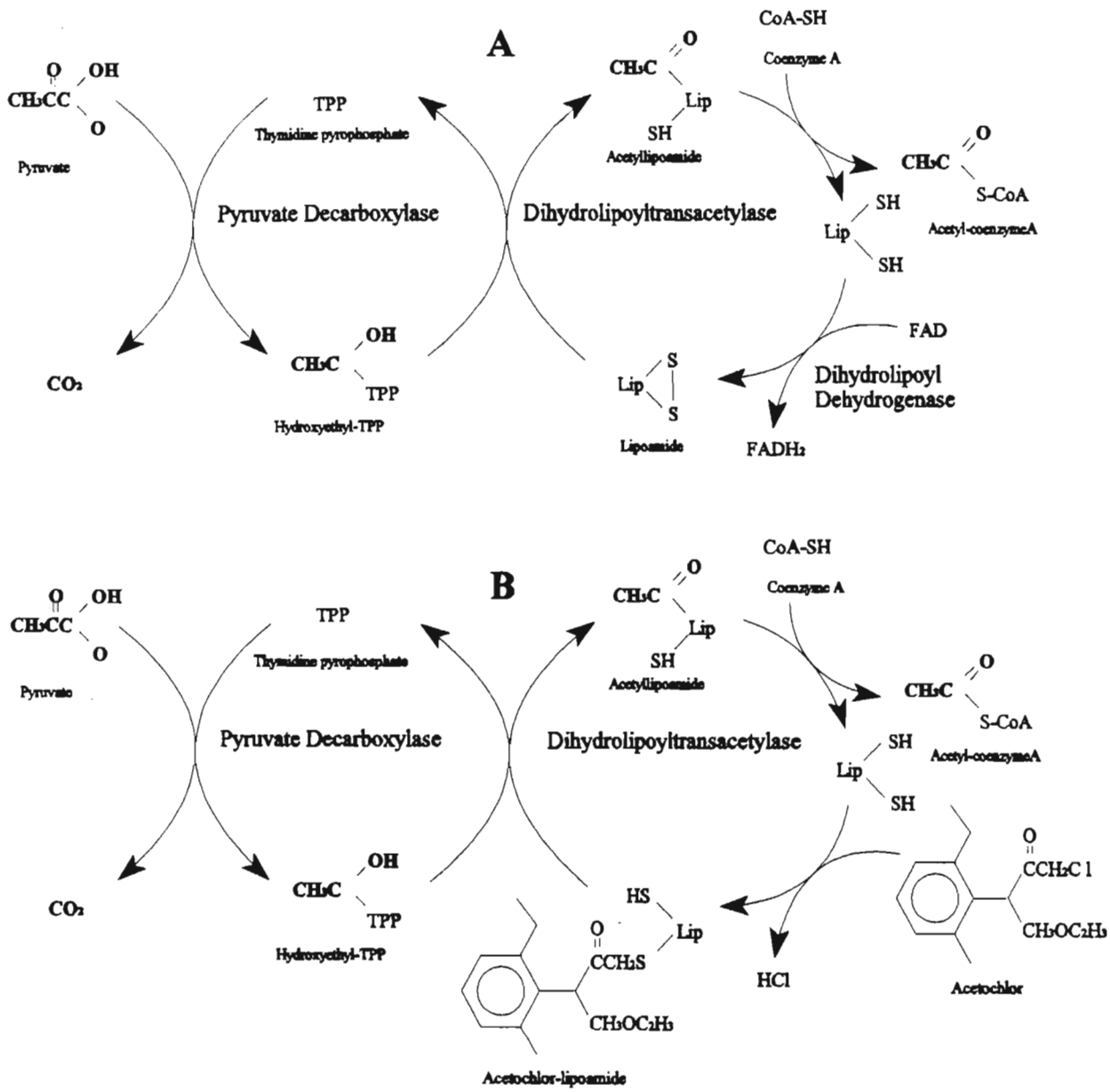

FIG. 5.15. Proposed mechanism whereby the chloroacetanilide herbicide, acetochlor, may interact with and thus inhibit the activity of the dihydrolipoyltransacetylase component of the pyruvate dehydrogenase complex. (A) Normal reaction mechanism whereby pyruvate is converted to acetyl-coenzyme A and CO_2 by the pyruvate dehydrogenase complex. (B) Possible mechanism by which acetochlor, a known alkylating agent, may prevent this reaction from occurring.

coenzyme A. It has been noted that the chloroacetanilide, acetochlor, binds non-enzymatically with coenzyme A *in vitro* (Leavitt and Penner, 1979) and it would, therefore, be possible that attachment of acetochlor to dihydrolipoyltransacetylase near to a position where coenzyme A interacts with the enzyme may have resulted in an elevated rate of non-enzymatic acetochlor-coenzyme A conjugation. By bringing coenzyme A and acetochlor into close contact and thus enhancing the probability of conjugation occurring between these compounds, increased expression of dihydrolipoyltransacetylase could possibly enhance acetochlor detoxification.

The significant difference in the rate at which A108 and *E. coli* XL1Blue containing pR5 detoxified acetochlor in NM.1 medium could have occurred for any one or a combination of a number of possible reasons. Firstly, lack of either expression sequence homology or common codon usage between the cloned A108 gene and *E. coli* genes may have resulted in reduced expression of the cloned gene in recombinant cells (Deretic *et al.*, 1989; Sayers *et al.*, 1994; personal communication[6]). Secondly, the rate of uptake of acetochlor molecules may have differed significantly between A108 and the *E. coli* strain in which acetochlor detoxification was tested. For example, it is possible that A108 contains transport proteins that are absent in *E. coli*, which, by enhancing the movement of acetochlor molecules into cells increases the rate at which detoxifying enzymes transform the herbicide. Thirdly, and probably most importantly, it is possible that the putative dihydrolipoyltransacetylase gene isolated in the present study is not the only A108 gene encoding the detoxification of acetochlor. Studies on microbial chloroacetanilide degradation have revealed that many microbes are capable of transforming chloroacetanilides via more than one pathway (Figs 1.5.1, 1.5.2, 1.5.3, and 1.5.4). An implication of this is that certain microbial species may contain more than one gene encoding primary chloroacetanilide transformation reactions. The acetochlor transformation products produced by A108 should be identified to obtain some indication as to the pathways used by this isolate in the detoxification of acetochlor. It should also be determined what, if any, are the acetochlor transformation products produced by *E.*

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coli cells containing pR5. By obtaining this information it should be possible to determine whether the dihydrolipoyltransacetylase gene of A108 plays a major role in acetochlor detoxification in this isolate or whether other transformation reactions, encoded by different genes, play a more active role in acetochlor detoxification.

The portion of the putative dihydrolipoyltransacetylase gene present in pR51 is homologous with the catalytic domain of this enzyme in *Azotobacter vinelandii* (Mattevi *et al.*, 1992). By extrapolating from data available on the extent of characterised bacterial dihydrolipoyltransacetylase genes it would seem to be possible to speculate on the extent of the coding region of the gene in pR5 (Fig 5.16). If these speculations are accurate it would be probable that, firstly, pR5 contains an entire copy of the gene which is possibly expressed under the control of its own promoter. Secondly, it would be probable that while pR53 contains an approximate portion of the putative gene encoding the first 47 N-terminal and last 116 C-terminal amino acids of the putative dihydrolipoyltransacetylase (with approximately 483 amino acids between deleted; Fig. 5. 16), pR52 contains a portion of the gene encoding only the first 320 N-terminal amino acids of the enzyme (326 amino acids from the C-terminal deleted; Fig 5. 16). It is possible that the acetochlor resistance afforded to *E. coli* by pR51, pR53 and pR52 may have occurred due to portions of the putative dihydrolipoyltransacetylase gene present on these plasmids complementing the analogous gene on the *E. coli* chromosome. The extra components of the enzyme available within cells containing these plasmids would possibly have given rise to either a greater number of pyruvate dehydrogenase complexes than are normally present in *E. coli* or complexes which were more herbicide resistant. The acetochlor resistance provided *E. coli* cells by pR51 and pR5 may have been greater than that provided by pR52 and pR53 due to the presence in these plasmids of the portion of the A108 gene encoding the catalytic domain of dihydrolipoyltransacetylase. The higher acetochlor resistance observed in cells containing pR51 relative to cells containing pR5 may have been through expression of the catalytic domain of the enzyme under control of the *lac* promoter. The catalytic domain of the putative A108 dihydrolipoyltransacetylase gene present on pR51 is directly downstream from the promoter, ribosome binding site and translation initiation codon of the *lacZ'* gene. Besides the basal uninduced expression of

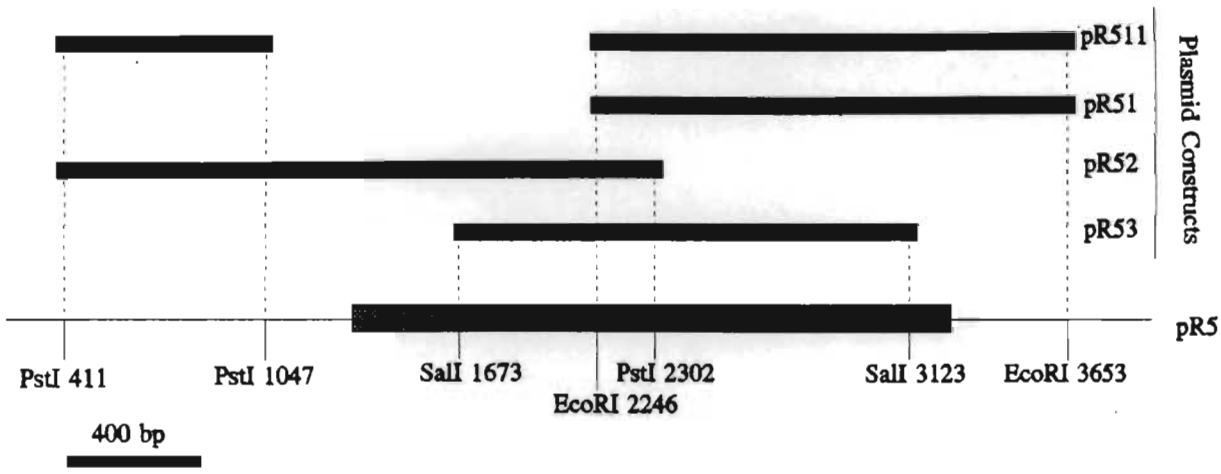

FIG. 5.16. The possible extent of the coding region of the putative A108 dihydrolipoyltransacetylase gene (lightly shaded region) on pR5. The size of the gene was estimated from sequences determined for the dihydrolipoyltransacetylase genes isolated from *Azotobacter vinelandii* (Hanemaaijer *et al.*, 1988) and *E. coli* (Stephens *et al.*, 1983). The partial sequence of the A108 gene (darkly shaded) was used to estimate the location of the entire putative dihydrolipoyltransacetylase gene on pR5 by aligning the nucleotide sequence determined with corresponding homologous regions of the dihydrolipoyltransacetylase genes of *Azotobacter vinelandii* and *E. coli*. The arrow indicates the direction in which the gene is transcribed. Bold black lines indicate deleted portions of the various plasmid constructs (pR51, pR52, pR53, and pR511) examined for their ability to provide *E. coli* with resistance to acetochlor. The numbering system used here is based on the first nucleotide of A108 DNA being situated at position 422.

the *lac* operon (Hastings, 1994; personal communication[7]), it is possible that slight induction of the operon may have occurred in cells grown on the undefined NA medium prior to their transfer to the acetochlor containing MS test medium. Marginal derepression of the *lac* operon on NA may have been the result of lactose traces present in the yeast or beef extracts used to make up this medium. It is, therefore, feasible that expression of the putative dihydrolipoyltransacetylase catalytic domain on pR51 under control of the *lac* promoter may have provided cells with acetochlor resistance in excess of the intact

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dihydrolipoyltransacetylase gene on pR5 (which was possibly being expressed under control of its native promoter). In this regard it would be of interest to determine the effects of using IPTG to induce the *lac* operons of the different plasmid constructs when testing the levels of acetochlor resistance in *E. coli* hosts containing these constructs. It is uncertain why pR511, which contains the same portion of the putative dihydrolipoyltransacetylase gene as pR51, was unable to provide *E. coli* with any significantly improved acetochlor resistance. The difference between the two plasmid constructs is that while pR51 contains the entire native downstream region of the A108 gene, much of this region in pR511 was deleted. It is possible that efficient expression of the portion of the gene encoding the catalytic domain of dihydrolipoyltransacetylase may require a suitable downstream transcription termination sequence. While such sequences are present immediately downstream from the translation termination codons of the genes encoding dihydrolipoyltransacetylase in *Azotobacter vinelandii* (Hanemaaijer *et al.*, 1988) and *E. coli* (Stephens *et al.*, 1983), it was not determined whether a similar sequence was present downstream from the putative dihydrolipoyltransacetylase gene isolated in the present study.

Although a gene apparently encoding the detoxification of acetochlor has been isolated from the soil inhabiting *Pseudomonas* strain, A108, it is uncertain whether the gene could be employed in engineering resistance to this herbicide in crops. In order to properly assess the usefulness of the gene in this regard it will be necessary to determine what, if any, is the acetochlor transformation which the gene encodes. By comparing the acetochlor transformation products produced by *E. coli* cells containing the plasmid constructs pR5, pR51, pR52, and pR53 with the herbicide transformation products produced by A108 it should be possible to determine, firstly, whether there are potentially any alterative acetochlor detoxification genes present in the A108 genomic DNA library and, secondly, whether these unisolated genes may be more suited to use in engineering plants for acetochlor resistance.

6 GENERAL DISCUSSION

The rate at which new herbicides are being developed is slower than the rate at which herbicide resistant weed varieties are arising and there is, at present, a severe shortage of herbicides which can be used effectively during the cultivation of important crops such as wheat (Gressel, 1992). The development of crops which are resistant to established broad spectrum herbicides which do not stimulate the evolution of resistant weed varieties, is a viable means of compensating for the decreased rate of new herbicide development (Lyon, 1991; Mazur and Falco, 1989). Because of a combination of the shortage of useful herbicide resistance genes in crops that are exploitable using conventional plant breeding practices, and the occurrence of resistance among important weed varieties to most of the herbicides in wide use, many crops such as wheat will require the acquisition of new herbicide resistance genes through genetic engineering (Gressel, 1992). Since the chloroacetanilides have a history of providing long term weed control without stimulating the occurrence of resistant weed varieties, a gene encoding resistance to a chloroacetanilide herbicide such as acetochlor would be of enormous agronomic value in the development of herbicide resistance in many important crops.

In the present study the isolation of a DNA fragment encoding resistance to acetochlor is described. This fragment of DNA, possibly encoding the dihydrolipoyltransacetylase component of the pyruvate dehydrogenase complex, is the first isolated from a microbial source which has been shown to provide a recombinant host organism with elevated resistance to a chloroacetanilide herbicide. It is, however, uncertain how a gene encoding dihydrolipoyltransacetylase might provided resistance to the *E. coli* cells in which it is present. The most obvious explanation would be that dihydrolipoyltransacetylase is an acetochlor target site and that the resistance afforded recombinant cells resulted from either the overexpression of the enzyme or its insensitivity to acetochlor. Since chloroacetanilides such as acetochlor almost certainly have multiple target sites in sensitive cells (LeBaron *et al.*, 1988; Sharp, 1988) it is unlikely that a gene encoding a single altered herbicide insensitive target protein would be of use for engineering resistance to the chloroacetanilides. Despite over 20 years of research no primary target sites of the chloroacetanilides have yet been identified. The discovery that a gene encoding an altered form of

dihydrolipoyltransacetylase can provide cells with resistance to a chloroacetanilide, could be an important development in efforts to determine the primary target sights of these herbicides.

One piece of evidence indicating that the putative dihydrolipoyltransacetylase gene may be of some use in providing plants with resistance to the chloroacetanilides is that it possibly encodes acetochlor detoxification. It should be the focus of further research to determine the mechanism by which the gene enabled acetochlor detoxification to occur in the recombinant *E. coli* cells containing it. If some transformation of the herbicide occurs in cells containing the gene then, at the very least, identification of these metabolites will help illuminate the interaction of acetochlor with the dihydrolipoyltransacetylase enzyme. From the results of these investigations it should be possible to determine whether the gene in question may be of use in engineering plants with acetochlor resistance.

If the resistance afforded to *E. coli* cells by the putative A108 dihydrolipoyltransacetylase gene is found to result from the reactivity of some component of the dihydrolipoyltransacetylase enzyme with acetochlor, it is possible that the gene could be employed as a true acetochlor detoxification gene in the production of herbicide resistant recombinant plants. It would then be important to determine whether activity of the dihydrolipoyltransacetylase in acetochlor detoxification was independent of other components of the pyruvate dehydrogenase complex. If the enzyme was capable of functioning independently of the complex it could operate in the cytoplasm of recombinant cells. The significance of this would be that, firstly, the gene would not require augmentation with appropriate plant organelle target sequences and, secondly, it would not interfere with the functioning of the native plant pyruvate dehydrogenase complexes which are found in mitochondria and plastids. Because even a slight increases in the rate of intracellular acetochlor detoxification could significantly improve the resistance of recombinant plants, it may still be possible to engineer acetochlor resistant plants using the gene.

It is unknown whether the putative A108 dihydrolipoyltransacetylase gene isolated in the present study would be of use in the production of recombinant plants with acetochlor resistance. Further research would be necessary possible to properly assess the value of the

gene in this regard. It is probable that investigations into the mechanisms that enable it to confer elevated acetochlor resistance to *E. coli* could yield some interesting insights into the mode of chloroacetanilide herbicide activity.

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