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Differential characterization of two novel antinematodal biological control agents : Streptomyces costaricus sp. nov. and a strain of Bacillus thuringiensis.

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DIFFERENTIAL CHARACTERIZATION OF TWO NOVEL ANTINEMATODAL BIOLOGICAL CONTROL AGENTS: *STREPTOMYCES COSTARICUS* SP. NOV. AND A STRAIN OF *BACILLUS THURINGIENSIS*

A Dissertation presented

by

JOSEPH ESNARD

Submitted to the Graduate School of the
University of Massachusetts Amherst in partial fulfillment
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Department of Plant Pathology

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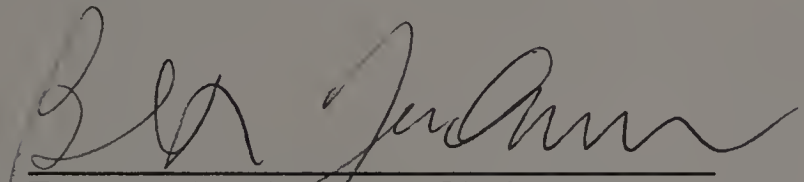

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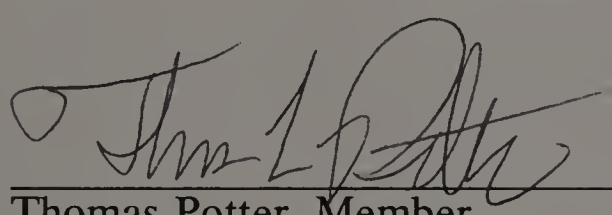

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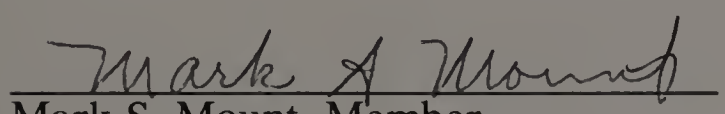

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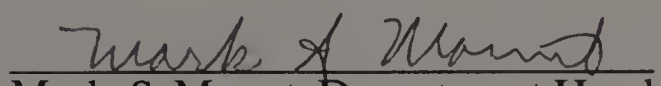

Mark. S. Mount, Department Head
Plant Pathology

DEDICATION

To my mother Celine and grandmother Eldra:

For their Love; for their Struggles;

and for the tribulations they endured.

To the memory of my father, Mr. Morrison Fedeé:

For his goodness and love.

To my dear wife Ann-Margaret and son Joshua:

the source of my happiness; gems of my life.

To the rest of my relatives, especially Andre, Junior, Maria, my mother-in-law Christine, Raphael...

For caring for me.

To Drs. Bert Zuckerman, Paul Hepperly, Antonio Sotomayor, Sean Carrington, Basil Williams and Steve Sinson, and, Mr. Mark Henecart:

For the important part that they played in my career.

To my pre-school, infant, primary and secondary school teachers:

For helping me lay a good foundation.

And to all others who encouraged me:

Because they helped to chart the course of my career.

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I have grown to love my wife and son even more after those trying times that my wife and I endured during our study towards doctorate degrees. My family's companionship was vital to my successful sojourn here at the University of Massachusetts.

ABSTRACT

DIFFERENTIAL CHARACTERIZATION OF TWO NOVEL ANTINEMATODAL BIOLOGICAL CONTROL AGENTS: *STREPTOMYCES COSTARICUS SP. NOV.* AND A STRAIN OF *BACILLUS THURINGIENSIS*

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Two novel antinematodal strains of bacteria, *Streptomyces* sp. (CR-43) and *Bacillus thuringiensis* (Bt) (CR-371) originating in Costa Rica were differentiated from other closely related strains and compared to rifampicin-resistant derivatives in greenhouse and field studies. The name *Streptomyces costaricus* is proposed for strain CR-43. The generic attribution was based on its typical morphology, production of LL-diaminopimelic acid and fatty acid composition. To clarify its taxonomic position, the isolate was compared with type strains of similar *Streptomyces spp.* The results of biochemical tests and profile analysis of hydrolyzable fatty acids indicated that CR-43 differed from previously described species and represents a new species. CR-43 (= ATCC 55274 = NRRL B-16897) is the type strain. A genotaxonomic approach to the differentiation of the streptomycetes was also taken. A modified RAPD analysis, conserved mini-sequence primed PCR (CMSP-PCR), was designed to target short

conserved sequences dispersed throughout the streptomycete genome. All species had distinct DNA profiles when DNA was amplified with either 15-mer primer 5SSU3 (5'-TGCGGCCGTACTCCC) or SSU5 (5'-CGGCAGGCCTAACAC). The DNA profile of CR-43 resembled most that of *S. h. decoyicus* using primer SSU5. Species delineation was easily achieved with CMSP-PCR DNA amplification without further enzymatic processing.

The differentiation of the novel Bt strain from five patented antinematodal Bt strains was required for patent prosecution. This was achieved by analysis of hydrolyzable fatty acid compositions of the strains by GC-MS and by DNA profile analysis. Thirty compounds in the total ion chromatograms were identified and analyzed using non-parametric statistics, the major fatty acids being i-15:0 (13.7-23.2%), i-13:0 (6.8-10.7%), i-14:0 (5.0-7.7%), 14:0 (3.0-4.1%), a-15:0 (3.9-9.9%), i-16:0 (3.6-7.2%), 16:0 (3.2-11.6%), i-17:0 (3.2-9.8%), 18:0 (tr-13.5%), a monounsaturated C16 (4.5-9.9%), a monounsaturated branched C17 (2.8-5.8%), a diunsaturated C18 (0-5.8%), and a monounsaturated C18 acid (0.3-4.8%). Molecular amplification of the DNA using a random nanomeric primer 5'-CCGAGTCCA (that could discriminate serovars) showed that RAPD profiles of the Bt's were different.

Spontaneous rifampicin-resistance (rif^+) was selected in CR-371 and CR-43. Rif^+ had a positively pleiotropic effect on the biocontrol activity of the Bt strain and a negatively pleiotropic effect on the novel streptomycete. Rif^+ was a useful marker for monitoring survival of CR-371 and CR-43 in soil.

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CHAPTER I

GENERAL INTRODUCTION

1.1 Background scenario

Chemical nematicides have been the primary means of control of plant-parasitic nematodes (along with the use of crop rotation and resistant cultivars). Strong reliance on chemicals is dramatically reflected in the $6-8 billion and $20 billion spent in the US and worldwide respectively on agricultural chemicals for plant protection (Anon. 1991). Phytonematodes cause an estimated 12% loss per annum in all major crops, costing about $8 billion to U.S. growers annually and an approximate $78 billion loss worldwide (Committee on National Needs and Priorities in Nematology, 1994). These values are certainly underestimates since part of the crop damage caused directly by nematodes are confused with other causes or go unnoticed (Seinhorst, 1981; Esnard and Zuckerman, 1996). Furthermore, significant damage occurs on home garden plants, forest trees, ornamentals, lawns, golf courses, turf grasses and greenhouse plants for which reliable estimates are unavailable.

Crop rotation, although effective, is not readily adopted by growers because in many instances the nonhost or alternative crops are not economically attractive. In countries, where the production of certain crops form the mainstay of the economy, for example banana export in St. Lucia and Dominica, crop rotation is not practiced because of institutionalized barriers and absence of markets for the

alternative crop. Plant breeding is time consuming and cultivars showing good resistance to nematodes are scarce especially among vegetables (Montalvo and Esnard, 1994). Although modern gene technology promises to shorten the time taken to develop nematode-resistant varieties (for example, through the use of NemaGene™ gene constructs developed by Advanced Technologies (Cambridge) Limited and Leeds University), such biotechnology is still mostly in experimental stages and may be an expensive enterprise for developing countries. Thus rotation and genetic improvement of crops may be hindered by technical, socioeconomic, educational, institutional and policy constraints (National Research Council, 1993).

There is continued imposition of restrictions or bans on use of most chemical nematicides in the United States, which means that they might not be an option for disease management in the near future since usage of the remaining nematicides are being re-examined and new nematicides are not being released with sufficient rapidity. For example, all granular formulations of carbofuran have been prohibited. Aldicarb and 1-3-Dichloropropene (1-3-D) have been banned on certain crops while their use on others are under special review. Methyl bromide, an effective general biocide, has been detected at unacceptable levels as an atmospheric pollutant and will most likely be banned within five years. Although chemical nematicides are effective for nematode control, in many cases they are expensive, toxic, environmentally incompatible or their effects do not last long enough. Many nematicides build up residues in food crops or infiltrate ground water.

The use of agrochemicals in developing countries will concurrently decrease as a consequence of the legislation enacted by many industrialized nations to regulate

the level of pesticides in imported food (Council on Environmental Quality, 1992; Zuckerman and Esnard, 1994a,b). The language of the recent North American Free Trade Agreement (NAFTA) suggests tighter regulation of pesticide residue levels in agricultural products imported from Mexico into the United States. Aldicarb, an effective nematicide/insecticide, was banned in St. Lucia after the chemical was detected in banana fruit exported to England.

There is therefore an urgent need for alternatives to chemical nematicides. This is consistent with the six national top ranking initiatives of the US viz. conservation and enhancement of air, soil and water resources; increased use of integrated and sustainable production systems; enhancement of food safety; protection of plants for sustained productivity; enhancement of agricultural and rural economies; and management of ecosystems to conserve and enhance biodiversity (Ken Barker, pers. comm.). Specific alternatives to chemical nematicides are offered by the development of effective biological controls (utilizing microbes and soil amendments such as green manures, chitin, collagen (Galper et al., 1990), wheat/barley mashes (areas in which the biocontrol program at University of Massachusetts has taken root)).

1.2 Current perspectives of phytonematode biocontrol

Despite concerted efforts at the development of plant disease biocontrols, there has been practically no successful commercial "live" biocontrol product available to growers for nematode management. *Bacillus thuringiensis* (Bt) has been an effective control for lepidopteran pests. Avermectins, derived from *Streptomyces*

avermitilis, are among the most potent anthelmintic, insecticidal and acaricidal compounds known (Fisher, 1993). At least eight companies have secured US patents for Bt strains (Feitelson et al., 1992). Our laboratory recently submitted a patent application (which was eventually approved) for a novel antinematodal Bt under license from the University of Massachusetts to Research Corporation Technologies, Tucson, Arizona. The characterization studies herein were prerequisites for the granting of the patent. A *Bacillus thuringiensis* (Bt) strain (UZ404-8d) significantly reduced soybean cyst nematode infestation (Zablotowicz et al., 1991 abstr.). Since the publication of the latter abstract, nothing is known of the strain UZ404-8d. *Pseudomonas aureofaciens* is also just mentioned as giving >50% reduction of ring nematodes (*Criconemella xenoplax*) on peach (Kluepfel and McInnis, 1991 abstr.).

Another product, Clandosan®, a formulation derived from chitin and urea, reached the commercial stage (Igene, Biotechnology, Columbia, MD). Although the product has given acceptable control of *Heterodera avenae* and *Tylenchulus semipenetrans* accompanied by significant yields (Spiegel et al., 1989), no significant control was achieved when tested in the northern, cooler regions of the U.S. (LaMondia, 1992; N. Jackson, University of Rhode Island, NE-171, 1994). Moreover the cost of applications of the most recent Clandosan® formulations is too high to be commercially viable (Zuckerman and Esnard, 1994).

Green manures generate considerable interest as they have been shown to be economically feasible as a substrate that allows effective colonization of the

biocontrol organisms in the soil environment and direct nematode control (Stirling, 1991). The use of antagonistic plants has received considerable attention in more tropical areas (Alam et, 1990; Rodriguez-Cabana, 1990; Vincente and Acosta, 1987). Many tropical legumes have been postulated to produced root exudates that act as confusates to host finding (Marban-Mendoza et al., 1992).

Pasteuria penetrans, an obligate parasite of root-knot nematodes, has given successful control under greenhouse (Stirling, 1990) and field plot conditions (Dickson, D., personal comm.). Its obligate nature presents a difficulty in producing sufficient biomass for commercial success. All attempts to date including the roller-bottle trixenic system of cultivation for the bacteria developed by Genetics Inst. (Cambridge, MA), later transferred to Ecogen, Inc., have thus far been unsuccessful. Despite these difficulties, the *P. penetrans-Meloidogyne incognita* system provides a useful model for studying specific attachment phenomena between microorganisms and thus the nature of biological control (Zuckerman et al., 1995 (submitted)).

Fungi have been tried but without any commercially successful biocontrol product yet. The egg parasite, *Paecilomyces lilacinus*, has given very variable results under field conditions (Alam, 1990; Cabanillas and Barker, 1989; Stirling, 1991). Asiatic Technologies (Manila, Philippines) markets a commercial product formulated by growing *P. lilacinus* on a coconut substrate (Anon. 1991). However several *Paecilomyces* spp. are known human opportunistic pathogens. Products containing *Arthrobotrys irregularis* and *A. robusta* are marketed in France (B'Chir et al., 1983) but

field results using the former (Royal 350) have been inconsistent for control of root-knot nematodes. The *A. robusta* strain (in Royal 300) was developed for control of *Ditylenchus myceliophagus* on mushrooms (Cayrol et al., 1978) but this biocontrol might not be necessary on this crop since sterilization is very effective in eliminating nematode problems. The original isolate of *Drechmeria coniospora*, another nematode parasite, lost its pathogenicity to root-knot nematodes and has not been recovered from the point of origin (Zuckerman et al., 1989b). *Hirsutella rhossiliensis*, another endoparasite, has been extensively studied in the US for its biocontrol potential (Jaffee and Zehr, 1985; G. Abawi, personal. comm.).

Natural reduction of nematodes in suppressive soils have been exploited by several researchers including the U. Mass. research team (Lumsden et al., 1987; Zuckerman et al., 1989a). Natural reduction of damage caused by *Heterodera avenae* on oats was among the first observed (Gair et al., 1969). Several fungal parasites of the eggs and cysts of this nematode were isolated by Kerry and co-workers (Forrest and Robertson, 1986, Kerry et al., 1982). Among the fungi, *Verticillium chlamydosporium* and *Nematophthora gynophila* gave successful control in greenhouse studies (Kerry, 1990; N. Viane, personal comm.). However, population increases of these natural antagonists have not been successful under field conditions (Kerry, 1990).

1.3 Antinematode biocontrol research at University of Massachusetts

Nematologists of the north-eastern region of the US have been pre-occupied for the past 3 years with research on the "design and evaluation of new approaches for the management of plant parasitic nematodes" under US Regional Research Nematology Project NE-171 (NE-171 1994 Annual Report). The University of Massachusetts (Amherst) research team (under the direction of Prof. Dr. Bert Zuckerman) started its search for alternatives to chemical nematicides by screening certain naturally suppressive soils in Mexico and Costa Rica for potential candidate microbes. The antinematodal effects of several soil isolates were tested and demonstrated. Two fungi (*Aspergillus niger* and *Paecilomyces marquandii*) and two novel bacteria were effective in field and greenhouse studies (Dicklow, 1993; Marban-Mendoza, 1992; Zuckerman et al., 1993, 1994). Because of the human pathogenicity of *A. niger* and *Paecilomyces* spp., we have de-emphasized research with these fungi and have focused on the use of the two bacteria with or without soil amendments.

A major problem in patenting the Bt strain, one of the bacteria discovered (Zuckerman et al., 1995), centered around a previous claim for nematicidal activity for five Bt strains patented in 1992 by Mycogen (Edwards et al., 1992). The existence of these prior patents by Mycogen resulted in an initial rejection of the patent application for Bt strain CR-371 by the US patent office because the examiner

claimed that granting of another patent for another antinematodal Bt would be redundant (Zuckerman and Esnard, 1994). To move the patent process forward, our laboratory was obliged to demonstrate that Bt CR-371 was novel and different from the five patented Mycogen Bt strains. The novel antinematodal *Streptomyces* species (CR-43) also faced problems in the patent process. APHIS required comparisons with plant pathogenic streptomycetes and the patent office required comparisons with the nematicidal *S. avermitilis* while the editorial board of the International Journal of Systematic Bacteriology required comparisons with six *Streptomyces* spp. showing close resemblance culturally and physiologically to the novel CR-43 strain.

In addition to pyro- and chemo-taxonomic methods for the characterization of novel strains, molecular taxonomy (based on DNA parameters) is now virtually a compulsory study for the description of novel organisms (Sigee, 1993) and is therefore part of the characterization studies herein. The molecular approach detects a greater amount of genetic variation than, for example, allozyme electrophoresis (Black, 1993) and gives a more direct relationship between organisms than conventional taxonomy which is based on phenotypes that reflect essentially only part of the genome (Goto, 1990). In fact, the ability of microbes to compete, survive and multiply in/on plants or in the soil environment to a large extent is determined by their genetic constitution (Sigee, 1993). In the genus *Streptomyces*, Beyazova et al. (1993) found that presently used physiological tests reflect too little of the genome to be universally useful in species characterization. Strain CR-43 and CR-371 had not been previously characterized or differentiated genetically from other closely related bacteria.

The following chapters therefore present the results of various approaches to the differentiation of these novel antinematodal microbes, *B. thuringiensis* strain CR-371 and *Streptomyces* sp. nov. CR-43, from already described microbes.

1.4 Objectives

The specific objectives of this study were:

1. To characterize the novel antinematode Bt strain CR-371 to the extent that would differentiate it from already-described closely related or anthelmintic strains by gas chromatographic-mass spectrometric analysis of their hydrolyzable fatty acids.

2. To describe the novel anti-nematodal/anti-fungal *Streptomyces* species (CR-43) as a species nova from Costa Rica using taxonomic criteria of the International Streptomyces Project (ISP) and to differentiate it from closely related species.

3. To design a novel molecular genetic approach for fingerprinting streptomycete genomes, based on the use of primers with rRNA gene mini-sequences and to use the approach for the differentiation of the streptomycete biocontrol from related species.

4. To differentiate the novel *Bacillus thuringiensis* strain CR-371 from closely related Bt's by generation and comparison of Random Amplified Polymorphic DNA (RAPD) profiles.

5. To produce rifampicin resistant CR-43 and CR-371 mutants to facilitate survival studies, assess efficacy and survival (persistence) of these marked biocontrols in greenhouse and field microplot experiments with or without soil amendments, and to detect any genetic differences between mutants and wild-types using arbitrary primer PCR.

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CHAPTER 2

DIFFERENTIATION OF NOVEL Bt (CR-371) FROM SIX STRAINS OF *BACILLUS THURINGIENSIS* BY HYDROLYZABLE FATTY ACID COMPOSITION

2.1 Chapter Abstract

Six potential biocontrol strains of *Bacillus thuringiensis* grown under identical conditions on fatty-acid-free King's B medium were differentiated on the basis of their hydrolyzable fatty acid content. Thirty four compounds were identified as their methyl esters in hydrolyzates among which the major fatty acids were i-15:0 (13.7-23.2%), i-13:0 (6.8-10.7%), i-14:0 (5.0-7.7%), 14:0 (3.0-4.1%), a-15:0 (3.9-9.9%), i-16:0 (3.6-7.2%), 16:0 (3.2-11.6%), i-17:0 (3.2-9.8%), 18:0 (tr-13.5%), a monounsaturated C16 (4.5-9.9%), a monounsaturated branched C17 (2.8-5.8%), a diunsaturated C18 (0-5.8%), and a monounsaturated C18 acid (0.3-4.8%). A cyclopropanoid fatty acid, cy17:0(9), was detected at low concentration in three strains including the anthelmintic strain CR-371. The presence or absence and the relative ratios of certain chromatographic peaks in combination with a Wilcoxon-signed-rank-test analysis of pair-wise differences in the total chromatographic profile distribution were used to differentiate the six bacterial strains.

2.2 Introduction

Bacillus thuringiensis (**Bt**) is the most widely used bacterial species for biological control of insects. Tanada and Kaya (1993) recently reviewed the importance of the Bacillaceae in biocontrol of insects. The potential of **Bt** products as alternatives to synthetic pesticides broadened with the discovery of **Bt** strains toxic to pathogenic protozoans, parasitic liver flukes and mites (Feitelson et al., 1992). Recently, Zuckerman et al (1993) demonstrated, for the first time in a field trial, the effective control of plant-parasitic nematodes by a novel strain of **Bt** (designated CR-371). Biocontrol methods using **Bt** are being applied in many countries throughout the world (Powell et al, 1990).

Increasingly new **Bt** strains specific for protozoan, liver fluke, mite, nematode and insect pest management are being discovered. Rapid, accurate and economically attractive methods for their differentiation from previously isolated **Bt** strains are needed. Among other considerations, definitive differentiation is needed when organisms are patented. In this study, gas chromatographic profile analysis of the hydrolyzable fatty acids from several biocontrol strains including CR-371 was explored. Because fatty acids are suitable for instrumental analysis and are synthesized by highly regulated enzyme systems, they have proved extremely useful in bacterial systematics (Komagata and Suzuki, 1987; Sasser, 1990; Siegel, 1993). Fatty-acid-based groupings agree with DNA homology (Roy, 1988; Huys et al., 1994).

Fatty acids have the general formula $CH_3(CH_2)_nCOOH$ where $n \geq 1$ i.e. all carboxylic acids above acetic acid (Lewis, 1993). They are classed among lipids, soap and waxes, and may be saturated or unsaturated (olefinic). Aromatic fatty acids also exist (e.g. phenyl stearic acid). When the active hydrogen of a fatty acid (RCOOH) is replaced (during esterification) by an alkyl group of a monohydric alcohol (R'OH) a fatty ester (RCOOR') is formed. Methanol is commonly used to form fatty acid methyl esters ($RCOOCH_3$). The latter have higher vapor pressures than the corresponding fatty acids and are distilled more easily. The bacilli and some actinomycetes characteristically contain substantially high levels of branched-chain fatty acids (Kaneda,1963, 1966, 1967, 1977; Minnikin et al, 1979; Saddler et al., 1987), that are in some cases unsaturated (Fulco, 1967; Kaneda, 1972; Fujii and Fulco, 1977). Unsaturated branched-chain fatty acids have been shown to be important components of *Bacillus subtilis, B. megaterium, Brevibacterium fermentans* and *Streptomyces griseus* in their adaptation to growth at low temperatures (Suutari and Laakso, 1992a, 1992b).

Virtually all studies reported thus far that have used fatty acid composition for characterization within the genus *Bacillus*, demonstrate successful differentiation of species but not strains. This study was undertaken to demonstrate the capability to differentiate **Bt** strains based on comparisons of their hydrolyzable fatty acid composition. It is essentially the differentiation of the novel antinematode strain (CR-371) from five of Mycogen's already-described **Bt** strains which are purportedly active

against plant-parasitic nematodes (Edwards et al., 1992) but which, to our knowledge, have not been demonstrated efficacious in authentic field trials.

2.3 Materials and Methods

2.3.1 Bacterial strains

Six strains of *Bacillus thuringiensis* were analyzed. **Bt** strain CR-371 (ATCC 55273) was a novel organism while NRRL B-18243, NRRL B-18244, NRRL B-18245, NRRL B-18246, NRRL B-18247 were five different patented strains of Mycogen (Edwards et al., 1992) obtained from the American Type Culture Collection. CR-371's potential to reduce population levels of several plant-parasitic and/or free living nematodes has been demonstrated (Zuckerman et al., 1993). All strains had been previously stored at -80°C in cryopreservation buffer [per 200 ml, 20 ml 1 M NaCl, 10 ml 1 M phosphate buffer, 60 ml glycerol; after autoclaving, 0.6 ml 0.1 M $MgSO_4$]. Each **Bt** strain was quadrant streaked onto solid fatty-acid-free King's B medium (*Pseudomonas* Agar F) in triplicate 6-cm Petri plates and grown in the dark for 48 h at 28 ± 0.1°C.

2.3.2 Extraction and derivatization of fatty acids

The fatty acid extraction procedure was a modification of that of Vannieuwenhuyze & Sandra (1987) and of Sasser (1990). It involved saponification of bacterial lipids with KOH and their methylation with methanolic HCL. Fifty milligrams wet weight of cells were carefully scraped from the forth quadrant of three plates of each **Bt** strain. King's B medium (50 mg in triplicate) was used as the

control. Each sample was placed in a 5-ml borosilicate serum bottle and 1 ml 5% KOH in 50% aqueous methanol was added. The serum bottles were crimped-sealed with a teflon-faced silicon rubber septa with one-piece tear-away aluminum caps, shaken rapidly for 2-3 s and then placed in a heating module at 100°C for 30 min with occasional shaking. The contents were allowed to cool to room temperature after which the aluminum caps were removed. One and a half milliliters of 25% hydrochloric acid (12.1 N) in methanol were added to each bottle which was sealed, agitated and then returned to the heating module for 10 min at 80°C. After cooling, methylated fatty acids were extracted by adding 1 ml of hexane to each serum bottle which was then shaken vigorously (with the teflon septum held in place by hand) for 15 s. The aqueous (bottom) phase was discarded and the hexane fraction was washed with 1 ml phosphate buffer [0.025M KH_2PO_4 + 0.025M Na_2HPO_4, pH 7] by agitating for 15 s. Each fatty acid solution was transferred to a small vial and assayed immediately by capillary gas chromatography or stored at -10°C.

2.3.3 Gas chromatography - mass spectrometry

A Hewlett Packard model 5989A GC-MS system (Avondale, PA, USA) was used. The chromatograph was equipped with a Hewlett-Packard polyimide coated fused silica HP-5 capillary column (30 m x 0.25 mm i.d.) coated with a 0.25 μm film of cross-linked bonded phenyl (5%)-dimethyl siloxane (95%) gum. Helium served as the carrier gas (column head pressure 70 kPa). Initial column temperature was held at 60°C for 1 min then increased 4°C/min to 250°C and held for 10 min. Injection was at 250°C in the splitless mode. The capillary column was directly coupled to the ion source through a heated transfer line. The mass spectrometer was operated in the

electron impact (70 eV) and ammonia chemical ionization modes (CI). Under CI conditions, the ammonia gas pressure was set at 0.9 Torr and the source temperature at 150°C.

2.3.4 Gas chromatography - flame ionization

To test the reproducibility of the results, analyses were repeated three times on a Varian 3700 gas chromatograph equipped with a flame ionization detector (FID, 250°C) at the end of a 50 m BP-5 column (SGE, i.d. 0.22 mm with 0.25 μm film). On-column injection techniques were used (Grob and Grob, 1978). The helium carrier gas head pressure was 141 kPa. The same temperature program in GC-MS analyses described above was followed. Peak areas were normalized on the largest peak, i-15:0. The coefficient of variation ranged from 0.64 to 1.01 % (n = 3).

2.3.5 Peak assignments

Peak assignments were made by comparing retention times and mass spectra to data obtained with a standard mixture of bacterial fatty acid methyl esters (Matreya, Cat. #1114). Relative retention times were determined by calculations relative to an internal standard ($C_{22}H_{46}$). Saturated branched-chain fatty acids were identified by cochromatographic comparisons of their relative retention times to those of the standards (Suutari and Laakso, 1993). **Bt** fatty acids not represented in the standard fatty acid mixture were identified by searching several mass spectral data bases (McLafferty and Stauffer, 1989; Ausloos et al., 1992; Mass Spectrometry Data Centre, Imperial Chemical Industries, U.K., 1993) and comparisons of their relative retention times with published results (Suutari and Laakso, 1993) and/or mass spectral interpretation.

2.3.6 Statistical analyses

A simple algebraic formula was introduced for pair-wise differentiation of the **Bt** strains based on F, the proportion of hydrolyzable fatty acid types in the elution profile common to both strains (s_1 and s_2):

$$F = \frac{n(s_1 \cap s_2)}{n(s_1 \cup s_2)} \qquad (1)$$

where $n(s_1 \cap s_2)$ = the number of common fatty acids shared by both sets (strain profiles), and $n(s_1 \cup s_2)$ = the total number of distinct fatty acids between both strain profiles (shared fatty acids counted only once). When F was less than one, the two **Bt** strains being compared were considered dissimilar. Theoretically, $0 \leq F \leq 1$. Brown et al (1979) had introduced a similar formula for DNA divergence studies:

$$F = \frac{n_{12}}{n_1 + n_2 - n_{12}} \qquad (2)$$

where n_1, n_2 = sizes of populations 1 and 2 respectively and n_{12} = number of restriction fragments common to both populations, for estimating the proportion of ancestral restriction sites that remain unchanged in two populations. The shared presence or absence of certain bacterial fatty acids (Lambert et al, 1983) and the relative ratios (Roy, 1988; Chase et al, 1992) of certain fatty acid methyl esters have been used to differentiate bacteria.

In addition, distributions of hydrolyzable fatty acids from the **Bt** strains were compared using Wilcoxon's signed rank test (Wilcoxon, 1945; 1947) which took into

account both the direction and size of the differences between two sets of paired measurements. Before applying Wilcoxon's test , the area counts in the profiles from each **Bt** strain were first "normalized" by dividing all peaks by the area corresponding to the same large peak (i-15:0) in each chromatogram. Statistical analyses were performed using MSTAT-C software (Michigan State University, MI). The motivation for using the Wilcoxon's signed rank test rather than more complex procedures was that it provided a means of analysis without making assumptions regarding the distribution underlying the population of measurements. An assumption of the existence of a normal distribution in chromatographic measurements or molecular genetic data for example may not be acceptable (Excoffier et al., 1992; Massart and Kaufman, 1983). Excoffier (1992) used a permutational approach to analyze the differences between DNA profiles. In commonly used statistical linear discriminant analysis or multivariate analysis of variance procedures, hundreds of samples are needed to describe a multivariate normal population adequately (Han et al., 1990). The number is dependent on the number of variables used in the comparison (Lavine et al., 1988). Other limitations are that values must be assigned for each observation in a sample's data set. This requires use of estimation techniques when observations for a given compound are below the analytical detection limit in some but not all data sets. The alternative is to reject the "non-detect" data and in the process lose information which may very effectively discriminate target populations.

2.4 Results and Discussion

For patenting purposes it became necessary to differentiate the antinematodal **Bt** from five already-differentiated **Bt** strains patented by Mycogen and nematicidal to plant-parasitic nematodes. GC-MS and GC-FID analyses of hydrolyzable fatty acids showed clear differences in all combinations of the six strains taken a pair at a time. It was possible to separate the six strains based on (i) the absence or presence of certain fatty acids, (ii) ratios of certain peaks within a chromatogram and (iii) results of the Wilcoxon matched-pairs Signed-Ranks Test.

2.4.1 GC profiles

Figures 2.1 A-F show the total ion chromatograms of the fatty acids from six **Bt** strains grown under identical conditions. These profiles appear similar and would probably lead to tight clustering if it were appropriate to use parametric statistical analysis (where a Type II error could be committed). Table 2.1 provides the peak assignments for 34 compounds in the profiles identified as their methyl esters.

Thirteen fatty acids were found in high amounts in the six strains: i-15:0 (13.7-23.2%), i-13:0 (6.8-10.7%), i-14:0 (5.0-7.7%), 14:0 (3.0-4.1%), a-15:0 (3.9-9.9%), i-16:0 (3.6-7.2%), 16:0 (3.2-11.6%), i-17:0 (3.2-9.8%), 18:0 (tr-13.5%), a monounsaturated C16 (4.5-9.9%), a monounsaturated branched C17 (2.8-5.8%), a diunsaturated C18 (0-5.8%), and a monounsaturated C18 acid (0.3-4.8%). Mass spectra of these undetermined compounds are in the appendix. The relatively high content of stearic acid (13.5%) in strain NRRL B-18247 was remarkable. To my knowledge, this is

Fig. 2.1 Total ion chromatograms of fatty acid methyl esters for **Bt** strains CR-371 (A), NRRL B-18243 (B), NRRL B-18234 (C), NRRL B-18245 (D), NRRL B-18246 (E) and NRRL B-18247 (F). Peak identities are given in Table 1. IS = internal standard.

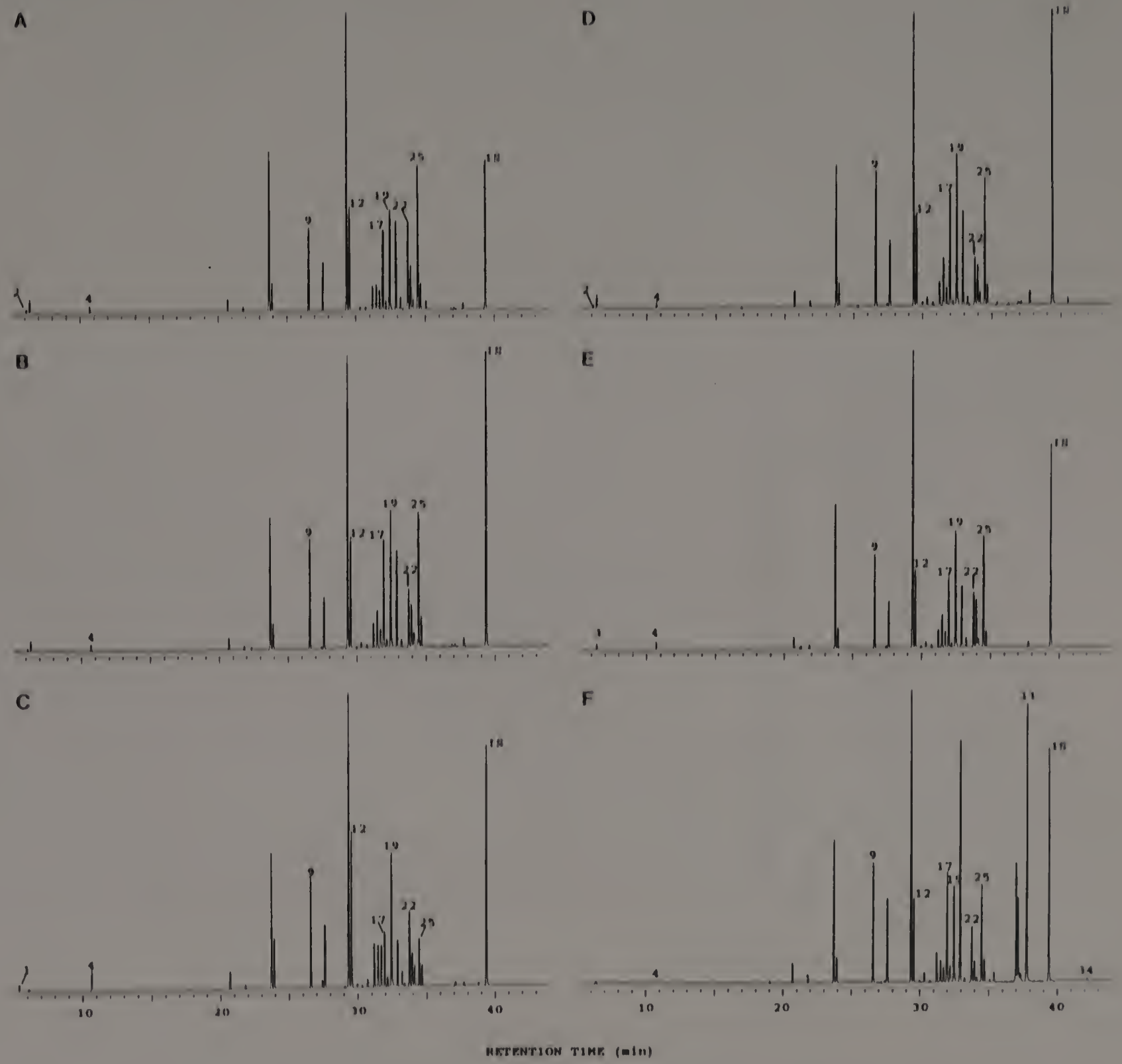
A
B
C
D
E
F
10
20
30
40
RETENTION TIME (min)

Table 2.1 Fatty acid composition of six *Bacillus thuringiensis* strains.

[a]No.	t_R[b]	Compound	MW[c]	ID[d]	%$_I$[e]	%$_{II}$	%$_{III}$	%$_{IV}$	%$_V$	%$_{VI}$
1	5.41	i-6:0	130	*	0.0	0.0	0.5	0.0	0.0	0.0
2	6.13	MTP[f]	134	*,#	0.4	0.2	0.2	0.3	0.0	0.0
3	6.37	BA[g]	146	*,#	0.9	0.5	0.0	0.9	0.4	0.2
4	10.68	BAA[h]	150	*,#	0.8	0.6	1.5	0.6	0.9	0.1
5	20.71	i-12:0	214	+,*	1.0	1.0	1.3	1.1	1.1	1.0
6	21.84	12:0	214	+,*	0.4	0.4	0.6	0.6	0.3	0.5
7	23.73	i-13:0	228	*,#	9.9	7.9	8.9	8.1	10.7	6.8
8	23.94	a-13:0	228	*,#	1.9	1.8	3.4	1.5	1.6	1.4
9	26.61	i-14:0	242	+,*	5.0	6.6	6.9	7.7	6.3	5.5
10	27.63	14:0	242	+,*	3.0	3.4	4.1	4.1	3.4	3.8
11	29.36	i-15:0	256	+,*	21.3	19.3	19.3	18.3	23.2	13.7
12	29.56	a-15:0	256	+,*	6.5	6.9	9.9	5.7	5.6	3.9
13	30.33	15:0	256	+,*	0.4	0.6	0.2	0.7	0.5	0.6
14	31.22	16:1	268	*	1.6	1.7	2.9	1.6	1.5	1.5
15	31.50	16:1	268	*	1.7	2.6	3.0	3.1	2.5	1.2
16	31.73	16:2	266	*	1.4	0.1	2.9	1.5	1.4	0.9
17	31.98	i-16:0	270	+,*	5.3	7.2	3.6	7.0	5.1	5.2
18	32.19	16:1	268	*	0.8	0.8	0.9	0.6	0.5	1.1
19	32.47	16:1	268	*	6.7	9.3	9.3	9.9	8.5	4.5
20	32.92	16:0	270	+,*	5.9	6.3	3.2	6.2	4.8	11.6
21	33.29	17:2	280	*	1.1	0.8	1.3	0.8	0.9	0.3
22	33.77	17:1	282	*	5.8	3.9	5.3	3.4	4.3	2.8
23	33.97	br17:1	282	*	2.9	3.1	2.6	2.8	3.7	1.1
24	34.14	br17:1	282	*	1.1	1.3	1.7	1.2	1.2	0.4
25	34.48	i-17:0	284	+,*	9.8	9.2	3.2	7.6	8.4	4.9
26	34.68	17:0	284	+,*	2.0	2.2	1.7	1.6	1.4	1.2
27	35.08	cy17:0(9)	282	+,*	0.8	0.2	0.0	0.0	0.0	0.3
28	35.38	br18:0	282	*	0.2	0.3	0.1	0.5	0.3	0.6
29	36.88	i-18:0	298	*,#	0.2	0.4	0.2	0.3	0.2	0.0
30	36.97	18:2	294	*	0.0	0.0	0.1	0.4	0.0	5.8
31	37.12	18:1	296	*	0.3	0.5	0.5	0.5	0.3	4.8
32	37.30	br19:0		*	0.2	0.1	0.3	0.3	0.2	0.8
33	37.75	18:0	298	+,*	0.6	0.8	0.3	1.3	0.5	13.5
34	42.21	20:0		*	0.0	0.0	0.0	0.0	0.2	0.2

[a]Peaks correspond to Figure 2.1; [b]Relative retention time in minutes; [c]Molecular weights of methyl esters based on chemical ionization mass spectra. [d]Identification based on (+) comparison with authentic standards, (*) mass spectral interpretation and data base search or (#) GC retention time; [e]Relative percentage fatty acid composition in **Bt** strain CR-371 (I), NRRL B-18243 (II), NRRL B-18244 (III), NRRL B-18245 (IV), NRRL B-18246 (V) & NRRL B-18247 (VI); [f]2-(methylthio)propanoic acid; [g]Butanedioic acid; [h]Benzylacetic acid.

the first report of such a high amount of stearic acid (18:0) in *B. thuringiensis*. Small amounts of the C17 analogue of lactobacillic acid, cis-9,10-methylenehexadecanoic acid (cy17:0(9)), occurred in three strains including the antinematodal CR-371.

Based on the types of fatty acids present or absent (Table 2.1), the nematicidal **Bt** (CR-371) was determined to be closest to Mycogen's strain NRRL B-18243, and, NRRL B-18244 most resembles NRRL B-18245. These similarities (F = 1 and 0.97 respectively) are reflected in Table 2.2 which lists the proportion (F) of fatty acids common to a pair of **Bt** strains.

2.4.2 Peak ratios

Despite this close resemblance, it was not difficult to differentiate these strains since the ratio of their fatty acid contents for certain peaks were very different (Table 2.3). The ratio of peak 22 (branched 17:1) to peak 17 (i-16:0) in strain CR-371 clearly differentiated it from strain NRRL B-18243 (Table 2.3; Fig 2.1). The ratio of these two peaks and that of peak 25 to peak 12 separate strain NRRL B-18244 from NRRL B-18245. All other pair-wise comparisons based on peak ratios showed strong differences between all **Bt** strains (Table 2.3). Quick and clear-cut separation of strains can be achieved therefore by using both the magnitude and direction (<1 versus >1) of the differences in peak ratios.

2.4.3 Statistical analyses

Non-parametric statistical analyses were used to rigorously test the significance of the differences between the six **Bt** strains. The test was selected based on the rationale of Han et al. (1990), Massart and Kaufman (1983) and Steel and Torrie (1980) that distribution-free statistics apply when the assumption of normality is unacceptable. Accordingly, both MS and FID data were analyzed by the Wilcoxon

Table 2.2 F values* from the pair-wise comparisons of the six strains of *Bacillus thuringiensis*.

STRAIN	CR-371	NRRL B-18243	NRRL B-18244	NRRL B-18245	NRRL B-18246	NRRL B-18247
CR-371	1	1	0.91	0.94	0.93	0.88
NRRL B-18243		1	0.91	0.94	0.93	0.88
NRRL B-18244			1	0.97	0.91	0.85
NRRL B-18245				1	0.93	0.88
NRRL B-18246					1	0.88
NRRL B-18247						1

*F value is the proportion of fatty acids common to any pair of strains.

Table 2.3 Ratios of certain diagnostic peaks in chromatogram of hydrolyzable fatty acids from six **Bt** strains.

Peak[a] Ratios	CR-371	NRRL B-18243	NRRL B-18244	NRRL B-18245	NRRL B-18246	NRRL B-18247
P12/P9	>1	>1	>1	<1	<1	<1
P17/P12	<1	<1	<1	>1	<1	>1
P22/P17	>1	<1	>1	<1	<1	<1
P25/P12	>1	>1	<1	>1	>1	>1
P25/P19	>1	<1	<1	<1	<1	>1
P25/P22	>1	>1	<1	>1	>1	>1
P33/P12	<1	<1	<1	<1	<1	>1

[a]Identities of peaks are listed in Table 2.1. P = Peak.

signed rank test. A test criterion and normal deviate were generated and tested for each comparison. All paired comparisons showed very highly significant differences ($p < 0.0001$; $n = 3$). These results corroborate previous conclusions from biochemical and genetic analyses that the Mycogen strains are different.

The detection of saturated iso- and anteiso-branched- and straight-chain fatty acids between C12 and C17 with i-15:0 most abundant in the six **Bt** strains agreed with the results of previous analyses on **Bt** (Kaneda, 1968). This study reports in addition C4, C6, C8, C18 and C20 hydrolyzable acids (Table 2.1) that were very useful for separation of the strains. Stearic acid (peak 33, Figure 2.1F) in particular was detected at an appreciable concentration in strain NRRL B-18247. To my knowledge this is the first report on the differentiation of **Bt** strains that utilized all components detected in each profile. Though fatty acid analyses are known to be extremely useful in bacterial systematics, (O'Donnell, 1985; Saddler et al, 1987; Komagata and Suzuki, 1987; Sasser, 1990), a majority of reports on species novae or strains presents only a few (rarely > 10) fatty acids with percentage composition. Bacterial fatty acid chromatograms usually contain more than 30 detectable peaks, the patterns of which have not been fully utilized in the differentiation of strains and species.

This study aimed to demonstrate that the novel antinematodal **Bt** strain (CR-371) is different from Mycogen's five **Bt** strains. In all cases claiming strain novelty, evidence of a difference is necessary for patenting purposes. Many studies on fatty acid content investigate the extent of clustering (or similarity) among species or

strains. It is expected that most bacterial strains of the same species would cluster to a substantial extent. However, in the absence of PCR-based methods (Brousseau et al, 1993) or DNA sequencing techniques which would give the definitive qualitative difference between strains, detection of the qualitative and quantitative differences in the total chromatographic pattern of bacterial fatty acids can be a useful tool for separation of strains.

The Massachusetts **Bt** isolate (CR-371) described herein originated from nematode suppressive soils in Costa Rica (Zuckerman et al, 1989), and was extensively tested for efficacy in controlling root-knot nematode (*Meloidogyne incognita*) and *Pratylenchus penetrans* (lesion nematode) in greenhouse trials in Massachusetts. The results of these studies and those of two-year large-scale field trials in Puerto Rico showing significant reduction in root-knot nematode injury and significant yield increases were reported by Zuckerman et al, (1993).

The claim for nematicidal activity for the five Mycogen **Bt** strains, as described by the 1992 Mycogen patent (Edwards et al., 1992), is based on laboratory trials showing nematicidal activity on the free-living nematode *Caenorhabditis elegans*. Those knowledgeable in testing for anthelmintic activity, utilize *Caenorhabditis* as a preliminary laboratory screen, indicating potential candidates for in vitro testing. In our Massachusetts laboratory, less than 1% of candidate anthelmintics which gave positive results against *Caenorhabditis* reach the field testing stage. In contrast, CR-371 is presented as different and having valid activity against plant nematodes on the basis of positive results in extensive greenhouse and field experiments.

Significant differences among the six **Bt** strains were demonstrated by a combination of mere absence/presence and relative peak ratios with a non-parametric statistical test of significance comparable in purpose to the t-test. Parametric statistical techniques were deemed inappropriate for the separation of the six strains mainly because of an otherwise inappropriate assumption of normality in each data set.

2.5 Conclusion

This study has demonstrated the capability to detect the intra-specific variation in the bacterial species *Bacillus thuringiensis* and to clearly differentiate strains on the basis of qualitative and quantitative differences in hydrolyzable whole cell fatty acid compositions. Moreover, the anti-nematode strain CR-371 is clearly differentiated from Mycogen's five nematicidal **Bt** strains. A non-parametric statistical analysis for paired comparisons was suitable for testing the significance of the differences between chromatographic profiles of the bacterial fatty acids and hence strain separation.

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CHAPTER 3

DESCRIPTION AND NAMING OF SPECIES NOVA, *STREPTOMYCES COSTARICUS*, ISOLATED FROM NEMATODE SUPPRESSIVE SOIL OF COSTA RICA

3.1 Chapter Abstract

A new bacterial strain CR-43 isolated from tropical soil and previously shown to have antinematodal and antibiotic properties is described. The name *Streptomyces costaricus* has been proposed. The generic attribution was based on its typical morphology, production of LL-diaminopimelic acid and fatty acid composition. To clarify its taxonomic position, the isolate was compared with type strains of similar *Streptomyces spp.* The results of a range of biochemical tests and profile analysis of hydrolyzable fatty acids indicated that CR-43 differed from previously described species and represents a new species. CR-43 (= ATCC 55274^T = NRRL B-16897^T) is the type strain of this new species.

3.2 Introduction

Nematode suppressive soils have been reported from several locations throughout the world. In our laboratory, microorganisms with antinematodal or antifungal properties have been isolated from suppressive soils in Mexico (Lumsden et al., 1987; Zuckerman et al., 1989) and Costa Rica (Dicklow et al., 1993). The biological basis of this suppressiveness was established by showing that heat

sterilization abolishes the suppressive effect. One of the organisms isolated from suppressive soil from Costa Rica showed both antinematodal and antifungal activity in laboratory, greenhouse and field trials (Dicklow et al., 1993). A culture of this organism (designated CR-43) was submitted for identification to the American Type Culture Collection (ATCC, Rockville, MD 20852) which concluded that the isolate was a member of the genus *Streptomyces* and could not be placed in a described species (ATCC report SC2285). Later the U. Mass. research team reported (Dicklow et al., 1993) that this isolate induced a biological effect previously unreported in that it specifically inhibited reproduction of the free-living nematode *Caenorhabditis elegans* **in vitro** under axenic conditions. This effect is referred to as antinematodal, as opposed to the nematicidal activity displayed against nematodes by organisms such as *Streptomyces avermitilis*.

Based on successful field trials for the control of several species of plant parasites, an application for a patent for CR-43 as a nematode biocontrol agent was filed by Research Corporation Technologies, Tucson, AZ 85710 under exclusive license from the University of Massachusetts at Amherst. Cryopreserved samples of CR-43 have been deposited with the ATCC (as ATCC 55274) and with the USDA/ARS Culture Collection (as NRRL B-16897). In this dissertation, a comprehensive review and description of CR-43 as a new species is provided, for which the name *Streptomyces costaricus* is proposed. Comparisons are made with plant pathogenic, anthelmintic and otherwise similar *Streptomyces* species.

3.3 Materials and Methods

3.3.1 Microorganisms

Characteristics of 9 described *Streptomyces* species were compared with those of strain CR-43, which was isolated from nematode suppressive soil in Costa Rica and stored at -80°C in cryopreservation buffer (Brenner, 1974). *S. avermitilis* (ATCC 31267^T) was obtained from the ATCC while *S. scabies* (ATCC 49173^T) and *S. acidiscabies* (ATCC 49003^T), both plant-pathogenic species, were supplied by Dr. R. Loria (Cornell university, Ithaca, NY). Comparison with plant-parasitic streptomycetes was required by APHIS since CR-43 was being introduced as a biological control agent for plant nematodes. *S. avermitilis* is a known anthelmintic species and was therefore appropriate for comparative study. Six other species, *S. hygroscopicus* (NRRL B-1865), *S. murinus* (NRRL B-2286), *S. griseoluteus* (NRRL B-1315), *S. hygroscopicus* subsp. *decoyicus* (NRRL ISP-5087), *S. rubiginosus* (NRRL B-3983) and *S. griseofuscus* (NRRL B-5429) have characteristics in common with CR-43, and were obtained from Dr. David Labeda (USDA, Peoria, IL). All bacterial strains were stored at -80°C as described above. Growth of all strains were initiated on ISP medium #2 (Shirling and Gottlieb, 1966) at 28°C. Comparisons were supplemented or confirmed with data on species characteristics already published.

3.3.2 Morphological and cultural characterization

Light microscopy was used to study the aerial mycelium and spore ornamentation of strain CR-43 which were then compared with the characteristics of the other type strains. Slide cultures were initiated in moist chambers and mycelium that was adhering to each cover slip was observed directly at 1,500x

magnification. Mycelia were also partially stained with methylene blue (as suggested by the ATCC). Standards described by Shirling and Gottlieb (1966) were used to describe spore chain morphology. Cultural characteristics of strain CR-43 and *S. hygroscopicus* subsp. *decoyicus* were determined by procedures recommended by the International Streptomyces Project (ISP) (Pridham and Gottlieb, 1948; Shirling and Gottlieb, 1966). The presence of soluble pigments was investigated on yeast extract - malt extract agar (ISP-2 = Difco yeast extract 4 g/L, Difco malt extract 10 g/L, dextrose 4 g/L, pH 7.3 before adding 20 g agar/L), oatmeal agar (ISP-3), inorganic salts-starch agar (ISP-4) and glycerol-asparagine agar (ISP-5) in this study and, on ATCC #172 medium by the ATCC. Melanoid pigment production was studied on peptone-yeast extract-iron agar (ISP-6) and tyrosine agar (ISP-7).

3.3.3 Carbon source utilization

Each carbon source, final concentration 1% (w/v), was prepared from a 10 % solution in glass-distilled deionized water sterilized by passing through a 0.22 μm Acrodisc filter. The basal salt medium used was previously described by Pridham and Gottlieb (1948). Absence or presence of growth on each medium was recorded. Data on carbon source utilization are from the current study. Data for species characteristics given in Table 1 were compared with those in the literature (Burg et al. 1979; Labeda and Lyons, 199l; Lambert and Loria, 1989a,b; Shirling and Gottlieb, 1968a,b,c, 1972). Ten carbon sources were tested viz. L-arabinose, D-fructose, D-glucose, D-mannitol, raffinose, rhamnose, sucrose, D-xylose, salicin and galactose. Acid production by strain CR-43 was tested (by ATCC) after growth in Nocardia Purple Broth (NPB) supplied with cellobiose, D-glucose, glycerol, maltose, galactose, D-mannitol, D-xylose, L-arabinose, D-fructose, lactose, sucrose, ribitol, galactitol,

erythritol or i-inositol. The feasibity of the commercial kit, BIOLOG, as an indicator of C-source utilization was explored and later abandoned because of inconsistent and unreliable results, a conclusion also drawn by Rüger and Krambeck (1994). BIOLOG relies on the irreversible reduction of a tetrazolium redox-dye to the purple formazan compound as an indicator for organic substrate oxidations.

3.3.4 Antibiotic and other inhibitory compounds

Growth was investigated on ISP medium 2 amended with several inhibitory compounds. Tests were performed on *S. costaricus, S. avermitilis, S. scabies*, *S. acidiscabies*, *S. hygroscopicus*; *S. murinus*, *S. griseoluteus*, *S. hygroscopicus* subsp. *decoyicus*, *S. rubiginosus* and *S. griseofuscus*. Data were from both this study and the published literature (Labeda and Lyons, 199l; Shirling and Gottlieb, 1968a,b,c, 1972). Sensitivity of the actinomycetes to streptomycin (20 μg/ml), penicillin (10 IU/ml), phenol (0.1%), thallium acetate (10 μg/ml), and crystal violet (0.5 μg/ml) was tested in ISP medium 2.

3.3.5 NaCl tolerance

Sodium chloride tolerance was tested on yeast extract-malt extract agar (ISP-2) supplemented with 5, 6 or 7% NaCl (wt/vol). Data are from this study and the literature.

3.3.6 pH sensitivity

The minimum pH that did not inhibit growth was determined on modified ISP medium 2 (yeast extract 4 g, malt extract 10 g, glucose 4 g, agar 20 g per liter of distilled H_2O). pH adjustments were made with 1 M HCl or 0.25 M KOH. Results were also compared with those reported in the literature.

3.3.7 Cellular fatty acid profile analysis

Streptomyces costaricus (CR-43), *S. hygroscopicus, S. murinus* and *S. griseoluteus* were quadrant streaked onto ISP-2 agar and grown aerobically in the dark for 96 h at 28 ± 0.1°C. In another experiment, *S. costaricus* (CR-43), *S. hygroscopicus subsp. decoyicus, S. rubiginosus* and *S. griseofuscus* were grown in ISP #2 broth by vigorously shaking for 92 h at 27-28°C. The fatty acid analysis was conducted (in duplicate) as described by Esnard et al. (1994) with slight modifications. Briefly, fatty acid methyl esters were prepared from 50 mg of wet cells by saponification of the bacterial lipids with KOH followed by methylation with methanolic HCl. ISP#2 media served as the control. The methyl esters were extracted in 1 ml hexane and analyzed by gas chromatography-mass spectrometry (using a Hewlett Packard HP model 5890 series II Plus GC-MS system coupled to an HP 5972 series Mass Selective detector). The GC was equipped with a glass capillary HP-5 column (30 m x 0.25 mm i.d.) and programmed to run from 60°C for 1 min, then increased 4°C/min until 250°C. Injection was at 280°C in the splitless mode. The mass spectrometer was operated in the electron impact (70 eV) and isobutane chemical ionization modes (CI). The percentage fatty acid composition was expressed relative to the total peak area (Suutari and Laakso, 1993). To differentiate closely related species, a Wilcoxon matched-pairs signed-ranks test was used to compare total ion chromatograms (Esnard et al., 1994). Parametric statistics were deemed inappropriate (Esnard et al., 1994) based on the rationale that an assumption of normality in the distribution of chromatographic measurements may not be correct (Massart and Kaufman, 1983) and that, in linear discriminant analysis or multivariate analysis of variance procedures, several hundred samples of data are necessary to adequately describe a

multivariate normal population (Han et al., 1990). The Wilcoxon signed-ranks test takes into account the size of rank order differences within corresponding pairs of peaks.

3.3.8 Cell wall composition

The method of Becker et al. (1964) was slightly modified to determine the presence/absence of the DL- or LL- form of the diaminopimelic acid (DAP) isomer in the cell wall of strain CR-43. Cells of CR-43 (test strain) and *S. hygroscopicus* (positive control) were grown for 72 h in yeast extract-malt extract-glucose medium at 28°C on a rotary shaker at 250 rpm. They were collected by filtration, washed in 75% ethanol, and air-dried at room temperature. Hydrolysates were prepared by autoclaving the dried biomass (10 mg) for 30 min at 1 atm in 6 M HCl in a 10-ml borosilicate serum bottle crimped-sealed with a teflon-faced silicon rubber septa with one-piece tear-away aluminum caps. The hydrolysates were cooled, filtered through Whatman #1 paper, washed with 3-4 drops of sterile glass distilled, deionized water (SGDDW), and dried (three times) on a boiling water bath to remove most of the HCL. 300 μl of SGDDW was added to the dried hydrolysate and 20 μl of liquid was spotted at the top of Whatman #1 paper. Ten μl of 0.01 M DL-α,ϵ-diaminopimelic acid (Sigma Chemical Co.) which contains both the L- and meso-DAP isomers were also spotted to serve as the reference standard. Descending chromatography was run for about 13 h with the solvent system methanol-SGDDW-6 M HCL-pyridine (80:26:4:10 v/v) (Staneck and Roberts, 1974). Spots were detected by dipping the paper in 0.2% ninhydrin in acetone and heating at 100°C for 2 min. DAP spots (with the L-isomer ahead of the meso-isomer) stained olive green fading to yellow while the other amino acids (which migrated ahead of DAP) stained purple.

3.4 Results and Discussion

3.4.1 The antinematodal strain

CR-43 was found to be distinctly different from related *Streptomyces* type strains used for comparison viz. *S. hygroscopicus* (NRRL B-1865), *S. murinus* (NRRL B-2286) and *S. griseoluteus* (NRRL B-1315), *S. h.* subsp. *decoyicus* (NRRL ISP-5087), *S. rubiginosus* (NRRL B-3983) and *S. griseofuscus* (NRRL B-5429). It was also shown to be different from the plant pathogens, *S. scabies* ATTC49173^T and *S. acidiscabies* ATCC49003^T, and from the nematicidal species *S. avermitilis* ATCC31267^T. Morphological, cultural and physiological characteristics are summarized in Table 3.1.

3.4.2 Morphological characteristics

The spore-bearing aerial hypha of strain CR-43 was simple (non-verticillate) and formed tightly coiled spiral spore chains on standard ISP agar media (ISP-2, 3, 4 and 5). Each spore chain consisted of 10-50 smooth spores and agrees with the observation of the ATCC. No sclerotia-like bodies, sporangia, flagellated or conidia-like spores were observed in the aerial or submerged mycelium. Fragmentation of the substrate mycelium was not observed.

3.4.3 Pigmentation

The color of the aerial mycelial mass up to or after 14 days was reddish-gray to gray-brown on yeast-malt agar, oatmeal agar, inorganic salts-starch agar and glycerol-asparagine agar. A yellow color was produced on ATCC medium #172 (N-Z amine with soluble starch and glucose (ATCC report SC2285)). On ISP-2 and ISP-5 media the substrate mycelium was light yellow and yellow to yellowish brown, respectively, (golden on ATCC medium #172); there were no distinctive pigments

Table 3.1 Differential characteristics of ten *Streptomyces* species.

Characteristics	[*]*costaricus*	[a]*avermitilis*	[b]*scabies*	[b]*ascidiscabies*	[c]*hygroscopicus*	*h. decoyicus*	[d]*murinus*	[d]*griseoluteus*	[d]*rubiginosus*	[d]*griseofuscus*
Spore mass/substrate mycelial color[e]:										
ISP medium 2	GB/Y[e]	BW/dB	G/G	W/B	G-B	G/CY	R'/Mo	G/±	G	R,G
ISP medium 3	GB/B	BG/dB	G/G	W/B	G-B	W-CG/B	R.G/GY	G/±	G	R,G
ISP medium 4	GB/Y	BG/GY	G/G	W/B	G-B	GˆB/BG'	G/Mo	G/±	G	R,G
ISP medium 5	GB/Y-YB	BG/YB	G/B	W/B	G-B	G/GY	R.G/GY	+-/±	G	R,G
Spore chain morphology[f]	sp	sp	sp	f	sp	*sp	sp	rf	sp	sp
Spore number per chain	10-50	>15	≥20	≥20	*>10	*>10	10-50	≈<10	10-50	10-50
Spore ornamentation[g]	sm	sm[g]	sm	sm	r	ND	sm	sm	sy	sm
Melanoid pigment on:										
ISP medium 6	-	+	+	-	-	-	-	-	-	-
ISP medium 7	-	+	+	-	-	-	-	-	-	-
Soluble pigment in:										
ISP medium 2	Y	B	-	ND	-	-	Y	ND	-	-
ISP medium 3	-	B	-	ND	-	-	Y	ND	-R	-
ISP medium 4	-	YB	-	Y/R[h]	-	-	Y	R-BG	-	-
ISP medium 5	Y	YB	-	Y/R	-	-	Y	ND	-	-
pH effect on soluble pigment	-	ND[e]	-	+	*-	-	-	+	-	-
Carbon utilization:										
L-arabinose	-[i]	+	+	+	+	*-tr	-/tr	+	*-	+
D-fructose	+	+	+	+	+	*+	+	+	+	+
D-glucose	+	+	+	+	+	*+	+	+	+	+
D-mannitol	+	+	+	+	+	*+	+[i]	+	+	+
Raffinose	-	+	+	-	-	*-	-/tr	-/tr	-tr	-tr
Rhamnose	-	+	+	+	+	*-tr	-/tr	-/tr	+	-tr
Sucrose	-	+	+	+	-	*-tr	-/tr	-/tr	+	-tr
D-xylose	+	+	+	+	+	*-tr	+	+	+	+
Salicin	+	*-	+	*+	*-tr	*-	*+	*-tr	*-tr	*-
Growth with										
5% NaCl	-	*+	+	*+	+	*-tr	*+	*+	*-	*-
6% NaCl	-	*-tr	+	*+	+	*-tr	*+	*-tr	*-	*-
7% NaCl	-	*-tr	-	-	+	*+[j-tr]	*+	*-tr	*-	*-
Phenol (0.1%)	-	*-tr	-	+	*-tr	*-	*-tr	*+	*-	*+
Thallium acetate (100 μg/ml)	-	*-tr	-	-	*+	*-	*+	*-	*-	*-
Crystal violet (1 μg/ml)	+	*-	-	+	*+	*-	*+	*-	*-	*-
Streptomycin (20 μg/ml)	-	*-	-	+	-	*+[j-tr]	*-	*-	*-	*-tr
Penicillin (10 μg/ml)	+	*-	-	+	*+	*+	*+	*-	*-tr	*+
Minimum pH for growth	3.5	*4.5	5.0	4.0	*3.5	*4.5	*3.5	*4.2	*4.9	*3.3
LL-Diaminopimelic acid	+	+	+	+	+	+	+	+	+	+

[a]Morphological and cultural data also reported by Burg et al. (1979); [b]Lambert and Loria (Lambert & Loria, 1989a,b); [c]Labeda and Lyons (1991), Pridham and Tresner (1974); and [d]Shirling and Gottlieb (Shirling and Gottlieb, 1968a,b,c, 1972). [e]GB, gray brown; BG, brownish grey; BW, brownish white; Y, yellow; Go, golden; CY, creamish yellow; dB, dark brown; G, gray; CG, carbon grey; BG', bluish grey; GˆB, grey-black with white specks; W, white; B, brown; G-B, gray to brown; R', red series; Mo, grayed yellow or yellow modified by red; R.G, red or gray; GY, grayed yellow; Y-YB, yellow to yellow brown; R-BG, red to bluish gray; R,G, typically light grayish reddish brown to light grayish brown; YB, yellowish brown; ±, not distinctive (GY to YB or olive brown); +-, aerial mycelium poorly developed or absent; ND, not determined. [f]sp, spiral; f, flexuous; rf, rectiflexuous; w, warty; ≈, common. [g]sm, smooth; r, rough; sy, spiny. [h]Color on acid/alkaline media respectively. [i]+, positive reaction; -, negative reaction; tr, trace. [j]Pridham and Tresner (1974). * Novel results from this study only.

produced on ISP-3 or ISP-4 agars although a brown or yellow color was observed. A yellow diffusible pigment was produced on ISP-2, ISP-5 and ATCC medium #172 that was not sensitive to pH changes effected by addition of HCl or NaOH. No melanoid pigments were observed on tyrosine agar and peptone-yeast extract-iron agar media.

3.4.4 Physiological characteristics

Observations in each replication were consistent for each *Streptomyces* species tested. On basal medium (Pridham and Gottlieb, 1948), CR-43 utilized D-fructose, D-glucose, D-mannitol, D-xylose, salicin and galactose but not L-arabinose, raffinose, L-rhamnose and sucrose as sole carbon sources. This pattern of carbon source utilization was different from that of all comparison strains (Table 3.1). CR-43 tolerated pH ranges below 4.0, grew in the presence of 1 ug/ml crystal violet or 10 IU/ml penicillin. Growth was inhibited in media containing NaCl at ≥5%, thallium acetate at 100 ug/ml, phenol at 0.1% or streptomycin at 20 μg/ml while type strains of other spp. responded differently. An additional reaction of strain CR-43 growing in NPB (ATCC report SC2285) was acid production when the medium was supplemented with cellobiose, glucose, glycerol, maltose, galactose, mannitol or xylose but not with L-arabinose, fructose, lactose or sucrose. There was no growth in NPB supplemented with ribitol, galactitol, erythritol or i-inositol. Strain CR-43 exhibited detectable antibiosis to *Rhizoctonia solani* and antinematodal activity against *Caenorhabditis elegans*. The antinematodal activity was also previously reported from laboratory, greenhouse and large-scale field trials (Dicklow et al., 1993).

3.4.5 Chemical properties

Analysis of whole cell hydrolysates showed the presence of the LL-diaminopimelic acid isomer ($R_{LL\text{-}DAP}$= 0.8) in the cell walls of strain CR-43 and

other strains. This provided additional justification for placing CR-43 in the genus *Streptomyces*. The fatty acid pattern was characteristic of the genus *Streptomyces* (Kataoka and Nojima, 1967; Kroppenstedt, 1985; Saddler et al., 1987) consisting mainly of a-15:0, 16:0, a-17:0, i-15:0, i-16:0 and i-17:0 on agar media (Table 3.2; Fig. 3.1, 3.2).

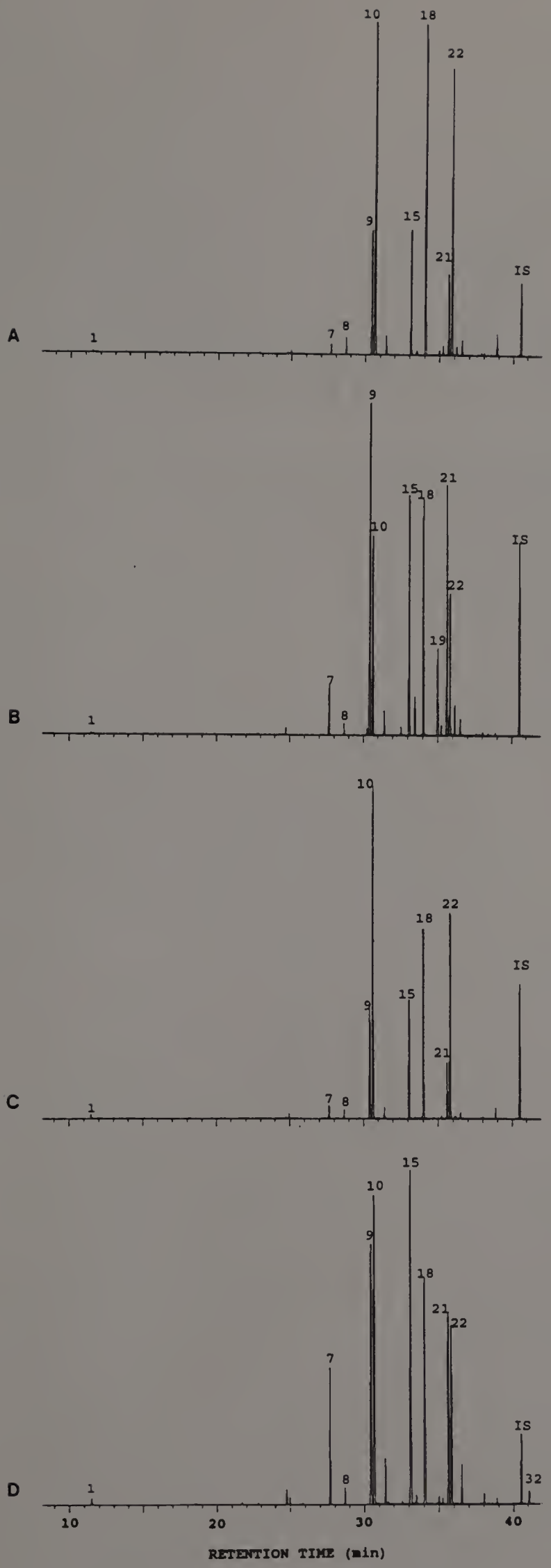
3.4.6 Differentiation from described species

Strain CR-43 was compared with described species chosen on the basis of their potential relatedness. A subculture of CR-43 sent to the ATCC could not be placed in a described species (ATCC report SC2285). After surveying the various species of *Streptomyces* described in Bergey's Manuals (William et al., 1989) and by the International Streptomyces Project (Shirling and Gottlieb, 1966, 1968a,b,c, 1972) and in discussions with those conversant with *Streptomyces* taxonomy, strain CR-43 was found most similar to six species viz. *S. hygroscopicus, S. murinus, S. griseoluteus, S. hygroscopicus* subsp. *decoyicus*, *S. rubiginosus* and *S. griseofuscus*. However, CR-43 differed from these species tested in their total reactions in physiological and cultural tests, fatty acid profiles and/or gross morphology (Tables 3.1, 3.2; Fig. 3.1). Culturally, CR-43 differs from the species that has the closest fatty acid profile from agar culture, the type strain *S. murinus*, in the absence of a soluble pigment when grown on oatmeal agar (ISP-3) or inorganic salts-starch agar (ISP-4) media. In addition the color of *S. murinus* spore mass is in the red-color series on ISP-2, red or gray on ISP-3 or ISP-5, and gray on ISP-4 while CR-43 produces a distinct and consistent gray-brown color when it sporulates on ISP medium #2, 3, 4 or 5 after 14 days. The substrate mycelium of CR-43 is brown on oatmeal and yellow on inorganic salts starch agar media while that of the type strain of *S. murinus* is grayed yellow on the former and modified by red on the latter medium. CR-43, but not *S. rubiginosus*

Table 3.2. Fatty acid composition of *Streptomyces costaricus* (CR-43) grown on ISP #2 agar and broth media.

Peak No.	Compound[a]	Culture Agar[b] %	Culture Broth[c] %
1	BAA[d] (t_R[e] = 1.00)	0.1	0.1
2	i-12:0	nd[g]	0.1
3	12:0	0.1	0.2
4	i-13:0	0.1	0.2
5	a-13:0	0.2	0.2
6	13:0	<0.1	0.1
7	i-14:0	0.6	3.8
8	14:0	1.0	2.4
9	i-15:0	8.4	6.7
10	a-15:0	25.2	13.6
11	br14:0	<0.1	nd
12	15:0	1.2	2.3
13	ND[f]	0.1	0.1
14	16:1	0.1	nd
15	i-16:0	8.1	12.5
16	i-16:1	0.3	0.2
17	ND	nd	0.3
18	16:0	24.2	22.5
19	i-17:1	0.3	0.2
20	a-17:1	0.6	0.2
21	i-17:0	5.5	2.5
22	a-17:0	20.3	6.7
23	br17:0	nd	0.3
24	cy17:0(9)	0.6	1.4
25	17:0	1.0	nd
26	ND	<0.1	nd
27	18:1	<0.1	nd
28	ND (MW = 298)	0.2	5.0
29	i-18:0	0.2	4.9
30	ND (MW = 296)	<0.1	0.6
31	18:0	1.4	12.8
32	ND (MW = 290)	0.1	0.1

[a]Identification based on a combination of comparisons with authentic standards, mass spectral interpretation and data base search, and relative GC retention times. br = branched.
[b,c]Mean of 3 extractions from growth on ISP #2 agar and broth media (CV ranged from 0.90% to 2.99%). Peaks correspond to Fig. 3.1 and 3.2 respectively.
[d]Benzylacetic acid
[e]Relative retention time (min).
[f]ND, Not Determined (MW = Molecular weights of methyl esters based on chemical ionization mass spectra; mass spectra in Appendix). [g]nd, not detected.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RETENTION TIME (min)

or *S. griseofuscus*, produces soluble pigments when grown on ISP #2 and #5 media. In addition, *S. rubiginosus* produces spiny spores and utilizes L-rhamnose and sucrose as sole carbon sources. CR-43 does not utilize L-arabinose while *S. griseofuscus* does so.

For more critical examination of the species, non-parametric statistical analyses were used to identify the significantly different fatty acid profiles, in pair-wise comparisons with CR-43. Differences in total ion chromatograms were analyzed by the Wilcoxon signed rank test in which a test criterion and normal deviate for normalized percent compositions were generated and tested in each comparison using MSTAT-C software (Michigan State University, MI). All paired comparisons among species grown on solid or liquid medium yielded highly significant differences ($p < 0.001$). The closest species to CR-43 culturally and physiologically that had been grown on solid agar, viz. *S. murinus* (NRRL B-2286), was easily differentiated from the new strain by comparing the ratios of i-14:0 to 14:0, a-15:0 to 16:0, and of 16:0 to a-17:0 in *S. murinus* and CR-43, respectively (Fig. 3.1, Table 3.3). Among the broth cultures, *S. hygroscopicus decoyicus* (NRRL ISP-5087) gave the closest fatty acid profile to that of CR-43. The ratio of i-17:0 (peak 21) to i-15:0 (peak 9), and i-16:0 (peak 15), a-17:0 (peak 22), or i-18:0 (peak 29) (which is <1 in CR-43 but >1 in *S. hygroscopicus decoyicus*) clearly differentiated the two species (Fig. 3.2, Table 3.3). Also, after 7 days of growth on ISP medium #2 agar, CR-43 appeared yellow while a white coloration was observed for *S. h.* subsp. *decoyicus*.

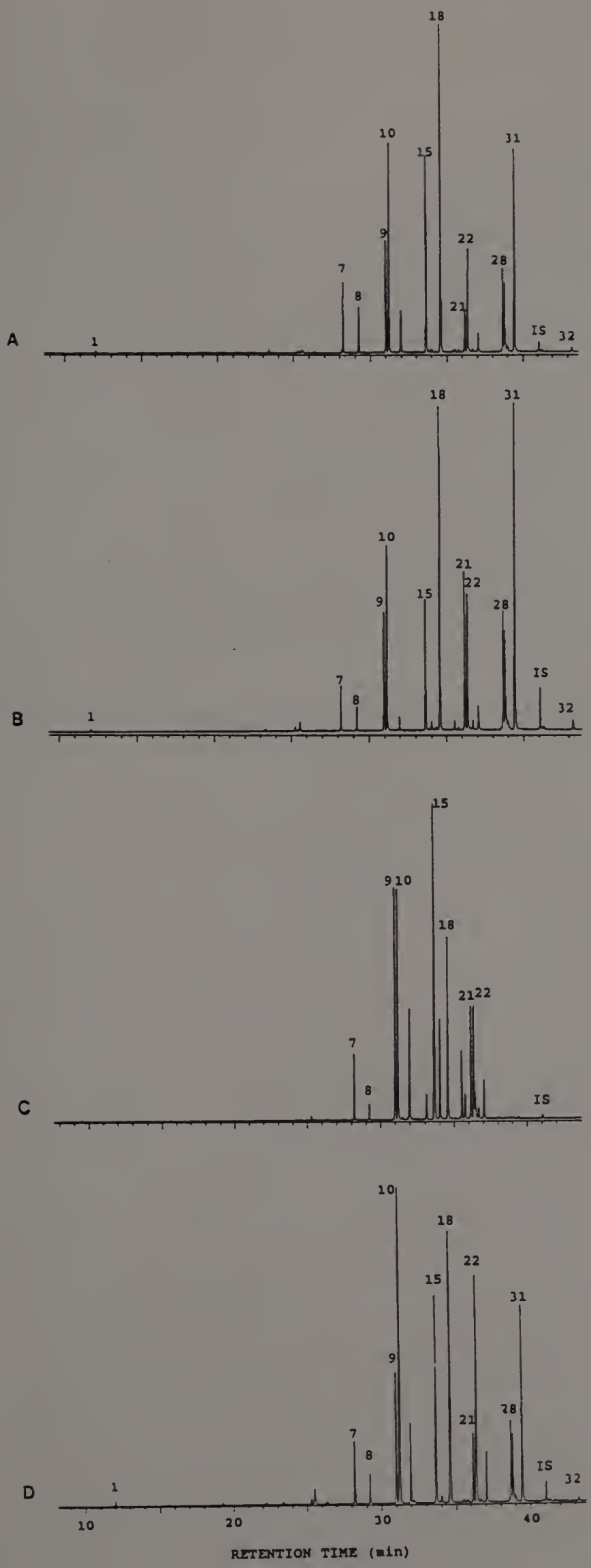
Based on observations above on morphological, cultural and whole-cell chemical characteristics, CR-43 is a member of a new species of *Streptomyces*, for which the name *Streptomyces costaricus* Esnard and Zuckerman is proposed. Isolate CR-43^{T}, the type strain of *S. costaricus*, has been deposited in the American Type Culture and USDA-ARS Culture Collections, under the accession numbers ATCC 55274 and NRRL B-16897, respectively.

3.4.7 Description of *Streptomyces costaricus* sp. nov.

Streptomyces costaricus (cos. ta. ri'cus. L. deriv. *costaricus*, indicating geographic origin in Costa Rica).

Mature spore chains are tightly-coiled spirals, with 10-50 spores per chain. Spores are smooth, and gray brown in mature colonies.

Aerial mycelial mass is grayish brown on yeast extract-malt extract agar, oatmeal agar, inorganic salts-starch agar and glycerol asparagine agar; yellow on N-Z amine medium containing soluble starch and glucose (ATCC medium #172). The substrate mycelium is light yellow on yeast extract-malt extract agar and glycerol-asparagine agar, golden on ATCC medium #172, brown on oatmeal agar, and yellow on inorganic salts-starch agar. A yellow pH-insensitive diffusible pigment is produced on yeast extract-malt extract agar and glycerol-asparagine agar. The pigment color is orange-yellow on ATCC medium #172. No melanoid pigment is produced on peptone-yeast extract-iron agar or tyrosine agar. Color of the reverse side of the colony also pH-insensitive.


A
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RETENTION TIME (min)

Table 3.3 Ratios of certain peaks in the chromatogram of hydrolyzable fatty acids from CR-43, *S. hygroscopicus* (S.h.), *S. murinus* (S.m.), *S. griseoluteus* (S.g.), *S. h.* subsp. *decoyicus* (S.h.d.), *S. rubiginosus* (S.r.) and *S. griseofuscus* (S.gf.) grown on agar and in broth media.

	Agar			Broth			
peak ratios[a]	CR-43	S.h.	S.m.	S.gl.	CR-43	S.h.d.	S.r.	S.gf.
P7/P8	<1	>1	>1	>1				
P10/P15	>1	<1	>1	<1	>1	>1	<1	>1
P10/P18	≈1	<1	>1	>1	<1	<1	>1	>1
P18/P22	>1	<1	<1	>1				
P18/P31					>1	<1	>1	>1
P9/P22	<1	>1	<1	>1	<1	>1	>1	<1
P21/P9					<1	>1	>1	>1
P21/P29					<1	>1	>1	≈1

[a]Identities of peaks are listed in Table 2. P = peak; ≈, approximately.

D-fructose, D-glucose, D-mannitol, D-xylose, salicin and galactose are utilized for growth, but L-arabinose, raffinose, rhamnose and sucrose are not. Acid produced from cellobiose, D-glucose, glycerol, maltose, galactose, D-mannitol and D-xylose but not from L-arabinose, D-fructose, lactose and sucrose. No growth in presence of ribitol, galactitol, erythritol and i-inositol. 7% NaCl is inhibitory. Tolerance to other toxic compounds are summarized in Table 3.1.

Cell walls contain LL-diaminopimelic acid. The most abundant hydrolyzable fatty acids are a-15:0, 16:0, a-17:0, i-15:0, i-16:0 and i-17:0 in cells grown on ISP #2 agar medium. Octadecanoic acid increased 9-fold in ISP #2 broth (Table 3.2). Isolated from a tropical soil. Exhibits antinematodal and antifungal properties. The type strain is strain CR-43^T (= ATCC 55274^T = NRRL B-16897^T).

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CHAPTER 4

CONSERVED MINI-SEQUENCE PRIMED PCR (CMSP-PCR) ANALYSIS SUGGESTS NOVEL *STREPTOMYCES* SPECIES

4.1 Chapter Abstract

A genotaxonomic approach to the differentiation of the streptomycete isolate CR-43 (originating in Costa Rica) from eight already-described species (*S. avermitilis*, *S. scabies*, *S. acidiscabies*, *S. murinus*, *S. griseoluteus*, *S. hygroscopicus* subsp. *decoyicus*, *S. rubiginosus* and *S. griseofuscus*) was taken to supplement cultural and biochemical characterizations. A modified RAPD analysis, conserved mini-sequence primed PCR (CMSP-PCR), was designed to target short conserved sequences dispersed throughout the streptomycete genome. All species had distinct DNA profiles when DNA was amplified with either 5SSU3 or primer SSU5. The DNA profile of CR-43 resembled most that of *S. h. decoyicus* only when molecular cloning was primed with SSU5. Species delineation was easily achieved with CMSP-PCR DNA amplification and without further enzymatic processing of the amplified products.

4.2 Introduction

Streptomycetes are of tremendous importance commercially for the great diversity of natural products they synthesize from secondary metabolism. In fact actinomycetes have supplied more than 70% of the bioactive secondary metabolites isolated from microbes (Gräfe, 1989). *Streptomyces* spp. have been tested against

several plant pathogens including nematodes (Dicklow et al., 1993; El-Abyad et al., 1993) and as plant growth promoters (El-Shanshoury, 1989). Despite this, only one bacterial product (viz. Mycostop biofungicide (Lahdenperã et al., 1991)), and about two derived products (viz. avermectin and streptomycin) have been commercialized for use on plants.

The genus *Streptomyces* is characterized by bacteria that are Gram positive, non-endospore forming, filamentous and producing spores both in chains and aerial mycelia. The vegetative mycelium is ≤1 μm in diameter. The species possess a very complex life cycle exhibiting both bacterial and "fungal-like" growth habits. Most differentiation-associated phenotypic characteristics studied to date such as formation of aerial mycelia, spores, pigments, antibiotics, exoenzymes and odorous substances are relatively unstable genetically (Gräfe, 1989) and present problems for species delineation. For example, *S. scabies* variants no longer differentiating aerial mycelium or spores frequently lose the ability to produce melanin or antibiotics (Schrempf et al., 1988). In the last two decades bacterial systematics have focused directly on genotypic analyses. A recent review by Leblond and Decaris (1994) provides new insights into the genetic instability within the genus *Streptomyces*, suggesting that it is related to large deletions or DNA amplifications which occur at the extremities of linear genomes present in several streptomycete species. For that reason genomic DNA only was analyzed in this study.

Restriction enzymes are not useful in delineating species because the genomic patterns generated are not specific at that taxonomic level (Crameri et al., 1983) even

when rare cutting enzymes (such as *Ase*I, *Dra*I) combined with pulse field gel electrophoresis are used (Leblond et al., 1990; Beyazova and Lechevalier, 1993). Gel electrophoresis of the complete enzymatic digest of *Streptomyces* genomic DNA generates only a distinct strain-specific pattern of bands after staining with ethidium bromide (Hopwood et al, 1985). The most widely used approach in microbial systematics and phylogeny is currently the comparison of ribosomal RNA gene sequences which have a combination of highly conserved and less conserved sequences that have well documented the history of evolution of prokaryotes and other living organisms (Ludwig and Schleifer, 1994). In earlier phylogenetic investigations, a bacterial species was pragmatically defined by a polyphasic approach in addition to a DNA reassociation of approximately 70% taken as the cut off point (Wayne et al., 1987). Currently phylogenetic studies concentrate mostly on 16S rRNA which raises the question about whether or not it is the organismal phylogeny or only a subset, the history of the molecule (rRNA gene), that should be relied on. Stackebrandt and Groebel (1994) warns that sequence analysis of 16S rRNA may not be an appropriate substitute for DNA reassociation for delineation of species and measurement of intraspecific variation. rRNA sequence variation between and within species and genera is not always sufficient to give specific amplification products (Grant et al., 1993). Certain DNA homology groups may not be distinguished by 16S rRNA probes (De Muro et al., 1992). In fact the 16S rRNA sequence of *Bacillus anthracis* is identical to that of *B. cereus*, a food-poison bacterium (Ash and Collins, 1992). The 16S rRNA-encoding gene sequence of *Frankia* is highly homologous to corresponding genes in *Streptomyces* spp. (Normand et al., 1992). Restriction analysis

of amplification products from rRNA gene sequences is useful but entails additional processing (Avaniss-Aghajani et al., 1994; Carlotti and Funke, 1994).

In this study, a modified approach to genotaxonomic differentiation of several *Streptomyces* species was attempted. The whole genome of the *Streptomyces* species was taken into consideration (not just the rRNA region). The genome was conceptualized as a mixture of evolutionary conserved and variable sequence elements and so a novel technique called Conserved Mini-Sequence Primed PCR (CMSP-PCR) was introduced in order to "scan" all of the genome to achieve species differentiation. It is similar to the RAPD technique except that instead of a random arbitrary primer sequence (Welsh and McClelland, 1990; Williams et al., 1990), use is made of a single 15-mer primer with a sequence that allows hybridization to a conserved sequence repeated throughout the genome. In this study the sequence of a relatively small portion of a conserved region of the small subunit (SSU) rRNA gene of bacteria was used as the primer. Thus, unlike RAPD's which randomly generate too much variation to allow differentiation at the species level (Williams et al., 1993), CMSP-PCR amplification of DNA of several species reflects the variation in relative locations of a particular conserved "priming" sequence within the different genomes. PCR is far simpler and more sensitive than RFLP detection by DNA blot hybridization which is laborious and incompatible with applications requiring high throughput (Rönner and Stackebrandt, 1994; Weising et al., 1995; Williams et al., 1993). In this dissertation, the term "oligotype" is introduced to mean the particular DNA profile generated by the PCR reaction. This study also provided additional evidence to show that CR-43 is a novel species.

4.3 Materials and Method

4.3.1 Streptomycete species and growth conditions

Nine actinomycetes of the genus *Streptomyces* viz. *S. costaricus, S. avermitilis, S. scabies, S. acidiscabies, S. hygroscopicus* subsp. *decoyicus*, *S. murinus*, *S. griseoluteus*, *S. rubiginosus* and *S. griseofuscus* were each grown aerobically for 56-60 h at 25-26°C in 50 ml of modified ISP medium #2 broth (4 g yeast extract, 10 g malt extract, 4 g dextrose, 100 g sucrose per L, adjusted to pH 7.3 before autoclaving, after autoclaving 2 ml of 2.5 M $MgCl_2.6H_2O$, 25 ml of 20% glycine). They were chosen for comparisons on the basis of suggestions of APHIS, the US patent office, the International Committee for Systematic Bacteriology, a *Streptomyces* authority Dr. David Labeda and reported species described in Bergey's Manual (8th edition) (See chapter 3).

4.3.2 DNA extraction

The streptomycete biomass was pelleted by centrifugation at 5,000 rpm (2,987 g) for 10 min in a Sorvall^R Superspeed RC-B refrigerated centrifuge. One ml of TE (10 mM Tris.Cl, 1 mM EDTA, pH 8.0) containing 10-mg/ml lysozyme solution + 200 μg/ml proteinase K was added to ≈50 mg of cells in 1.5-ml microfuge tubes, vortexed to break up clumps and incubated at 37°C with occasional agitation. After 1 h, the cells were collected by centrifugation for 2 min at 10,000 g. 600 μl of GSS [4 M guanidine thiocyanate + 25 mM sodium citrate (Anderberg et al., 1995) + 0.5% v/v sarkosyl] were added followed by vigorous vortexing until cells were resuspended. Cell lysis was completed by incubation at 50-56°C for 1 h. Tubes were gently

inverted once and then cooled on ice. Cold sodium acetate (200 μl, 7.5 M) was added and tubes were left on ice for 10 min. Each lysate was mixed thoroughly but gently for 15 s with 700 μl of cold equilibrated phenol-chloroform-isoamyl alcohol (25:24:1). The aqueous (top) phase (500 μl) was retrieved after a 15-min high-speed centrifugation at 10,000 g. The DNA was precipitated by addition of 0.54-0.6 volume ice-cold isopropanol, mixed by inverting tubes, cooled on ice (or at -20°C for 5 min), spun at low speed for only 20 s and then washed thrice in 0.8 ml cold 70% ethanol (centrifugations: 10,000 x g, 5 min). The supernatant was discarded and the remainder was removed with a thin-tipped pipet. The pellets were air-dried for 10 min and carefully redissolved in 100 μl TE or nuclease-free water. DNA samples were diluted (1/50 x) in TE to determine the concentration of the DNA based on the assumption that 50 μg/ml of DNA has an OD of 1 at 260 nm. An OD_{260}/OD_{280} ratio between 1.65 and 1.85 was used as an indicator of sufficient purity of the DNA sample (Zyskind and Bernstein 1992). Quantification of the DNA was also carried out using a DNA Mass Ladder (Gibco BRL, Gaithersburg, MD, USA) since OD_{260}/OD_{280} ratios may be incorrect (Glasel, 1995).

4.3.3 PCR amplification with SSU rRNA-sequence-derived primers

Two 15-mer primers SSU5 (5'-CGGCAGGCCTAACAC-3') and 5SSU3 (5'-TGCGGCCGTACTCCC-3') that are respectively homologous to sequences within the conserved 5' end and central region of the SSU rRNA gene of most bacteria, were custom synthesized by standard phosphoramidate chemistry (National Bioscience Inc., Plymouth, MN). T_m values of the primers were respectively 50°C and 52°C respectively by the "2*(A+T) + 4*(G+C)" method. Both primers exhibited no

significant 3'-terminal dimer and hairpin loop formation, $\Delta G < 1.4$ kcal/mol. These conserved specific primer minisequences were tested singly (CMSP-PCR). Primer SSU5 was used to differentiate CR-43 from *Streptomyces* spp. (viz. *S. hygroscopicus* subsp. *decoyicus*, *S. murinus*, *S. rubiginosus* and *S. griseofuscus*) that were deemed closest to it based on similarities in cultural, fatty acid and other biochemical characteristics (Chapter 3).

PCR was performed using Perkin-Elmer's GeneAmp® PCR Core Reagents (Roche Molecular Systems, Branchburg, NJ, USA). Components of the PCR reaction (50 μL) were added in the following order: 1x reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), glycerol (5%), dATP, dGTP, dTTP, dCTP (each 100 μM), AmpliTaq® DNA polymerase (0.025 U/μl), primer (0.2 μM), $MgCl_2$ (2.1 mM), and approx. 2-5 ng template DNA in TE. Lu and Nègre (1993) had previously shown that glycerol added to the PCR reaction at 5% or 10% improves DNA amplification. DMSO has the same effect but is more toxic. Two control reactions for the PCR were set up: one with the media-only extraction control (zero template control) and the other with all reagents except the primer. Each reaction was overlaid (to reduce evaporation) with ≈50 μL of Nujol mineral oil (Perkin Elmer) and placed in an automated GTC-2 Genetic Thermal Cycler (GL Applied Research Inc, Grayslake, IL) programmed for cycle 1 to be 3 min at 94 C, 5 min at 36 C, 40 min at 72 C, as suggested by Myers and Chiu (1994) for increasing the amplification of specific products, followed by 44 standard cycles of 1 min at 94 C, 2 min at 40°C (for primer 5SSU3) or 45°C (for primer SSU5), 2 min at 72°C and a final hold at 72°C for 7 min. The reaction was held at 4°C until assayed. Amplification products (14 μl) were analyzed by electrophoresis in 1.7% UltraPure agarose (at 90 V for 1 h, 3.5 cm) in 1X TBE (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA, pH 8.0), detected by

staining with 0.1 μg/ml ethidium bromide and photographed on Polaroid 665 film by UV transillumination through an orange filter (Kodak # 23A). One eighth volume of tracking dye [50% bromophenol blue, 50% glycerol in TBE] was added to each DNA digest before loading the gel. The molecular size of DNA fragments resolved by the 1.7% agarose gel was determined by comparing their mobilities with the mobilities of a 1-μg/μL kilobase ladder composed of fragments ranging in size from 0.134 to 12.216 kilobases (Kb) [Gibco BRL Life Technologies Inc., Gaithersburg, MD]. Extraction and amplification were done twice to ascertain reproducibility of the technique.

4.4 Results and Discussion

CMSP-PCR amplification is a simple and rapid method for the reliable differentiation of closely related *Streptomyces* species (Fig. 4.1, 4.2). The final set of PCR parameters that worked successfully was determined by repeated trial, optimization and trouble shooting, essential steps before beginning PCR work (Cobb and Clarkson, 1994; Wernar and Heuvelman, 1993; Yu and Pauls, 1994). Amplified polymorphic DNA methods based on PCR generally use single short primers and work best when lower annealing temperatures are used (viz. 30-40 C). Good amplification is not achieved with 10-mers at annealing temperatures above 40°C (Williams et al., 1993). On the other hand, use of low annealing temperatures decreases the specificity of the PCR producing anonymous but reproducible DNA fragments from complex genomes (Bowditch et al., 1993). Annealing temperatures of 40°C and 45°C with the 15-mer primers worked well in CMSP-PCR (Fig. 4.1, 4.2).

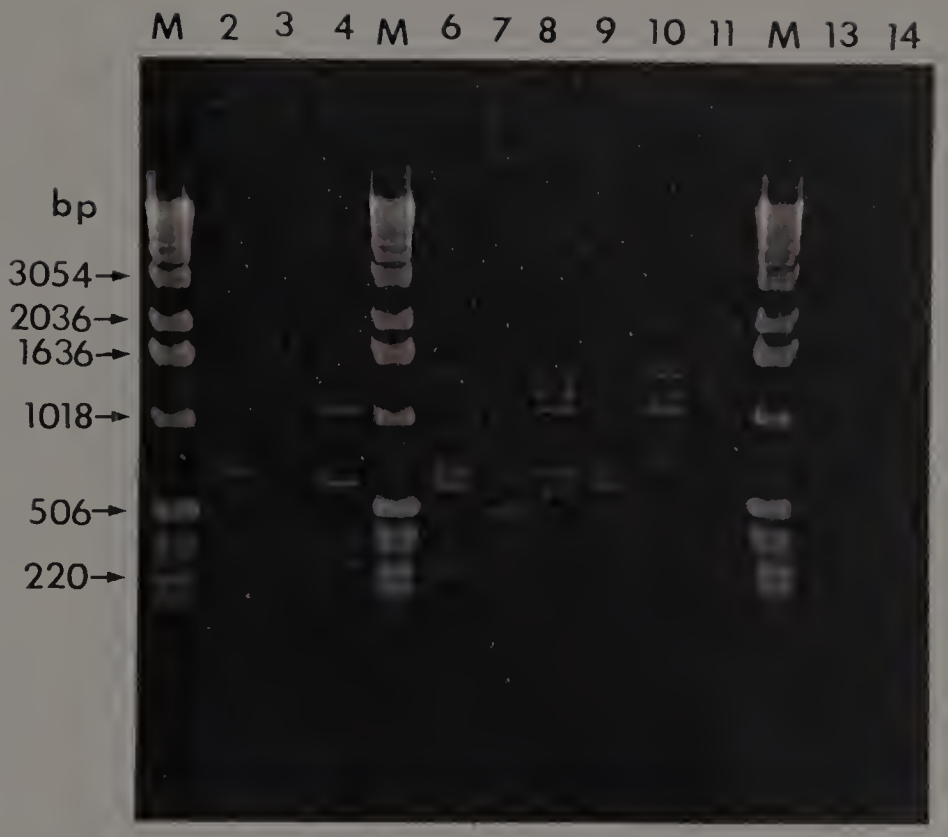

Fig. 4.1 DNA profiles of *Streptomyces* spp. generated by conserved mini-sequenced primed PCR using primer 5SSU3 (T_m = 52°C) at annealing temperature of 40°C. Amplification products were resolved in a 1.4% agarose gel which was stained with ethidium bromide (0.1 μg/ml). Lanes M = 1 kb DNA marker, lane 2 = *S. avermitilis* (ATCC 31267), lane 3 = *S. scabies* (ATCC 49173), lane 4 = *S. ascidiscabies* (ATCC 49003), lane 6 = CR-43 (=ATCC 55274), lane 7 = *S. hygroscopicus* subsp. *decoyicus* (NRRL ISP-5087), lane 8 = *S. murinus* (NRRL B-2286), lane 9 = *S. griseoluteus* (NRRL B-1315), lane 10 = *S. rubiginosus* (NRRL B-3983), lane 11 = *S. griseofuscus* (NRRL B-5429), lane 13, 14 = template DNA only or primer only respectively omitted in control PCR reactions to determine whether any bands were associated with primer or degraded DNA template artifacts.

Fig. 4.2 DNA profiles of five biochemically similar *Streptomyces* spp. generated by conserved mini-sequenced primed PCR using primer SSU5 (T_m = 50°C) at annealing temperature of 45°C. Amplification products were resolved in a 1.7% agarose gel which was stained with ethidium bromide (0.1 μg/ml). Lanes M = 1 kb DNA marker, lane 2 = *S. murinus* (NRRL B-2286), lane 3 = *S. griseofuscus* (NRRL B-5429), lane 4 = *S. hygroscopicus* subsp. *decoyicus* (NRRL ISP-5087), lane 6 = CR-43 (=ATCC 55274), lane 7 = *S. rubiginosus* (NRRL B-3983).

Short sequences (≥4 bases) located in highly conserved genes (such as rRNA genes) are also differentially located within the genome of an organism and polymorphisms generated by PCR amplification of sequences between some of these sequences were sufficient for species differentiation (Fig 4.1, 4.2). McDermott et al. (1994) working with fungi, used specific primer pairs to amplify and detect variation at genetically defined loci whereas this study focused on differences in the locations of hybridization of a single 15-mer primer to a specific conserved mini-sequence repeated within the genome of each species. Haun and Göbel (1987) and Lin et al (1994) designed six taxon specific hybridization probes complementary to the 16S rRNAs of members of the bacterial genera *Proteus* and *Fibrobacter* for identification at the genus, species and subspecies levels. Their method differs from the present one in that they used dot-blotting and southern hybridizations instead of PCR.

Currently, molecular genetic approaches are virtually compulsory for delineating novel taxons from already described ones even though a stable phenotypic feature of value might be unveiled (such as the cyclic component in menaquinone of the MK-8(H_4) type in *Nocardia* that is absent in the closely related genus *Rhodococcus* (Howard et al., 1986; Rainey et al., 1995)). Phenotypic clusters are known to correlate well with ribotypic clusters (Elomari et al., 1994). The major drawback of species characteristics based on phenotypic features (such as fatty acid, isozyme profiles, etc.) is variation during growth. Since the genetic material of organisms is a constant feature, genotypic profile analyses are more suitable for differentiating closely-related species particularly when they require different growth conditions.

The characteristics, behavior and ecological relationships of an organism are determined by a large volume of information within the "total" genome. A single gene, (such as for an rRNA (Woese, 1987), an exotoxin (Klan and Cerniglia, 1994) or a methane monooxygenase (McDonald et al., 1995)), can not predict the cultural, physiological and biochemical characteristics to a greater extent than a "scan" of the "total" genotype. rRNA gene sequences are very useful for determining prokaryote phylogeny (see Woese, 1987 and other journals of systematics). However, in species delineation ribotyping focuses on one or a few gene sequences whereas CMSP-PCR and RAPDs reflect information from the whole genome which means that the latter techniques are of more predictive value in terms of the characteristics of an isolate than mere focus on a single molecule. Moreover, analysis of the total genome instead of single gene sequences that are highly conserved may be useful for differentiating closely-related but different isolates. In fact, incongruence between results of DNA-DNA hybridization and rRNA sequence analysis have been reported in the International Journal of Systematic Bacteriology (Fox et al., 1992; Martínez-Murcia et al., 1992).

The DNA profile of CR-43 generated by CMSP-PCR using primer 5SSU3 is different from all other *Streptomyces* species tested (Fig. 4.1). Further species delineation was achieved when the closest species DNA profiles were compared using another primer (SSU5) at increased stringency (at an annealing temperature of 45 C, five degrees higher than that used for primer 5SSU3) (Fig. 4.2). These primers annealed to at least the conserved regions within the 16S rRNA gene and also to other regions with homologous sequences. Amplification of regions within rRNA genes with two primers (simultaneously) produces one or a few DNA products only

and usually does not show differences between species and between genera (Avaniss-Aghajani, 1994).

Based on the characteristics listed in Table 3.1 in chapter 3, a review of Bergey's manual (8th edition, Pridham and Tresner, 1974), and the opinion of *Streptomyces* expert Dr. David Labeda (USDA-ARS, NCAUR, Peoria, Il), CR-43 supports a novel species although it is close to *S. griseofuscus* (NRRL B-5429) and *S. rubiginosus* (NRRL B-3983) physiologically. The DNA profile, however, of CR-43 (lane 6) is closest to (although clearly different from) that of *S. hygroscopicus* subsp. *decoyicus* (NRRL ISP-5087) (Fig. 4.1 lane 7, Fig. 4.2 lane 4). This result is consistent with the observations that fatty acid profiles of *S. h.* subsp. *decoyicus* and of *S. murinus* are closest to those of CR-43 (See chapter 3). The approximate 500-bp band (in Fig. 4.1) amplified from strain NRRL ISP-5087 but absent in the DNA profile of CR-43 clearly differentiates the two actinomycetes. Phenotypically CR-43 differs from strain NRRL ISP-5087 in its production of a consistent grey brown spore mass in ISP media #2, 3, 4 & 5 whereas the latter presents a grey color on ISP media #2 and #5, white to carbon grey on ISP medium #3, and a grey to brown color on ISP medium #4 after 14 days. Also, CR-43 can utilize salicin (1 μg/ml) as a sole C-source and can grow on crystal violet (1 μg/ml) whereas strain NRRL ISP-5087 cannot.

The copy number of the rRNA gene cluster varies among *Streptomyces* spp.: e.g. there are four in *S. ambofaciens* (Pernodet et al., 1989) and six in *S. coelicolor* (Baylis and Bibb, 1988). The rRNA genes of *Streptomyces* are thought to be in the usual order of most bacteria (viz. 16S-23S-5S). No tRNA-encoding sequence has been

located in the gene cluster to date (Pernodet et al., 1989). Members of the genus *Streptomyces* have produced the majority of known antibiotics. Resistance to some of these antibiotics may involve rRNA modifications (Cundliffe, 1986). The 16S rRNA sequence of *Streptomyces* however, shows regions that are highly variable among species (Pernodet et al., 1989). It was envisaged that CMSP-PCR amplification (using a single primer with sequences homologous to conserved 15-nt stretches within the rRNA genes) would produce polymorphisms sufficiently varied to allow differentiation of the species. DNA amplified from the 16S rRNA gene in the absence of further processing seldom generates enough bands to differentiate all species compared (Avaniss-Aghajani et al., 1994). In earlier phases of this research, an AT-rich 9-mer primer (JE-1: 5'-ATTAATCCA) was also designed but it failed to generate any DNA product (results not shown). It had been chosen on the basis of Beyazova and Lechevalier 's (1993) rationale that AT-rich sequences might be more conserved than GC-rich sequences.

CMSP-PCR-generated DNA polymorphisms reflect the degree of similarity between base sequences of the microbes tested. Base sequence drift varies from being highly conserved in rRNA genes to regions in the genome that code for highly variable amino acid sequences. Streptomycetes in particular form a very genetically unstable group owing to large deletions and DNA sequence amplifications that occur at high frequencies (Leblond and Decaris, 1994; Schrempf et al., 1988). The use of numerical taxonomy for delineation of *Streptomyces* species on the basis of phenotypic characteristics only may even result in the inadvertent grouping of isolated variants in a cluster different from that of the corresponding wild-type strains

(Schrempf et al., 1988). Therefore the amplification of DNA sequences from both the rRNA and other genes throughout the genome simultaneously could provide a useful yet rapid approach to species differentiation and avoid such misclassification.

On the basis of the differential hybridization of two 15-mer primers (used singly) within the genomes of CR-43 and 8 related *Streptomyces* species, CR-43 (proposed name *Streptomyces costaricus*) is suggested as a novel species. (See chapter 3 for phenotypic characteristics).

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CHAPTER 5

ARBITRARY PRIMER (AP) POLYMERASE CHAIN REACTION (PCR) DIFFERENTIATES CR-371 FROM SIMILAR Bt STRAINS

5.1 Chapter Abstract

The polymerase chain reaction (PCR) was used to generate DNA profiles of CR-43, five Bt strains patented by Mycogen, and Bt strain Shy1-1 by the random amplified polymorphic DNA (RAPD) technique. A nanomeric primer, 0955-03, previously shown to clearly differentiate Bt serovars, was used. All strains produced distinct DNA profiles which demonstrated that Bt strains patented by Mycogen (as nematicidal) are different from the novel antinematodal Bt CR-371 originating in Costa Rica. The RAPD technique used with the primer 0955-03 (5'-CCGAGTCCA) proved to be a useful and promising tool for differentiating Bt strains.

5.2 Introduction

Bacillus species are probably the most important spoilage and contaminating bacteria from an industrial and medical point of view (Berkeley et al., 1984). Although they are poor colonizers of soil, these aerobic, endospore-forming rods could be the dominant bacteria isolated from the rhizosphere (van Elas et al., 1989; Tedla and Stanghellini, 1992; Stanghellini and Rasmussen, 1989). *Bacillus thuringiensis* (Bt) is the most commercially successful biological control agent for arthropod pests

which include dipteran vectors of human diseases, lepidopteran forest and agricultural pests and the beekeepers wax moth pest. Ninety percent of the biocontrol market (a value of ≥50 million dollars) is taken up by Bt sales (Lal and Lal, 1993). More strains are being discovered and improved as exemplified by patents granted to Mycogen Corporation (Edwards et al., 1992, US Patent 5,093,120) and University of Massachusetts (Zuckerman et al., 1995, US Patent 5,378,460) for Bt's showing antinematodal activity.

Initially, the US patent office was reluctant to accept CR-371, which originated in Costa Rica, as a novel antinematodal Bt with the argument that antinematodal Bt strains had already been introduced (viz. the Edwards et al. 1992 patent) and that CR-371 , as presented, was indistinguishable (Zuckerman and Esnard, 1994). Thus the existence of differences between antinematodal Bt strains had to be demonstrated.

Chemical profiles generated by gas chromatography combined with mass spectrometric analyses of hydrolyzable fatty acids demonstrated that real differences exist between Mycogen's Bt's and CR-371 (Esnard et al., 1994; also see chapter 2). Although this analytical approach is a proven method for bacterial identification and was sufficient for the US examiner to grant the patent, DNA profiles were also generated and compared using random amplified polymorphic DNA (RAPD) and is the subject of this chapter. Other methods such as bacteriophage typing, H-serotyping or other biochemical tests were not explored because of their insufficient discrimination of intraspecific variation or their time-consuming nature (Berkeley et

al., 1984). The BIOLOG system presented problems for the identification of this Gram positive bacterium and would certainly not have been useful at the strain level.

Since the genetic material of an organism is more stable than phenotypic characteristics, DNA profiles are very suitable for comparing bacterial isolates and strains. Despite their complex array and mostly cryptic nature, Bt plasmids are maintained and inherited with remarkable stability (Lereclus et al., 1993). The RAPD method is a very powerful but quick modern method of analyzing the genome of an organism. It is based on the differential extension of DNA between short random arbitrary primer DNA sequences that anneal at several positions on separated strands of template DNA (Welsh and McClelland, 1990; Williams et al., 1990). The method is more suitable for comparisons at the strain level because of the high level of variation generated (Williams et al., 1993). Generation of RFLPs is laborious requiring DNA fragment labelling. The feasibility of the RAPD method for differentiating bacterial strains has been clearly demonstrated (Brousseau et al., 1993; Mazurier and Wernars, 1992; Stephan et al., 1994) and was pursued in this study of antinematodal Bt strains.

5.3 Materials and Methods

5.3.1 Bacteria and growth conditions

Eight strains were tested viz. the novel Costa Rican Bt strain CR-371 (ATCC 55273), a spontaneous rifampicin resistant mutant derivative (CR-371rif^{+}), 5 Bt strains patented by Mycogen (NRRL B-18243, NRRL B-18244, NRRL B-18245,

NRRL B-18246, NRRL B-18247 obtained from ATCC) and Shy1-1 (which is a Bt strain our program had shown to have antinematodal effects against animal parasites (results unpubl.)). Shy1-1 was supplied by a collaborator, Dr. Frank Cannon (Head of Biotechnology/Microbiology, U. Mass.). The strains were previously stored at -80°C in cryopreservation buffer [per 200 ml, 20 ml 1 M NaCl, 10 ml 1 M phosphate buffer, 60 ml glycerol; after autoclaving, 0.6 ml 0.1 M $MgSO_4$]. One volume of Nutrient Broth (NB) was prepared and was aliquoted in 50-ml quantities in 250-ml Erlenmeyer flasks before autoclaving. Cultures were initiated by inoculating cooled NB with 1 sterile loopful of the thawed Bt suspension. The cultures were grown in the dark at 22-25°C on a rotary shaker at 270 rpm for 17 h.

5.3.2 DNA extraction

Chromosomal DNA from each *B. thuringiensis strain* was prepared from 1 ml of medium transferred to a 1.5-ml microfuge tube. Cells were pelleted by centrifugation at 10,000 g for 3 min. The supernatant was discarded and the pellet was resuspended in 1.5 ml of saline (9.0 g/L) by vortexing. The cells were collected by centrifugation again. The saline wash was done three times. 100 ml of lysozyme (2 mg/ml in TE) was added to the pellet followed by incubation at 37 C. After 45 min, 1.5 ml of saline was added and cells were washed once as before. The pellet was resuspended in 0.5 ml of sterile glass distilled deionized water and incubated in a boiling water bath for 30 min to release the DNA into solution. The boiled suspension was immediately cooled on ice for 5 min and then cleared by centrifugation at 10,000 g for 30 s at room temperature. The supernatant was transferred to another sterile microfuge tube and used immediately after cooling on ice or stored at -20°C until amplification. DNA concentrations were determined by using a DNA Mass Ladder (Gibco BRL, Gaithersburg, MD, USA).

5.3.3 PCR amplification with a random primer

An arbitrary oligonucleotide primer that had been previously shown to clearly differentiate Bt strains and even serovars (Brousseau et al., 1993), was used in this work. The primer, 0955-03, with sequence 5'-CCGAGTCCA, was synthesized by standard phosphoramidate chemistry as before (National Bioscience Inc., Plymouth, MN). T_m value of the primer was 30°C by the "2*(A+T) + 4*(G+C)" method. The primer exhibited no stable 3'-terminal dimer and hairpin loop formation.

PCR was performed using Perkin-Elmer's GeneAmp® PCR Core Reagents (Roche Molecular Systems, Branchburg, NJ, USA). Components of the PCR reaction (25 μL) were added in the following order: 1x reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), glycerol (5%), dATP, dGTP, dTTP, dCTP (100 μM), AmpliTaq® DNA polymerase (0.625 U), primer (0.2 μM), $MgCl_2$ (2.1 mM), and 12 μl of supernatant (containing approx. 1-5 ng template DNA in sterile glass-distilled deionized water). Lu and Nègre (1993) had shown that glycerol added to the PCR reaction at 5% or 10% improves DNA amplification. DMSO has the same effect but is a toxic compound. Two control reactions for the PCR were set up: one with the media-only extraction control (zero template control) and the other with all reagents (including CR-371 template DNA) except the primer. The reaction was overlaid with 50 μL of Nujol mineral oil (Perkin Elmer) and placed in an automated GTC-2 Genetic Thermal Cycler (GL Applied Research Inc, Grayslake, IL) programmed for 1 cycle of 3 min at 94°C, 5 min at 33°C, 40 min at 72°C (as suggested by Myers and Chiu (1994) for increasing the amplification of specific products) followed by 44 cycles of 1 min at 94°C, 2 min at 36°C, 2 min at 72°C and a final hold at 72°C for

7 min. DNA extraction and PCR was repeated but the thermal cycler was programmed to increase the stringency of the reaction (viz. 1 cycle of 3 min at 94°C, 2 min at 40°C, 2 min at 72°C followed by 44 cycles of 1 min at 94°C, 2 min at 40°C and 2 at 72°C). At the end of both programs, the reactions were held at 4°C in the thermal cycler until assayed. Amplification products were analyzed by electrophoresis in 1.4% UltraPure agarose (at 90 V for 1 h, 3.5 cm) in 1X TBE (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA, pH 8.0) and detected by staining with 0.1 μg/ml ethidium bromide and photographed on Polaroid 665 film by UV transillumination through an orange filter (Kodak # 23A). One eighth volume of tracking dye [50% bromophenol blue, 50% glycerol in TBE] was added to each DNA digest before loading the gel. The molecular size of DNA fragments resolved by the 1.7% agarose gel was determined by comparing their mobilities with the mobilities of a 1-μg/μL kilobase ladder composed of fragments ranging in size from 0.134 to 12.216 kilobases (Kb) [Gibco BRL Life Technologies Inc., Gaithersburg, MD]. Extraction and molecular amplification were done twice.

5.4 Results and Discussion

The primary goal of this research was to compare CR-371 with other closely related antinematodal Bt's, in particular those patented by Mycogen. The RAPD analysis showed that each strain had a distinct polymorphic DNA profile with primer 0955-03 producing bands between 220 and 2,500 bp (Fig. 5.1). Zero primer and zero template controls yielded no detectable amplified product. The number of DNA bands in each oligotype ranged from 2 (in CR-371) to 10 (NRRL B-18246).

Differences in band intensities were observed. Shy1-1 (from F. Cannon) was also distinctly different from strains studied (lane S in Fig. 5.1) and may not face a difficult prosecution when a patent application is submitted.

Primer 0955-03 was previously shown to anneal to the chromosomal DNA (and not plasmid DNA) of *B. thuringiensis* strains and serovars (Brousseau et al., 1993) and so it was not necessary to heat-cure Bt strains in this study. In the gels, co-migrated amplified DNA products are not necessarily of identical nucleotide sequence. The RAPD technique is attractive because it can produce amplified DNA products without prior knowledge of the nucleotide sequence of the template DNA. Unlike Brousseau et al. (1993), DNA fragments greater than 1.5 kb were readily amplified and were among the most intense bands (Fig. 5.1). The efficient amplification of specific bands >1.5 kb might be related to the lowered stringency conditions for which the thermal cycler was pre-programmed during temperature cycle 1 that preceded more stringent thermal conditions for primer annealing to have a more specific amplification (Myers and Chiu, 1994). RAPD analysis of Bt using primer 0955-03 (6 G+C% 66.7%, 3 A+T 33.3%) is highly reliable since strains, indistinguishable on the basis of biochemical or serological examination, have been shown to be easily separated (Brousseau et al., 1993).

Conditions for the PCR reaction were relatively stringent since the temperature for the later cycles was set 6°C above T_m of the primer. When the PCR reaction was repeated with annealing temperature 10°C above T_m, the number of bands decreased with no bands being detectable in the PCR reaction for CR-371 (Fig. 5.2). Under both conditions discrimination of all strains was achieved. Arbitrary primary PCR using other primers have been reported to discriminate between

Fig. 5.1 RAPD profiles of *Bacillus thuringiensis* strains with primer 0955-03 (T_m = 30°C) at annealing temperature of 36°C. DNA amplification products were resolved in a 1.4% agarose gel which was stained with ethidium bromide (0.1 μg/ml). Lanes M = 1 kb DNA marker, lane CR = strain CR-371, lane Cr = CR-371rif$^+$, 3 = NRRL B-18243, 4 = NRRL B-1824, 5 = NRRL B-18245, 6 = NRRL B-18246, 7 = NRRL B-18247, S = Shy1-1. Lanes 0_t, 0_p, = template DNA only and primer only respectively omitted in control PCR reactions to determine whether any bands were associated with primer or degraded DNA template artifacts.

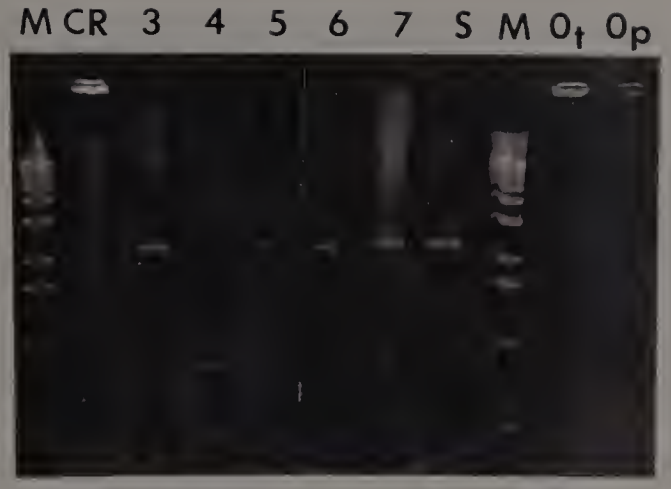

Fig. 5.2 Amplified RAPD products of *Bacillus thuringiensis* strains with primer 0955-03 (T_m = 30°C) at annealing temperature of 40°C. DNA amplification products were resolved in a 1.4% agarose gel which was stained with ethidium bromide (0.1 μg/ml). Lanes M = 1 kb DNA marker, lane CR = strain CR-371, lane Cr = CR-371rif$^+$, 3 = NRRL B-18243, 4 = NRRL B-1824, 5 = NRRL B-18245, 6 = NRRL B-18246, 7 = NRRL B-18247, S= Shy1-1. Lanes 0_t, 0_p, = template DNA only and primer only respectively omitted in control PCR reactions to determine whether any bands were associated with primer or degraded DNA template artifacts.

isolates of opportunistic pathogens sharing the same serotype (Mazurier and Wernars, 1992). The method is also attractive for the fact that extraction or purification of the DNA prior to amplification is not necessary and, since oligomeric primer sequences can easily be synthesized on every continent, exchange of materials as is for antisera, phage sets, etc is also not necessary.

Selection of a spontaneous rifampicin mutation in CR-371 could carry pleiotropic effects. RAPD analysis using primer 0955-03 discriminated between the wild type and CR-371rif$^+$ (Fig. 5.1, lanes CR, Cr). The rif$^+$ mutation is known to be chromosomal and represents a deletion (Sebald, 1993). The mutation affects the ß-subunit (Prescott and Baggot, 1993) of the lone RNA polymerase which transcribes all major RNA classes viz. rRNA, mRNA, tRNA in bacteria (Wolfe, 1993). The detection of different RAPD's in CR-371rif$^+$ and CR-371 under the particular PCR conditions described herein is not surprising since one of the inventors of this technique observed that even single nucleotide changes (and that could include rif$^+$ effects) in the target genomic DNA sequence may change the pattern of amplified DNA segments (Williams et al., 1990). Polymorphisms in both streptomycete and Bt rif$^+$ mutants are treated further in chapter 6.

In eukaryotes, RAPD's are known to be inherited usually in a dominant (not codominant) fashion which means that allelic states and allele frequencies of bands cannot be determined easily (Weising et al., 1995). Because co-migrating bands might not be homologous and separated bands (characters) might be linked, estimation of genetic relatedness based solely on band sharing might be incorrect. In bacterial systems, this problem is of much less importance.

In summary, the novel Bt strain CR-371 was distinguishable from all of Mycogen's patented antinematodal Bt's. In fact all strains produced distinct profiles using RAPD primed by the nanomeric oligomer, 0955-03, previously used by Brousseau et al. (1993). The strain is deposited in the American Type Culture Collection as ATCC 55273.

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CHAPTER 6

EFFECT OF SPONTANEOUS RIFAMPICIN RESISTANCE (Rif$^+$) MUTATIONS ON THE BIOCONTROL ACTIVITY OF TWO NOVEL BACTERIAL STRAINS AND USE OF RIF$^+$ TO MONITOR SURVIVAL IN SOIL

6.1 Chapter Abstract

Rifampicin-resistant mutants were generated and compared to the wild-types *Bacillus thuringiensis* (Bt) strain CR-371 (=ATCC 55273) and *Streptomyces costaricus* sp. nov. type strain CR-43 (=ATCC 55274) in bioassay, survival and molecular genetic studies. The rif$^+$ mutation had a positively pleiotropic effect on the biocontrol activity of the Bt strain and a negatively pleiotropic effect on the streptomycete strain. CR-371rif$^+$ significantly decreased nematode (*Caenorhabditis elegans*) activity in vitro assays, whereas CR-43rif$^+$ had no effect or increased nematode activity ($p = 0.05$). In greenhouse trials in two consecutive years, soil drenches of the marked Bt biocontrols gave a significant increase in only height of tomato cv. Rutgers ($p = 0.05$) pre-inoculated with root-knot nematodes *Meloidogyne incognita* race 3 but no difference in shoot weight or root-knot severity compared to the wild-type and controls. In survival studies in the green house, both CR-371rif$^+$ and CR-43rif$^+$ initial population levels respectively declined progressively from approx. 7 and 6 log cfu to <20 cfu/g soil over 7 weeks in soil planted to Rutgers tomato. In field microplots of lettuce at Geneva, New York, initial population also declined to low levels after 7 weeks viz. 10-25 cfu/g soil. In Costa Rica, CR-371rif$^+$ declined from 10^6-10^7 cfu/g to

≤5.5 log cfu/g soil planted to bananas within 5 days of application. Pleiotropy in both mutants was detectable at the molecular genetic level using random amplified polymorphic DNA analyses.

6.2 Introduction

In our biocontrol program, research is being conducted towards the development of commercial anti-nematode biocontrol products utilizing a novel *Bacillus thuringiensis* Bt (CR-371) and *Streptomyces costaricus sp. nov.* (CR-43). In addition, substrate enhancement studies have been initiated in our program which show promise for increasing the efficacy of the biocontrol organisms. A small scale trial on tissue cultured bananas has been initiated in Costa Rica but application of biocontrol agents for agricultural purposes will most likely be on a much larger scale. The impact of CR-371 and CR-43 on the environment and their success as commercial products will depend on their ability to survive and remain effective long enough in the agroecosystem (Meadows, 1993). *Bacillus* spp., although poor root colonizers (Liang et al., 1982; van Elas et al., 1989), could be the dominant bacteria isolated from rhizosphere soil (Tedla and Stanghellini, 1992; Stanghellini and Rasmussen, 1989). A few studies have shown that Bt added to unamended soil does not grow under most conditions (West et al., 1985). In fact, viable log phase vegetative cells of Bt added to untreated soil disappeared rapidly (evidenced within 24 h by a 91% loss) followed by an exponential loss between day 1 and 10 (West et al., 1984). Among several possible factors, predatory bacteria have been identified that may degrade Bt spores (Casida, 1988). Under nutrient-rich conditions however,

Bt grows well in soil (Martin and Reichelderfer, 1989; West et al., 1985a,b). Soil pH has been shown to have little effect on spores that survived the first 2 weeks of incubation (Petras and Casida, 1985). The novel biocontrol strains in our program might be different since significant nematode control and increases in yield were obtained in greenhouse trials and in large-scale field trials in 1990-91 in Puerto Rico (Dicklow et al., 1993; Zuckerman et al., 1993). Residual growth media added inadvertently with CR-43 and CR-371 during drench applications could provide nutrient-richer conditions for population increase and/or increase the antinematodal effect directly. A necessary requirement for adequate study of the fate of biocontrols is the ability to monitor microorganisms in soil after application.

Several methods have been used to monitor bacterial population dynamics in soil and rhizosphere viz. antibiotic resistance markers, genetically engineered markers, and the polymerase chain reaction (Kloepper and Beauchamp, 1992; Michell, 1992). Antibiotic-resistant, marked bacteria have been used in field persistence studies (Martin and Reichelderfer, 1989) and might be the most successful to date. Genetically engineered markers, such as lacZY constructs, have utility in root colonization studies but result in underestimates of microbial population densities (Zablotowicz and Parke, 1992). Flemming et al. (1994) showed that the *lac* marker was not suitable for monitoring *Pseudomonas aeruginosa*. One study showed that the lacZY marker is, at best, similar to the rif^+ marker in terms of its effects on movement and persistence of *Pseudomonas fluorescens* (Parke et al., 1992). Another chromogenic marker system, using *xyl*E, requires an additional step (viz. spraying plates with catechol) to identify colonies (Clayton and Bidd, 1990). The

lux gene marker system depends on bioluminescence and is primarily qualitative rather than quantitative and requires special detection equipment (Mahro et al., 1991). The DOPA-melanin biosynthesis (*mel* gene) system is not reliable (especially for *Streptomyces* spp.) because the melanin pigment is not always produced on the isolation media being tested and also because it may not be stably maintained (Elliott et al., 1994). Another system using the pigmented actinorhodin antibiotic biosynthesis genes (*act*) is not well-known or widely used.

Rifampicin resistance (rif^+), however, could serve as a useful selective trait since the marker can be easily selected and used to facilitate investigation of persistence of biocontrol organisms in the environment and root colonization capacity (Gemell and Roughley, 1993; Kloepper and McInroy, 1992; Weller and Saettler, 1978). Spontaneous rif^+ resistance is easy to obtain in most anaerobes, usually at a frequency of 10^{-8} to 10^{-9} (Sebald, 1993). The mechanism of this mutation is unique among antibiotics in that it involves the inhibition of RNA polymerase, the enzyme that catalyses the transcription of DNA to RNA (Prescott and Baggot, 1993). Rifampicin specifically binds to the ß subunit of RNA polymerase and causes abortive initiation of RNA synthesis. Gram negative bacteria are much less permeable to rifampicin. There is no known cross-resistance with other antibiotics. Rifampicin sensitivity is dominant (Ausubel et al., 1992) . The rif^+ mutation is chromosomal and very stable, not being able to revert to sensitivity either spontaneously or following mutagenesis, a situation compatible with a deletion (Sebald, 1993). Rif^+ resistance is known to remain stable for at least 5 years in *Bacillus megaterium* strain B153-2-2 (Liu and Sinclair, 1992). Persistence studies in

greenhouse and microplot soil planted to a crop, had not been previously conducted for the two antinematodal strains using rif$^+$ resistant isolates generated in our laboratory.

Studies were conducted (A) to study plant response and efficacy of nematode control using the rif$^+$ mutants; (B) to determine the survival of the rif-marked Bt and streptomycete biocontrols when applied to non-pasteurized and pasteurized soil with or without organic amendments; and (C) to detect any genetic differences between the mutant and wild-type isolates using the polymerase chain reaction. As a preliminary to all experiments, a culture purity check was conducted on stored cultures.

6.3 Materials and Methods

6.3.1 Checking for changes in bacterial cultures

In the earliest phases of this work, a culture purity check of the supplied cryo-frozen samples (viz. CR-371 = CR-450 = *Bacillus thuringiensis* and CR-43 = *Streptomyces sp. nov.*) was conducted during fatty acid analyses (See chapters 2 and 3). A Varian model 3700 gas chromatograph (Los Altos, CA, USA) was used to separate the derivatized bacterial fatty acids. The chromatograph was equipped with a fused silica capillary column (60m x 0.25 mm i.d.) coated with a 1-μm film of cross-linked methyl silicone gum (DB-1) in conjunction with a split/splitless injector (250°C) and an FID (250°C). Helium served both as the carrier gas (linear velocity 27 cm/s) and as detector make-up gas (30 ml/min). Initial column temperature was held at 100°C for 1 min then increased 8°C/min to 260°C for approx. 30 min. The

sampled injected consisted of 1 μl of extracted fatty acid methyl esters in hexane flushed with 1 μl dichloromethane. Integrator functions were set at chart speed 0.4 cm/min, attenuation 7 and peak rejection at 10,000 counts.

6.3.2 Generation of spontaneous rif$^+$ resistant mutants

Rifampicin-resistant mutants of the wild type Bt (CR-371) and *Streptomyces* (CR-43) strains were selected on solid media amended with 50 (Wiebe and Campbell,1993), 100, 200, 300 μg/ml rifampicin by the method of Liu and Sinclair (1992). Rifampicin (Sigma) was dissolved in the smallest volume of methanol and then added to cooled (50°C) nutrient agar (Liu and Sinclair, 1992) and ISP#2 agar which had been autoclaved for 15 min at 121°C. Media preparation and incubation were done in the dark (the latter being at 26°C) (Wiebe and Campbell, 1993). Selected Bt and Streptomycete colonies were increased in Nutrient (NB) and ISP#2 broths respectively, and then replated on solid agar. Surviving colonies were individually transferred to fresh agar media. Stock cultures of the rif$^+$ mutants were stored at - 80°C in cryopreservation buffer (Brenner, 1974).

Effective antagonism of the rif$^+$ mutants to nematodes (*Caenorhabditis elegans*) was checked against the wild type by a bioassay which involved addition of 10 μl of 20-h and 3-d old NB- and ISP #2 (or PDB) broth-cultures (used to grow the Bt and streptomycete respectively) to 50 μl of a 30-40 nematode/ml suspension in Heme medium (McClure and Zuckerman, 1982). For larger volumes, the same culture/suspension (v/v) ratio was used. Nematodes were at various developmental stages. Bioassays were done in 96- or 24-well microplates, or in 10-ml vials held at 21°C and examined under a dissecting microscope.

6.3.3 Bacterial inoculum for greenhouse and field trials

6.3.3.1 Greenhouse experiment

The Bt and streptomycete inocula were prepared by incubating a 0.5-ml of a cryopreserved cell suspension in 50-ml batches of NB for 18-20 h and in ISP #2 broth (50 ml) for 4 days, respectively, with vigorous shaking at 250 rpm on an orbital shaker. Temperatures ranged from 26-28 C. Final concentration of the Bt and streptomycete strains ranged from 1-3 x 10^7 cfu/ml and 0.5-1.5 x 10^6 cfu/ml, respectively, using the spreader plate technique. The cells were pelleted at 7000-8000 rpm for 6 min, quickly washed with 40 ml glass distilled deionized water, re-centrifuged, collected and then brought back up to 50 ml with glass distilled deionized water (Dicklow et al., 1993). The top 2 cm of soil around the stem of each tomato plant cv Rutgers was removed using a sterile spatula before application of the bacterial drench. The point of application was covered with the soil that was dug up. Only one inoculation per plant was done for each experiment (in 1993 and 1994).

The soil used in this study was a non-pasteurized clayey loam sampled from a field in North Amherst that was previously known to be heavily infested with *Meloidogyne incognita*. The plants were grown in a green house (35.0 - 38.2°C), watered every 2 days and fertilized biweekly by wetting soil with 10-30-20 Peters Professional® soluble plant food (3.347 g/L) (Grace-Sierra Horticultural Products Co., Milpitas, CA). Greenhouse whiteflies were controlled with the chalcidoid wasp *Encarsia formosa*. There were five replications of each bacterial treatment. A soil sample (5 g) was taken near the roots when the plants were first inoculated with the biological control agents and then weekly in the same way.

6.3.3.2 Field microplot experiment

The Bt and streptomycete strains were tested in 1994 in field microplots at the New York State Agricultural Experiment Station (Geneva, N.Y.). The experiment was arranged in a split-plot design where soil was untreated (NP) or pasteurized (P) (at 60°C just enough to maintain actinomycete and other spore formers but not plant pathogenic fungi and bacteria). Each of these main plots was divided into chitin-treated (C) and non-chitin (NC) treated soil. All plants were inoculated with the Northern Root-Knot Nematode (NRKN), *Meloidogyne hapla*, at a rate of 20 eggs per cc of soil. Each microplot consisted of lettuce cv. Montello directly seeded (¼ inch deep) in muck soil placed in an unglazed cylindrical drainage clay tile (25-cm-d, 30 cm long) with its rim slightly raised above ground level and its base ≈10 inches below ground. Treatments were replicated 5 times and included *M. hapla* alone, nematicide (Vydate L), CR-371rif$^+$ (≈5 x 10^7 cfu), CR-43, and CR-371rif$^+$ + CR-43 to give a total of 100 microplots. Only the control and CR-371rif$^+$ (only) treatments were used to study the rate of survival of the bacteria in this report. CR-371rif$^+$ survival in soil was also studied in Costa Rica.

6.3.4 Nematode inoculum

6.3.4.1 Greenhouse experiment

All plants were pre-inoculated with root-knot nematodes. The root-knot nematode (*Meloidogyne incognita* race 3) inoculum was previously increased on tomato cv. Rutgers in the green house. Eggs were extracted by the method of Hussey and Barker (1973) while the Baermann funnel method modified to improve aeration was used for larval extraction. Five-ml suspensions each containing 8000 eggs + 1075 juveniles were used to inoculate the roots of 20-d old plants.

6.3.4.2 Field microplot

At Geneva, the plants were inoculated with *M. hapla* at a rate of 20 eggs per cc of soil.

6.3.5 Population dynamics and survival of biocontrols

6.3.5.1 Greenhouse

For population studies, rif^+ mutants were inoculated by infiltration into subsurface soil around the stems of 27-d old potted tomato seedlings in the greenhouse. 5 g rhizosphere soil was sampled immediately after inoculation (0 days) and at 7-d intervals for 7 weeks. Each soil sample was agitated in 20 ml of sterile phosphate buffer [0.025M KH_2PO_4 + 0.025M Na_2HPO_4, pH 7], allowed to settle 5 min after which three 100-μl aliquot from each step of a 10-fold dilution series were spread on rifampicin/cycloheximide-amended NB and ISP#2 plates (Legard and Schwartz, 1987). Rifampicin and cycloheximide (5 mg/ml) were added to the media at 100 and 50 μg/ml respectively. Plates were incubated in the dark at 28°C for 48 h and 96-120 h respectively after which bacterial colonies mostly from the 10^{-6} and 10^{-7} dilution were counted under a stereomicroscope at 10x magnification. Colonies of *Bacillus* and *Streptomyces* spp. were identified by their distinct morphology on NA and ISP#2 medium respectively. Counts (cfu) were represented as log number of bacteria per g of soil. The population study was done in 1993 and repeated in 1994.

6.3.5.2 Field microplot

At the end of the trial, 40-60 grams of muck soil were sampled from the NRKN biocontrol experiment on lettuce in Geneva, N.Y. All plots with treatment

3 (CR-371rif$^+$-treated soil) which included chitin or non-chitin-amended pasteurized or non-pasteurized soil were sampled. Untreated (no bacteria) plots in chitin or non-chitin-amended pasteurized or non-pasteurized soil were analyzed as controls. At the end of the experiment, a 30-g portion of the soil was vigorously stirred with 20 ml tap water. The suspension was passed through a series of sieves of varying pore size viz. 425 μm → 75 μm → 45 μm → 25 μm (500 mesh). Five ml of a phosphate-glycerol buffer (per 200 ml, 20 ml 1 M NaCl, 10 ml 1 M phosphate buffer, 60 ml glycerol; after autoclaving, 0.6 ml 0.1 M $MgSO_4$) were added immediately to reduce the incidence of cell burst. The sieved suspensions were centrifuged at 3000 rpm for 2 min and 10^{-0} to 10^{-6} dilutions were made in mini-well plates. 100 μl of each dilution was spread in triplicate on rifampicin/cycloheximide amended nutrient agar and incubated for 20-36 h at 25°C in the dark. Only dilutions yielding between 30-300 colonies were counted (Klement et al., 1990).

6.3.6 Plant responses

The plant response to the biocontrols and Rif$^+$ mutants were measured for the green house experiment only. After 7 weeks, plants were cut at the base at the point where the highest root emerged. Height (cm) and dry weight (g) of the above-ground parts of the plants were measured. Root gall indices were recorded on a scale of 0 to 5 according to Taylor and Sasser (1978) where 0 galls = 0, 1-2 = 1, 3-10 = 2, 11-30 = 3, 31-100 = 4, and >100 = 5. As a class excercise, the root-knot nematode species was identified using perineal patterns (Taylor and Sasser, 1983; Riggs, 1990).

6.3.7 Experimental design

In the green house experiment, the plants were arranged in a completely randomized design (on a bench raised about 1 m high) with 5 replications and 2 runs (1993 & 1994). Means for height, dry weight and gall index from each experiment were analyzed by ANOVA and separated by Duncan's Multiple Range Test using MSTAT-C (Version 1.4) statistical software. For the microplot experiment at Cornell University Experiment Station at Geneva, the complete experiment was a split plot design with 5 replications but ANOVA was done only on the CR-371rif$^+$ and untreated control treatments for the 4 main plots viz. N/C, N/NC, P/C and P/NC analyzed as 4 randomized treatments (main plots).

6.3.8 Molecular genetic difference between mutants and wild-types

6.3.8.1 Bacteria and growth conditions

The rif$^+$ mutant and wild type isolates of Bt strain (CR-371) and streptomycete (CR-43) were grown at 25-26°C in the dark in NB and ISP#2 (or PDB) broth for 20 h and 3 d respectively at 270 rpm on a rotary shaker. A portion (0.5 ml) of a cryopreserved suspension of each isolate was used to inoculate 50 ml of broth medium in a 250-ml Erlenmeyer flask.

6.3.8.2 DNA extraction

6.3.8.2.1 Bacilli

DNA from each *B. thuringiensis* isolate was prepared from 1 ml of medium transferred to a 1.5-ml microfuge tube. Cells were pelleted by centrifugation at 10,000 g for 3 min. The supernatant was discarded and the pellet was resuspended in 1.5 ml

of saline (9.0 g/L) by vortexing. The cells were collected by centrifugation again. The saline wash was done three times. 100 ml of lysozyme (2 mg/ml in TE) was added to the pellet followed by incubation at 37 C. After 45 min, 1.5 ml of saline was added and cells were washed once as before. The pellet was resuspended in 0.5 ml of sterile glass distilled deionized water and incubated in a boiling water bath for 30 min to release the DNA into solution. The boiled suspension was immediately cooled on ice for 5 min and then cleared by centrifugation at 10,000 g for 30 s. The supernatant was transferred to another sterile microfuge tube and used directly or stored at -20°C until amplification.

6.3.8.2.2 Actinomycetes

Chromosomal DNA from the Rif^+ mutant and wild type isolates of CR-43 was prepared from 50 ml broth. Cells were pelleted by centrifugation at 2,987 g for 4 min, subjected to 2 freeze-thaw cycles (-80°C/5 min in an ultrafreezer followed by 85°C/10 min) and then resuspended in 250 ml TE (10 mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0). Lysozyme (40 mg/ml in TE) was added to a final volume of 10 mg/ml and held at 37°C. After 45 min SDS (10%) was added to 4% and cell lysis was completed by incubation at 85°C for 15 min. Each lysate was gently mixed by inverting tube and then cooled on iced. The lysate was gently shaken with an equal volume of equilibrated phenol-chloroform-isoamyl alcohol (25:24:1, adjusted for genomic DNA to pH 8.0±0.2 with Sigma buffer B-5658) for 30 min. The aqueous (top) phase was retrieved after a 15-min centrifugation at 10,000 g. The phenol extraction of this aqueous phase was repeated. The DNA was precipitated by addition of 0.6 volume of ice-cold isopropanol (centrifugation: 2 min) and then

washed twice in 0.8 ml 70% ethanol (centrifugation: 5 min). The pellets were air-dried for 10 min and carefully redissolved in 100 or 200 μL TE. DNA was quantified as described in chapter 4.

6.3.8.3 PCR amplification with a random primer

Brousseau et al. (1993) had previously demonstrated the suitability of the random primer 0955-03, 5'-CCGAGTCCA, for differentiating serovars of *B. thuringiensis*. The primer was synthesized by standard phosphoramidate chemistry (National Bioscience Inc., Plymouth, MN). T_m of the primer was 30°C by the "2*AT + 4*GC" method. Primer exhibited no stable 3'-terminal dimer and hairpin loop formation.

PCR was performed using Perkin-Elmer's GeneAmp® PCR Core Reagents (Roche Molecular Systems, Branchburg, NJ, USA). Components of the PCR reaction (50 μL) were added in the following order: reaction buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl), glycerol (5%), dATP, dGTP, dTTP, dCTP (100 μM), AmpliTaq® DNA polymerase (1.0 U), primer (0.2 μM), $MgCl_2$ (2.1 mM), and approx. 2-10 ng streptomycete template DNA in TE or 5 μl of boiled Bt supernatant. Lu and Nègre (1993) had shown that glycerol added to the PCR reaction at 5% or 10% improves DNA amplification. DMSO has the same effect but is more toxic. The reaction was overlaid with ≈50 μL of mineral oil (Sigma) and placed in an automated GTC-2 Genetic Thermal Cycler (GL Applied Research Inc, Grayslake, IL) programmed for 1 cycle of 3 min at 94°C, 2 min at 30°C, 2 min at 72°C followed by 44 cycles of 1 min at 94°C, 2 min at 30°C, 2 min at 72°C and a final hold at 72°C for 7 min to

allow for full-length DNA extensions. The reaction was held at 4°C until assayed. Amplification products were analyzed by electrophoresis in 1.7% UltraPure agarose (at 120 V for 1.5 h, 4 cm) in 1X TBE (89 mM Tris-base, 89 mM boric acid, 2 mM EDTA, pH 8.0) and detected by staining with 0.1 μg/ml ethidium bromide.

6.4 Results and Discussion

6.4.1 Culture purity check

Figure 6.1 shows the elution patterns of the bacterial fatty acids extracted from the first set of bacterial colonies that had been grown on solid media. All cultures had been previously stored at -80°C. The chromatograms are different and distinguish the three tested cultures on the basis of component retention times and fatty acid concentrations. A much broader spectrum of fatty acids was observed in Bt strain CR-371 than in Bt strain CR-450 (Fig. 6.1a,b). In fact this observation was unusual since such large intraspecific variation in fatty acid profiles is not known to occur in bacteria (Esnard et al., 1994).

Over several weeks, cultures of Bt strain CR-371 changed progressively from a glossy to a more dry "starchy" appearance. In several points of the culture, aerial mycelium was observed. Microscopic analysis in our laboratory revealed a hyaline budding sparse mycelium of the *Aureobasidium* or *Candida* type. To confirm the presence of a possible contaminant, the fatty acids of strain CR-371 were extracted and analyzed using gas chromatography by the method of Esnard et al (1994) part of which is also described in chapter 2.

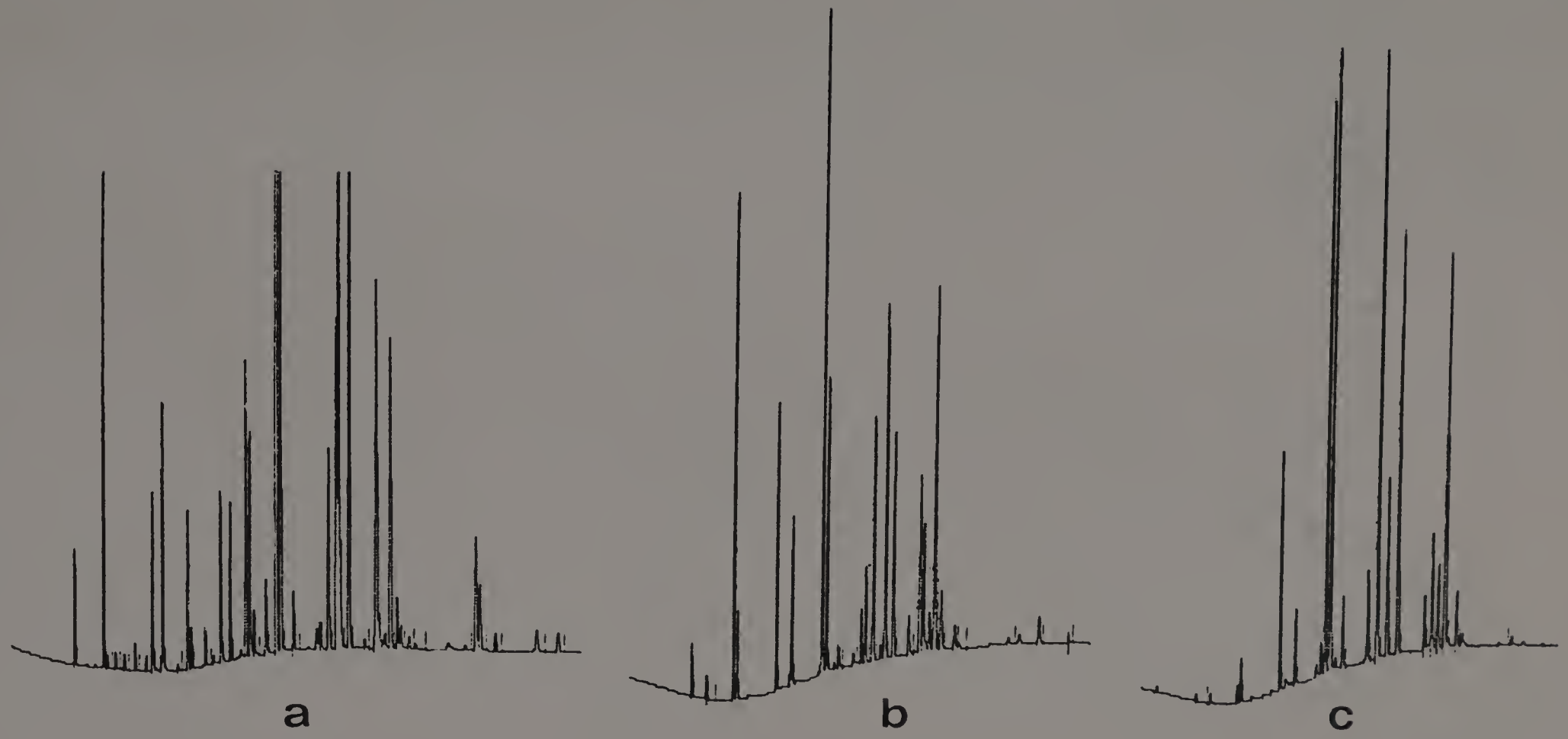


Fig. 6.1 Gas chromatograms of methylated fatty acids from *Bacillus thuringiensis* strains CR-371 (**a**) & CR-450 (**b**), and *Streptomyces* sp. strain CR-43 (**c**).

Fatty acid profiles of Bt strains CR-371 dated 4/89, 4/91, 7/92 (Fig. 6.2, 6.3, 6.4) showed presence of the contaminant in earlier cultures of **Bt** strain CR-371. Gas chromatographic analyses are extremely sensitive and should not vary significantly for a pure culture. The contaminant might have been present in some of the original cultures since 1989, probably growing more slowly than the more aggressive **Bt**.

To demonstrate the reliability of the gas chromatograph, fatty acid profiles were generated from two different sub-cultures of CR-371 stock culture dated 7/92 on two consecutive days (Fig. 6.3). No significant change in the profiles were observed demonstrating also the slow growth of the contaminant. However, 15 months earlier the fatty acid profile of a sub-culture from the same CR-371 stock culture dated 7/92 (Fig. 6.2) was very different. Normalizing peak areas of figures 6.2C and 6.3A revealed a dissimilarity in profiles (i.e. ratios were not equal to 1 and the Wilcoxon sign-ranked test revealed a significant difference ($p = 0.05$). Little change was observed for profiles generated from **Bt** strain CR-450 in August and October 1992 (Fig. 6.4). However, when these **Bt** strains were sent back to Microcheck Inc.'s Bacterial Identification Laboratory (Vermont), **Bt** cultures designated CR-371 were confirmed to have a yeast contaminant and to be similar to cultures designated CR-450. Only pure cultures supplied by Microcheck Inc were used in subsequent studies. No contaminants were discovered in the isolates of streptomycete cultures (dated 1988-1994).

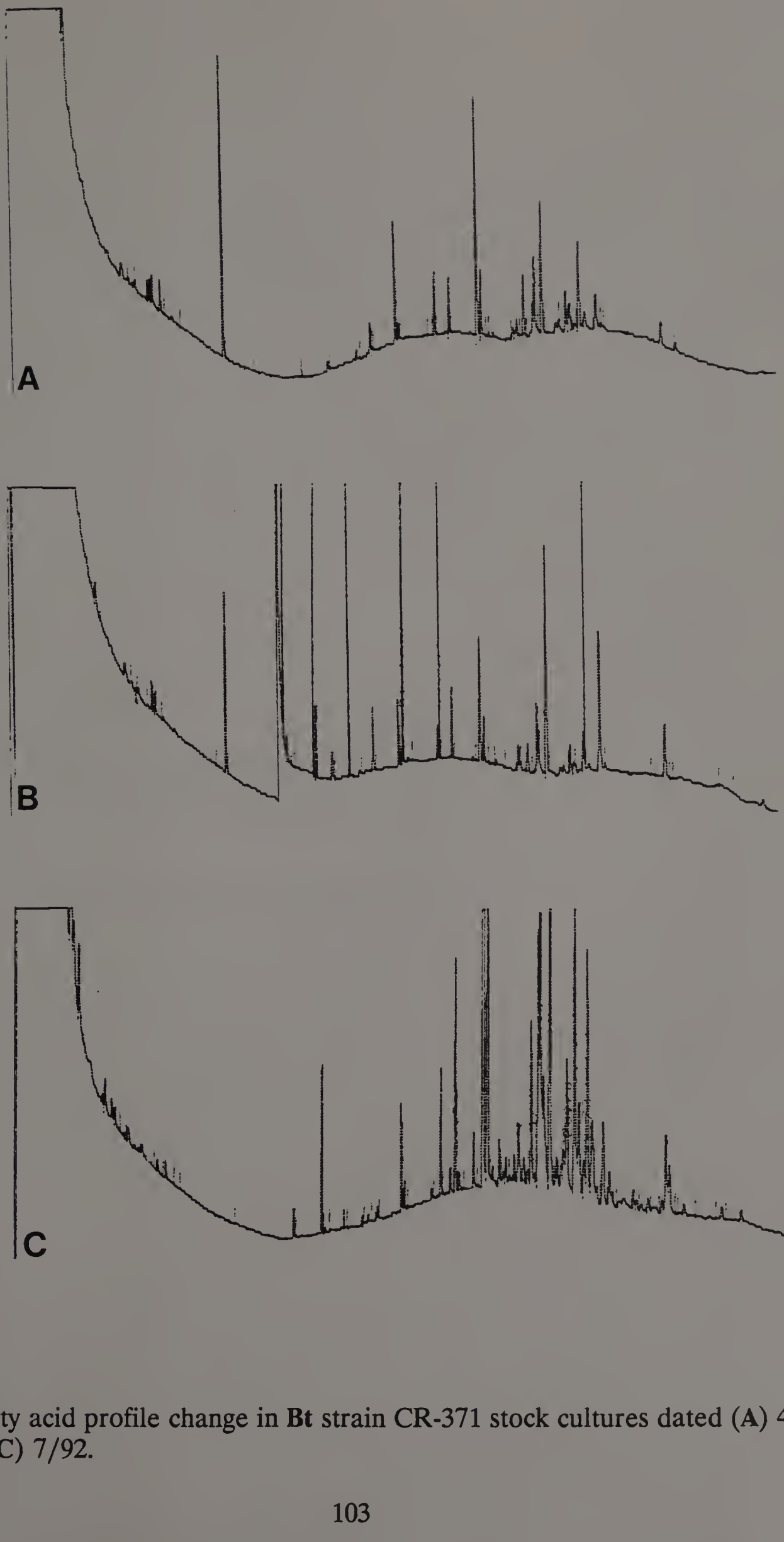
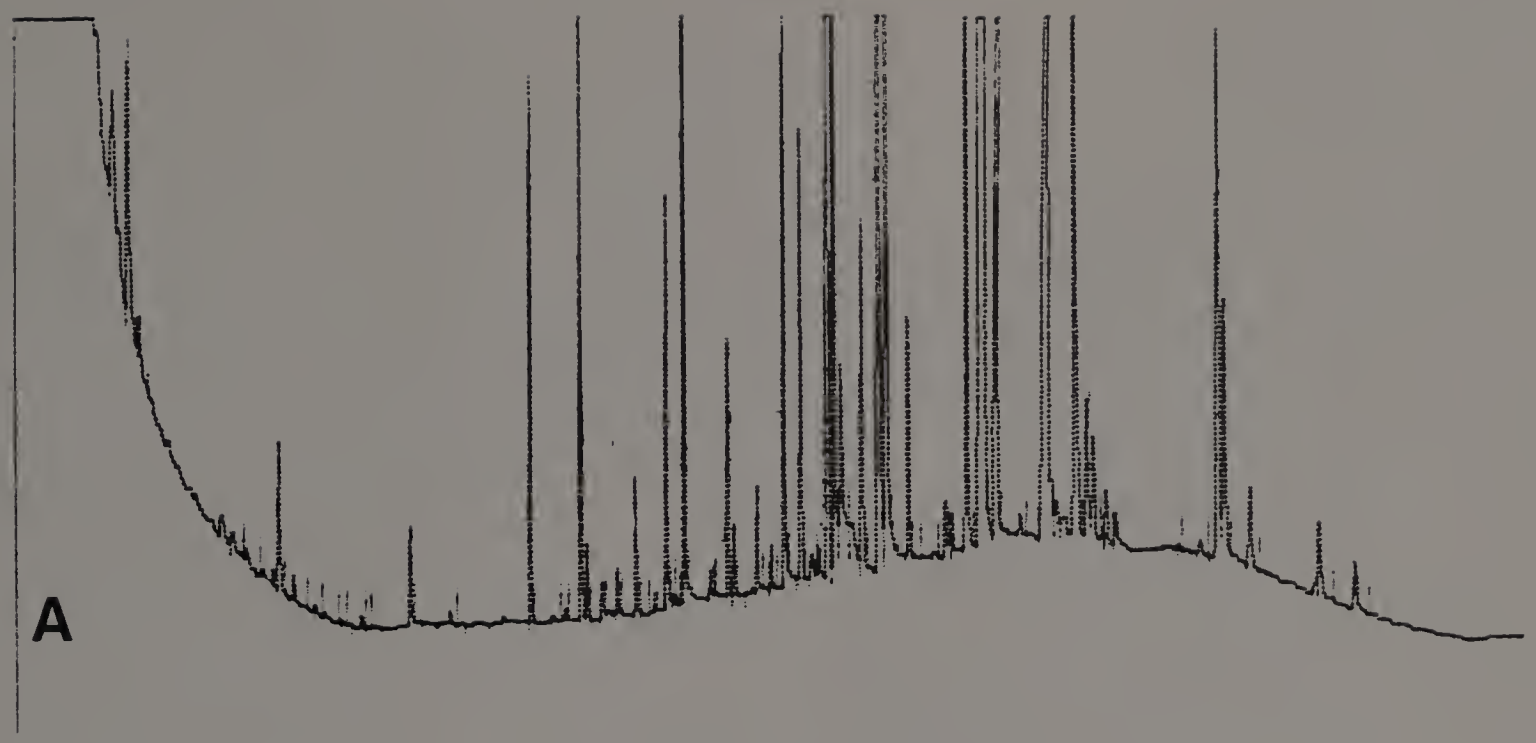


Fig. 6.2 Fatty acid profile change in **Bt** strain CR-371 stock cultures dated (**A**) 4/89, (**B**) 4/91, (**C**) 7/92.



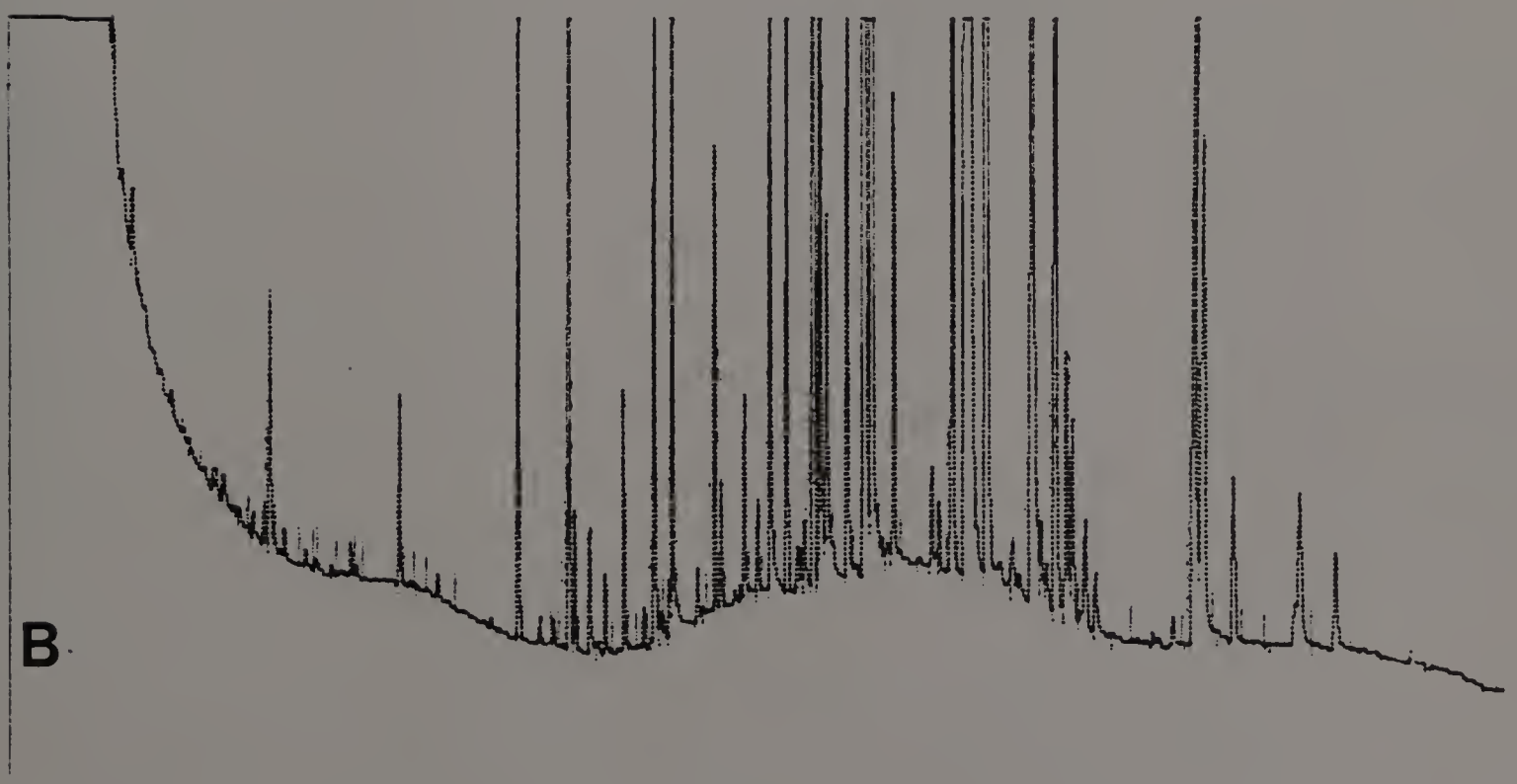


Fig. 6.3. Chromatograms of fatty acids isolated from two different sub-cultures of **Bt** strain CR-371 on two consecutive days (A,B).

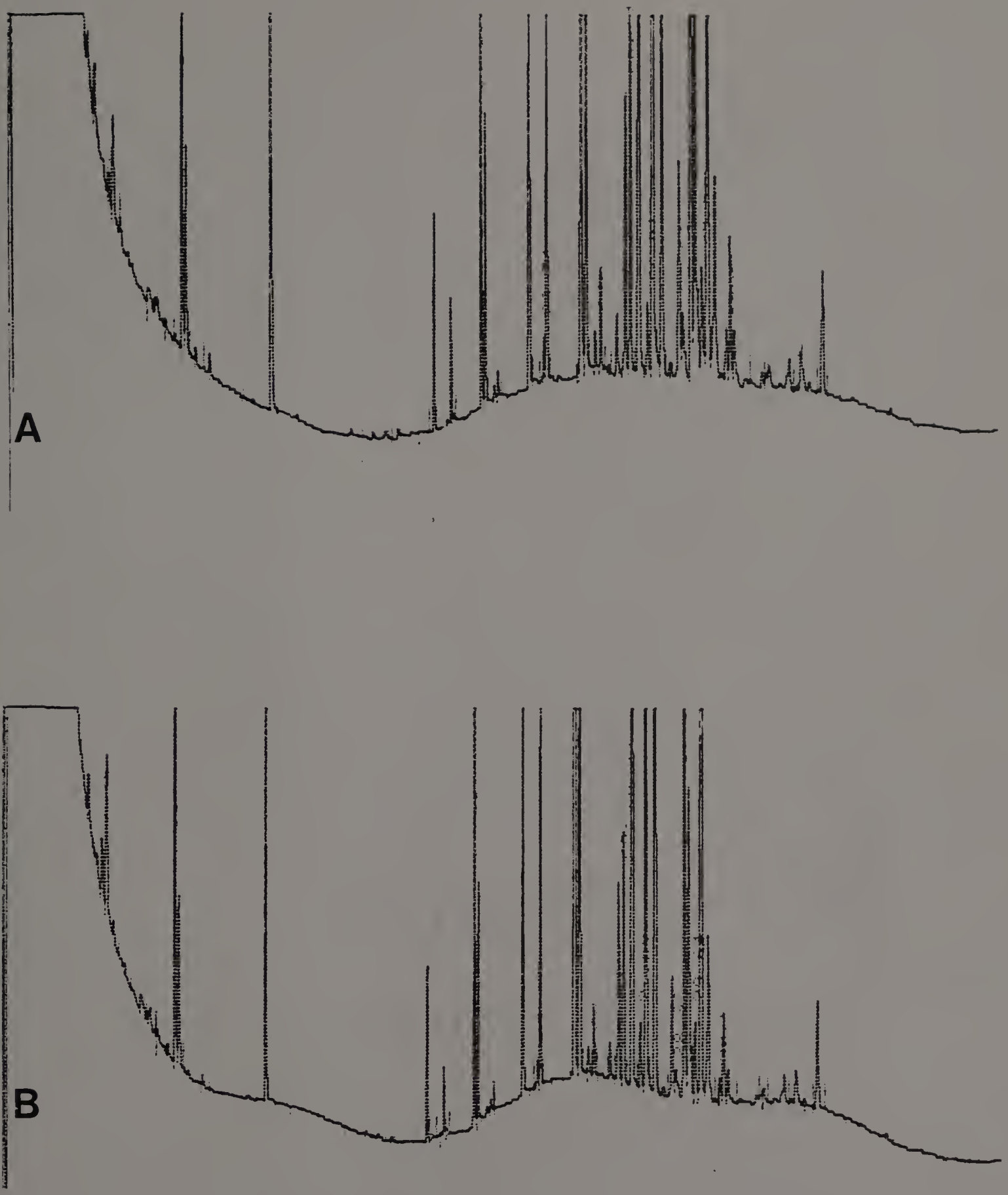


Fig. 6.4. Chromatograms of fatty acids isolated from two sub-cultures of **Bt** strain CR-450 in August 1992 (A) and October 1992 (B). (A) and (B) are similar.

6.4.2 Spontaneous rif$^+$ mutants selected

Colonies of CR-371rif$^+$ and CR-43rif$^+$ showing similar vigor, color, texture, morphology and size as the wild types were selected from solid media amended with rifampicin at 200 μg/ml and 100 μg/ml, respectively. They were grown in broth media without antibiotics and then selected again on solid media amended with rifampicin to select for bacteria that truly possessed spontaneous rifampicin resistance (rif$^+$) mutation. The rif$^+$ mutation occurred at a low frequency viz. 2-5 x 10^{-6} and 1-1.3 x 10^{-7} in the Bt and streptomycete strains, respectively. Most colonies of CR-43rif$^+$ that were not selected showed reduced colony size and sporulation, an observation also made by Elliott et al. (1994) working with *Streptomyces griseus.* Kloepper and McInroy (1992) reported that rifampicin resistance caused changes in growth rates, fatty acid profiles and C-source utilization of *Pseudomonas cepacia* rif$^+$ mutants but not its biocontrol activity against the damping-off fungus *Rhizoctonia solani* on cotton.

The existence of any pleiotropic effect of the rif$^+$ mutation on biocontrol activity was investigated by a bioassay of the rif$^+$ mutants against the free-living nematode *Caenorhabditis elegans*. The Bt CR-371rif$^+$ mutant had a significantly greater biocontrol effect on *C. elegans* than the wild type in vitro after 24 h (Fig. 6.5) and after 10 weeks (Fig. 6.6). In fact under the specific assay conditions, biocontrol activity of the wild Bt strain after 24 h was only slightly better than or equal to the controls (Fig. 6.5, unshaded bars) but over 10 weeks the nematode population was drastically reduced (Fig. 6.6). At 10 weeks, all nematodes cultured in the presence of the rif$^+$ marked Bt were dead (Fig. 6.6).

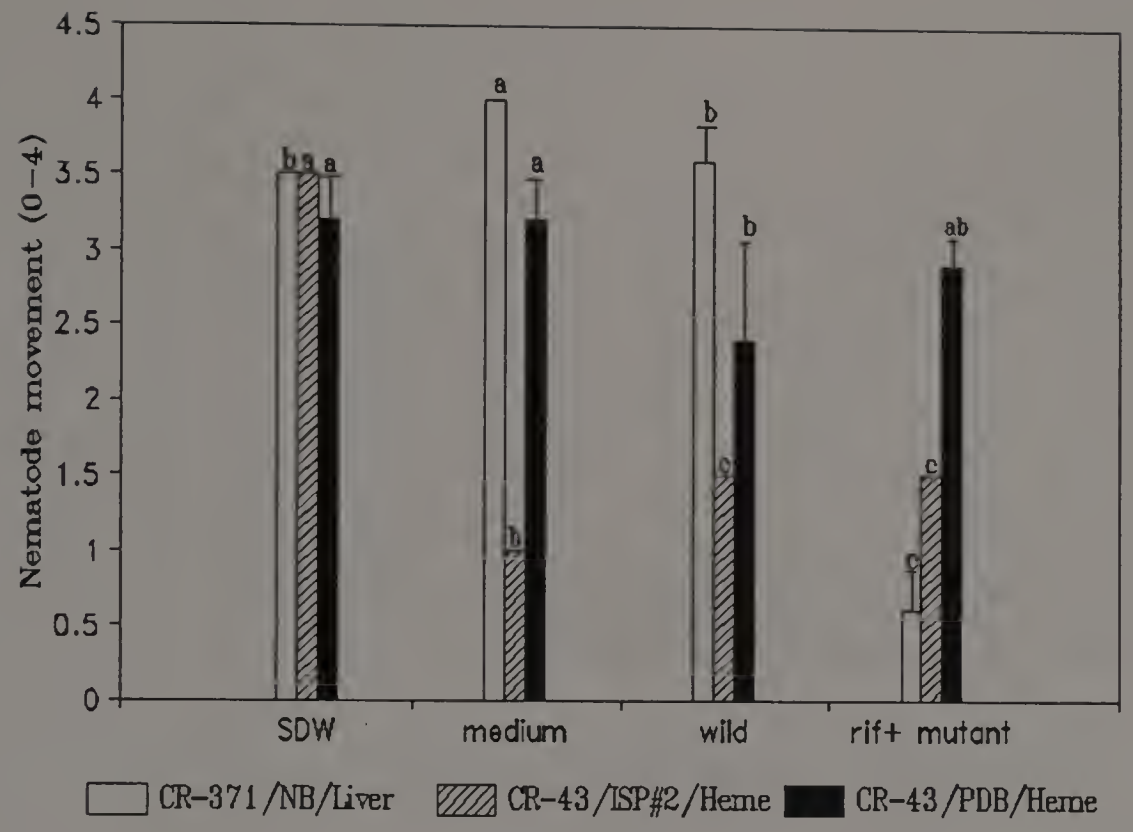


Fig. 6.5 Effect of wild and rif^{+} mutant strains on *C. elegans* activity on a scale of 0-4 where 0 = all dead, 1 = sluggish, 2 = av. 1:1 slow-fast, 3 = high, 4 = very high (all live). Values are means of 5 determinations. Bars with the same fill pattern and letter are not significantly different at p = 0.05. SDW = sterilized glass-distilled deionized water.

However, the rif^{+} mutation had a slightly pleiotropically deleterious effect on the biocontrol property of the streptomycete strain (Fig. 6.5, filled bars). The results of this assay, however, were confounded by the antinematodal effect of the ISP#2 medium (Fig. 6.5, 6.6, cross-hatched bars) which is probably contained in the malt or yeast extract component of this bacterial medium. Nematode densities increased significantly more in nutrient broth (used to grow the Bt strain) than in the putative nematode liver medium (Fig. 6.6).

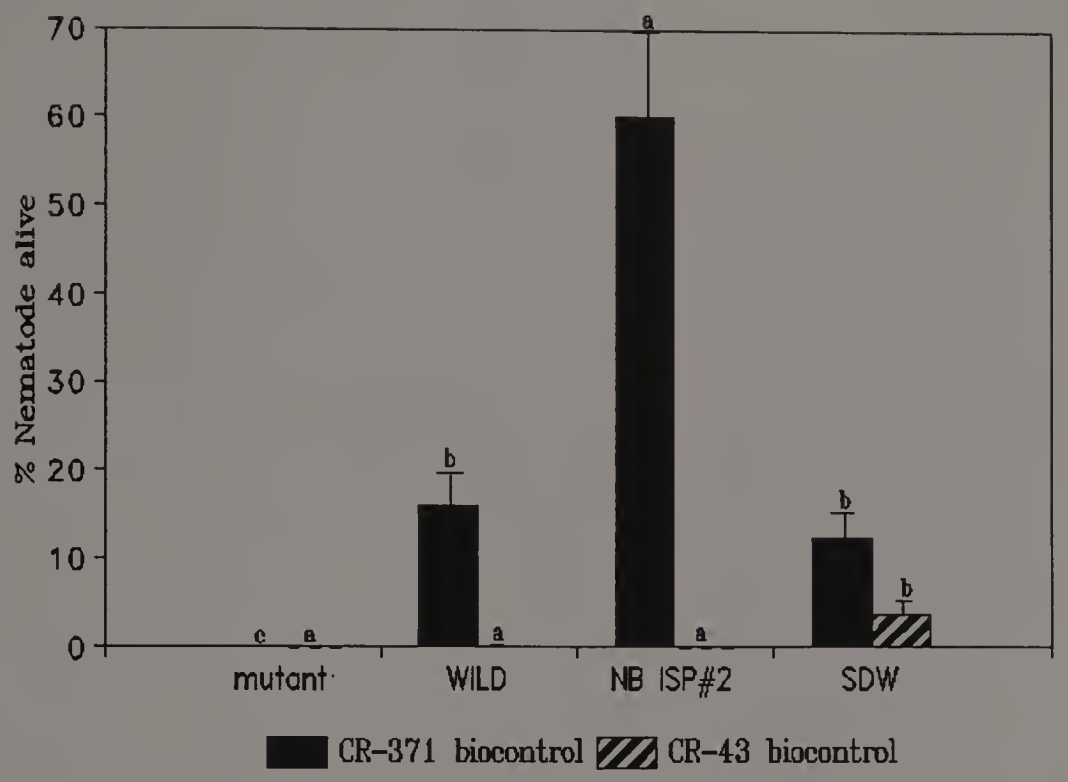


Fig. 6.6 Survival rate of *C. elegans* in presence of CR-371rif$^+$ & CR-43rif$^+$ mutants and wild types CR-371 & CR-43 for 10 weeks at 22.2°C. Values are means of 3 replications. Bar values with the same letter are not significantly different at p = 0.05. NB = Nutrient Broth (for Bt), ISP#2 = Streptomycete medium, SDW = sterilized glass-distilled deionized water.

6.4.3 Plant response and nematode control

The response of the root-knot susceptible tomato cv Rutgers to the addition of wild or rif$^+$ biocontrols was evaluated in two consecutive years. Among the parameters shoot dry weight, height and root-galling indices, only plant height was increased significantly and only in the treatment with the Bt CR-371rif$^+$ mutant (Fig. 6.7). The altered polymerase of CR-371rif$^+$ is probably also associated with an increased production by the bacterium of plant growth promoting metabolites.

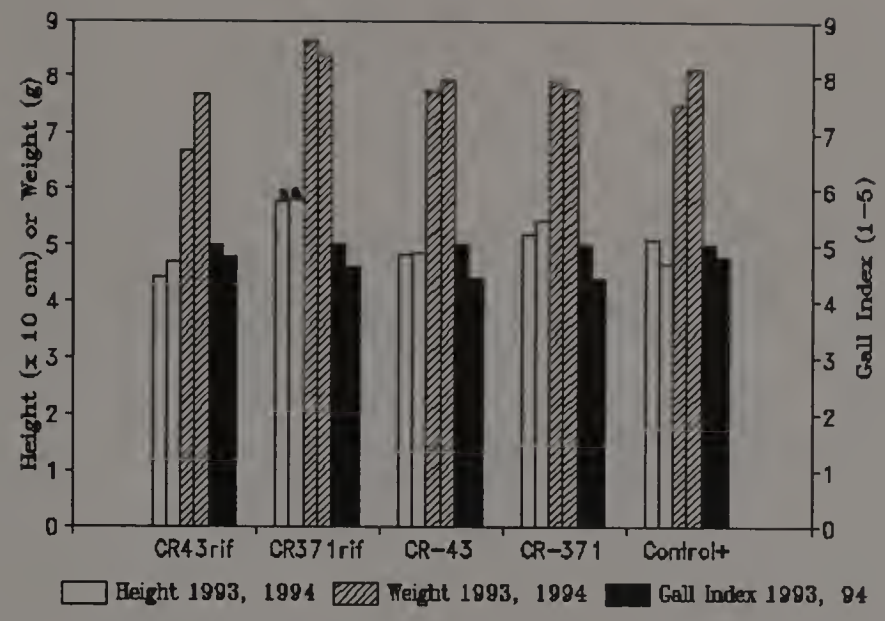


Fig. 6.7 Shoot dry weight, height and degree of *Meloidogyne incognita* galling of tomato plants treated with two rif$^+$ mutants and two wild-type bacteria over 7 weeks in the greenhouse in 1993 and 1994. Values are means of 5 replicates. Only mean height of CR-371rif$^+$ was significantly different (a).

Zuckerman et al. (1993) and Dicklow et al. (1993) obtained reduction of root-knot symptoms on tomato in greenhouse trials with the wild type CR-371 and CR-43 although the results were not statistically significant for the former. Shoot weights were not increased by both biocontrols, an observation also made in the present study (Fig. 6.7). Several differences in the conduct of the greenhouse trials might account for the absence of root-knot control. Only one 50-ml application of each biocontrol was made to each plant in this study whereas eight 50-ml Bt drenches at weekly intervals were applied by Zuckerman et al. (1993) and 2 applications of the streptomycete were made (Dicklow et al., 1993) which certainly afforded some nematode control by the repeated applications. Moreover, Bt and streptomycete

cultures in this study were grown respectively for no more than 20 h and 4 days (at 250 rpm) whereas in the previous trials, bacterial inocula were increased for >2 and 7-10 days (at 100 rpm) which probably produced greater spore numbers. The inocula in this study probably had a higher density of cells still in the vegetative stage and fewer spores to survive long enough after the bacteria were applied to the soil. Zuckerman et al. (1993) and Dicklow et al. (1993) removed the supernatant of each bacterial broth culture and replaced it with water in order to cancel nutrient effects. In this study, the bacterial inoculum was further washed once with water to eliminate any residual nutrient effect that the NB, ISP#3 or PDB medium might have directly on the nematodes (Fig. 6.5, 6.6) or on the soil microflora of antagonists or directly on the biocontrol inocula being applied so that any biocontrol activity would be associated with the organism only. It is likely that the extra washing reduced the concentration of residual nutrients or antinematodal factor(s) already in each volume of inoculum. The relative importance of these differences was not assessed.

6.4.4 Persistence and survival of biocontrols in soil

6.4.4.1 Greenhouse trial in natural soil

Rif$^+$-marked Bt and streptomycete biocontrols were used to monitor the survival of these organisms in soil in two years. Bt CR-371rif$^+$ and streptomycete CR-43rif$^+$ populations remained relatively high in the first 2-3 weeks but significantly declined after that period to <20 colony forming units per gram of soil at harvest, 7 weeks after biocontrol applications (Fig. 6.8). The fastest decline occurred within the second to third week from inoculation of the rif$^+$-marked streptomycete. Based

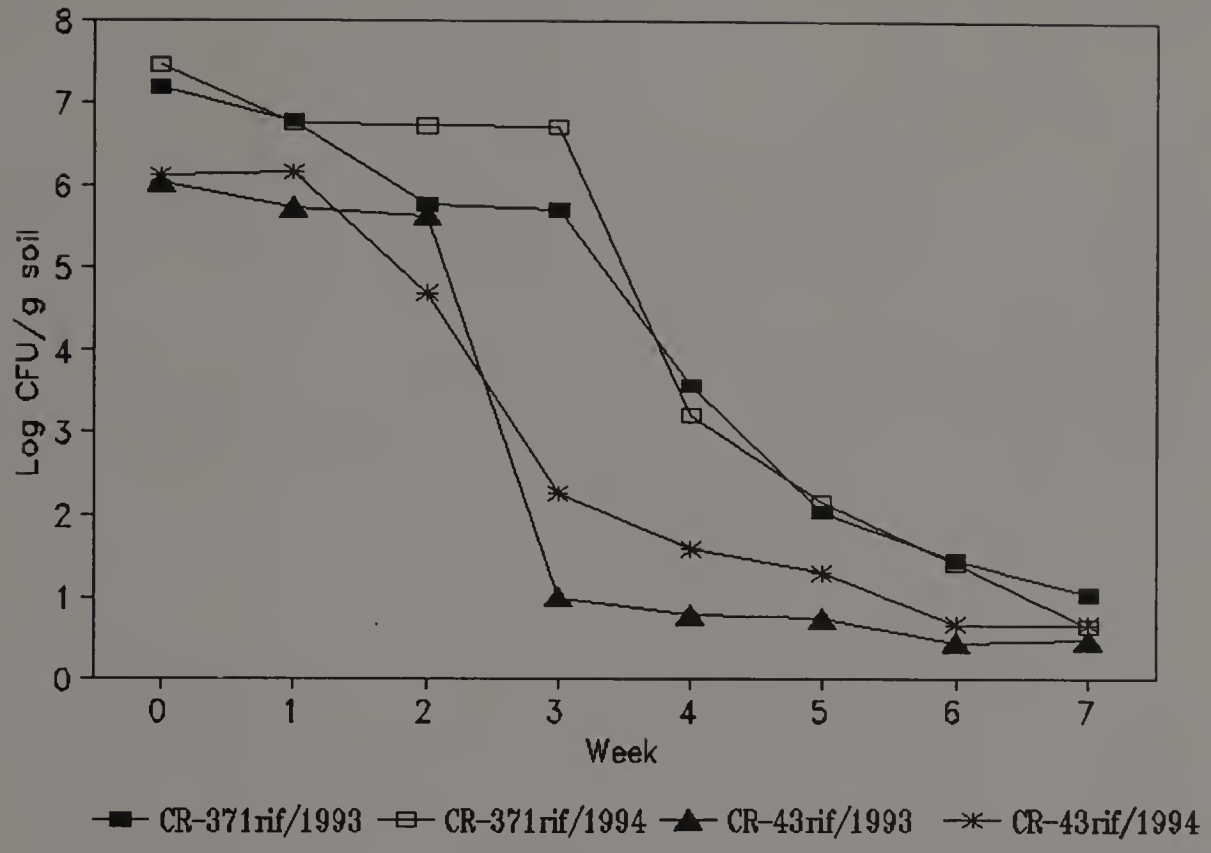


Fig. 6.8 Viable counts (per g of soil) for Bt and streptomycete rif^{+} mutants applied to soil at week 0 for control of *Meloidogyne incognita* over 7 weeks in the greenhouse in 1993 and 1994. CFU values are means of 5 replications.

on the trend in the survival curve, a biocontrol application at biweekly intervals or every 4 weeks would have helped to maintain a high enough bacterial population level and achieve the degree of root-knot control reported by Zuckerman et al. (1993) and Dicklow et al. (1993). The bacterial inocula in the latter reports probably also had a greater percent of cells that were in the survival (spore) stages.

6.4.4.2 Field persistence of rif-marked Bt

In a lettuce trial in Geneva, the survival of the rif-marked Bt (CR-371rif$^+$) in the field was quantified in muck soil

which was pasteurized or non-pasteurized (natural) and amended with (or without) chitin. At the end of 7 weeks the initial inoculum of CR-371rif$^+$ declined to a low level in all soil treatments (viz. ≈10-25 cfu per gram of soil, Fig. 6.9). Although there were no differences in the numbers of bacteria persisting in the various soil treatments at harvest, the greatest survival was in non-pasteurized chitin-amended (N/C) soil. Chitin-, starch-, or hemicellulose-amendments to soil are known to significantly increase viable counts of streptomycetes (Wellington et al., 1990) but the marked streptomycete was not studied in this trial because of the negative effects of rifampicin resistance in that microbe.

Preliminary results on survival of Bt CR-371rif$^+$ in Costa Rican soil were investigated in a Dole-UMass banana nematode biocontrol project and showed that Bt levels (≈10^6-10^7) declined to <5.5 log cfu/g soil in 5 d in natural tropical soils (Fig. 6.10). Bt usually sporulates in 4-5 d of culture. This would imply that some Bt inocula were still in the vegetative stage and were not able to survive at higher rates in natural soil. Survival is less at the higher dose. The efficacy of older inoculum of CR-371rif$^+$ is being investigated in our laboratory. Bt spore production can be assessed after 24 h (Salama and Morris, 1993) but Bt may take more than 1 week to complete sporulation if oxygen supply is low (Bernhard and Utz, 1993).

6.4.5 Biocontrol activity and survival of marked strains: a discussion

Overall the results suggest that rifampicin resistance is deleteriously pleiotropic in streptomycete strain CR-43rif$^+$ whereas the rif$^+$ mutation is an effective

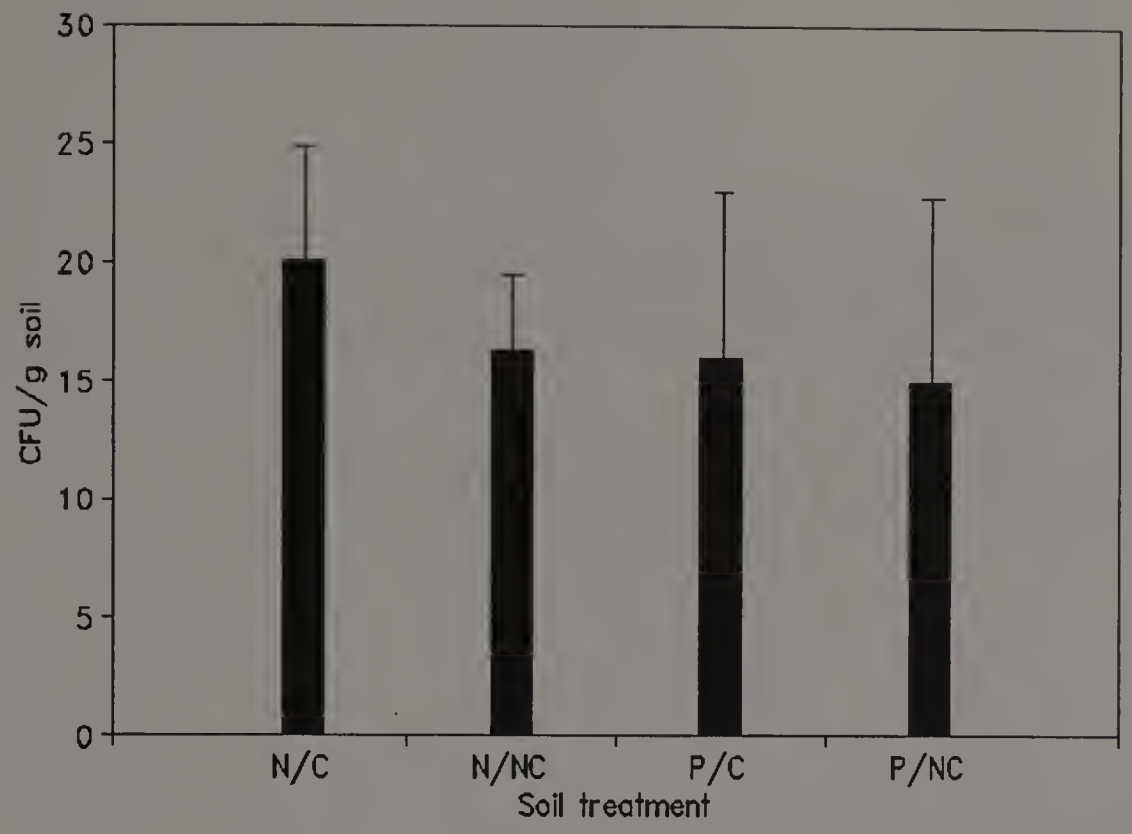


Fig. 6.9 Survival of Bt rif$^+$ mutant (CR-371rif$^+$) applied for control of *Meloidogyne hapla* over 7 weeks in field microplot at Geneva in 1994. CFU/g values are means of 10 determinations (2 counts for each of 5 microplot replications). Vertical bars indicate standard deviation, p = 0.2697, n.s.).

marker for monitoring the persistence and biocontrol effects of the Bt strain and might be positively pleiotropic in it. For both species the rifampicin resistance marker enable detection of the bacteria in amended and natural soil. Since CR-371 and CR-43 were being introduced as biological agents for control of nematodes, it was necessary to study the persistence of these biocontrols in the soil environment.

Although Bt is a common soil microorganism (Martin and Travers, 1989), it rarely grows in soil (Meadows, 1993). Bt might be deposited in soil by invertebrate cadavers and larvae that descend the plant to pupate or on fallen leaves. Bt may

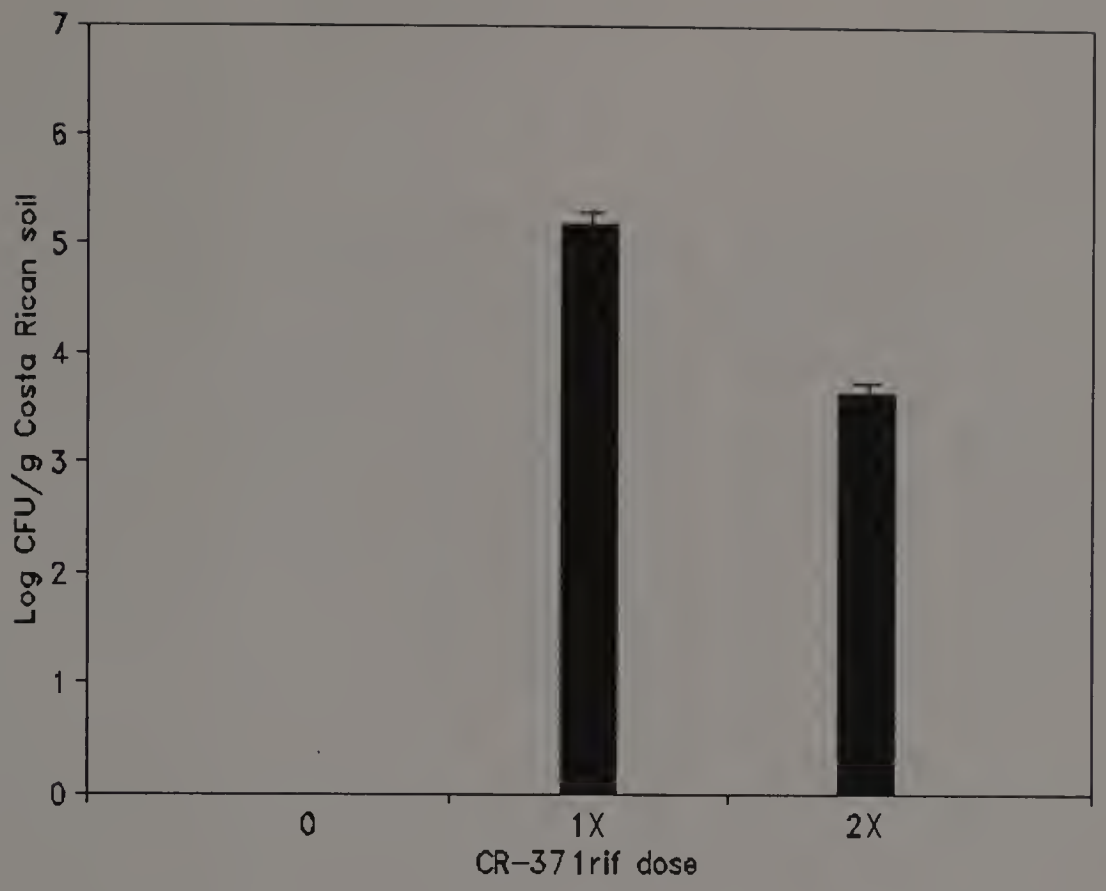


Fig. 6.10 Population levels of *Bacillus thuringiensis* strain CR-371rif^{+} 5 days after inoculation into soil planted to bananas in Costa Rica (1995).

grow in the soil when nutrients become more available as occurs when plants and animals decay and after rainfall (West and Berges, 1985; West et al., 1985a). The soil probably acts as a reservoir for Bt spores from where they may be disseminated over longer distances by the wind (Meadows, 1993). However, the recycling and role of Bt in the environment is still not fully understood (Bryne et al., 1992).

The presence of Bt in soil might be related to its close affinity to *B. cereus* which greatly outnumbers Bt in most soils and is frequently co-isolated (Meadows, 1993). Bt's without parasporal crystals (and therefore devoid of δ-endotoxin-coding plasmids) are indistinguishable microscopically from *B. cereus* and derived colonies

would be classified as *B. cereus*. The possibility that *Bacillus cereus* is derived from Bt is supported by the fact that out of about 7,000 Japanese isolates identified as belonging to the Bt/*B. cereus* group, only about 30% reacted with Bt flagellar antisera (Ohba and Aizawa, 1986c). "Bt"s remain as dormant spores in the soil until favorable growth factors occur. A few "Bt"s might have germinated under favorable conditions and, because of the maintenance cost (Godwin and Slater, 1979) to a cell for having to reproduce plasmids, synthesize RNA and proteins form plasmid-borne genes and carry out physiological functions directed by plasmid genes, spontaneous loss of plasmids would occur and help cells gain a selective advantage over the ones that harbored more plasmids. Perhaps addition of a closely related bacterium (viz. Bt) favors cells most different from *B. cereus* ecologically. A pioneering study with animals has shown that competition does promote divergence in adaptive radiation (Schluter, 1994) which may also operate in natural selection among microbes.

One study reported that the bacillus *B. megaterium* (B153-2-2) biocontrol for *Rhizoctonia solani* Kühn on soybean increased after introduction to soil, remained constant during the growing season and then decreased near the end of the year (Liu and Sinclair, 1992). However, it is well known that in soil *B. thuringiensis* has a very short survival time (Beer, 1991, and this study). Vegetative cells have been observed to disappear at rapid exponential rates (West et al., 1984). Applied parasporal crystals disappear also (but at a non-exponential rate) whereas spore numbers may remain unchanged for up to 13 weeks at 25°C with no germination (Pruett et al., 1980; West et al., 1984). Several studies show that, unlike Gram-negative bacteria, introduced *Bacillus* spp. are poor rhizosphere and soil colonizers existing mainly as spores (Liang et al., 1982; van Elas et al., 1989).

In this study, the Bt biocontrol strain declined to slightly lower levels in pasteurized than in non-pasteurized chitin-amended soil (Fig. 6.9). This agrees with the results of Amner et al. (1991) who observed that *B. subtilis* survival was greater in fresh than in sterile compost. In another study, however, West et al. (1985a) observed that Bt spore inocula of 10^3-10^5 spores/g of soil apparently failed to germinate in non-sterilized soil and viability declined by up to 55% during 90 d at 25°C. The researchers suggested that mortality of Bt spores in non-sterilized soil results from their inherent inability to survive in such soil environments. Inside insect larvae, antibiotic production, rather than pathogenicity, determined dominance of a particular crystalliferous Bt strain (Pendleton, 1969). Petras and Casida (1985) reported that 90% of Bt spores quickly disappeared in soil with the remaining 10% persisting for prolonged periods. Non-obligate predatory bacteria such as unnamed strain L-2 and *Cupriavidus necator* may be partly responsible for the reduction of Bt spores and crystals (Casida, 1988).

In nutrient-supplemented natural soils Bt and *B. cereus* are known to grow at rates comparable to other resident soil bacteria (West et al., 1985). Addition of $CaCO_3$ increased Bt and *B. cereus* growth rates in natural, autoclaved and nutrient-supplemented autoclaved soil. Decreased growth rates were observed in nutrient supplemented natural soil to which $CaCO_3$ was added, probably as a consequence of increased competition for nutrients from indigenous microbes. $CaCO_3$ probably increases soil pH above 5.2 which is suitable for growth of *Bacillus* isolates (West et al., 1985). Chitin added to soil releases ammonia which can increase soil pH and result in increased solubility of many nutrients and creation of a favorable environment for Bt growth. This was apparent in the non-pasteurized chitin-amended plots in the Geneva experiment (Fig. 6.9).

Rifampicin resistance was deleteriously pleiotropic in the streptomycete strain CR-43rif$^+$. *Streptomyces* spp., for example on wheat and turf grass roots, are quite sensitive to rifampicin (Drahos et al., 1986). Colonies appearing on media amended with 200 μg/ml rifampicin were severely folded, glossy and reduced in size compared to wild type colonies. Selections were therefore made from plates with 100 μg/ml rifampicin. Elliott (1994) reported that rif-marked *Streptomyces* have been rarely recovered when introduced into the rhizosphere. In this study on the contrary, the rif-marked streptomycete strain (CR-43rif$^+$) was easily recovered from soil (Fig. 6.8). A slightly slower rate of growth and sporulation was discernable in CR-43rif$^+$. Elliott (1994) used a very low concentration of rifampicin (10 μg/ml) for rif$^+$ selection and predictably observed only slight colony size reductions and no growth inhibition. This probably explains their generally lower recovery rate (Elliott, 1989) and degree of colony growth alterations than in this study. Elliott (1994) also observed reduced isolation of the *S. griseus* strain when multiple antibiotic resistance markers (including rif$^+$) instead of a single marker was used, but the recovery rate varied with the basal medium. Background contamination on plates in this study was not a problem since the concentrations of cyclohexamide and rifampicin suppressed fungi and other bacteria for at least the duration of the examination period.

Sporogenesis in microbes produces the "seeds" of survival and is a very complex process in streptomycetes that varies depending on the strain and environment (Coleman and Ensign, 1982; Kendrick and Ensign, 1983). Gross

morphology can vary greatly with media composition and physical factors. Actinomycetes form a closely interconnected ecological community with fungi and other bacteria where they continually experience substrate limitation. Their polymorphism is strongly correlated with the production of a variety of secondary metabolites which is usually favored by suboptimal growth conditions. Secondary metabolite production is known to be suppressed by inorganic phosphate and by C- and N-sources that tend to support vegetative growth rather than morphological differentiation (Gräfe, 1989). The RNA polymerase selected in the streptomycete strain CR-43rif^{r} probably functions in a manner that greatly affects sporulation and secondary metabolite production that are probably necessary for biocontrol activity. In the green-house experiments, phosphates released from the NPK fertilizer probably suppressed production of secondary metabolites that might have had antinematodal effects.

There is evidence that some secondary metabolites affect the producer as autoregulators of development processes (Gräfe, 1989) such as control of sporulation and aerial mycelium formation; maintenance of spore dormancy; control of spore germination; regulation of secondary metabolism; control of cell adhesive properties; maintenance of spore insensitivity to osmotic pressure, UV-radiation and heat; and regulation of genetic processes. Peptide antibiotics produced by *Bacillus* strains are known to regulate transcription specificity for sporulation and germination (Ristow and Paulus, 1982; Danders and Marahiel, 1981). In several streptomycetes, several antibiotics affect cytodifferentiation and secondary metabolism involving induction of mechanisms protecting the producer cell from its own toxic secondary metabolites

(Gräfe, 1989). In other microbes, acquisition of resistance to one antibiotic is often associated with hypersensitivity to others (Johanson and Hughes, 1995). The rif^{+} mutation might have affected the regulation of one or more of these processes associated with growth and survival in vitro and in soil.

Certain streptomycete species could transiently lose a trait (such as antibiotic production necessary for biocontrol) if passed through a complex natural substrate. This was the case with *S. thermoviolaceus* (Amner et al., 1993) which gave at first 80% recovery in autoclaved compost using its natural antibiotic marker (granaticin) but only 1-2% recovery up to 2 d after release into non-sterilized raw compost. After 3 days granaticin pigment was lost and *S. thermoviolaceus* could not be distinguished from indigenous species. The fact that the compost transiently affected the physiology of the bacterium highlights the importance of not extrapolating biological properties observed in the lab or in one locality to those exhibited in natural or remote field environments.

Perhaps the real reason for the low survival of the streptomycete in soil was that the inoculum was too young when it was applied to the soil. van Elas et al. (1994) found that starvation of *Pseudomonas fluorescens* prior to introduction resulted in enhanced survival as compared to a population of vegetative cells.

6.4.6 DNA polymorphisms in wild and rif-marked biocontrols

Molecular genetic differences were detected in DNA cloned from Bt strains CR-371 and CR-371rif^{+} and from the streptomycete strains CR-43 and CR-43rif^{+}

(Fig. 6.11). Williams et al. (1990), who first demonstrated the usefulness of DNA polymorphisms amplified by arbitrary primers, indicated that it might be possible for the RAPD assay to detect single base changes in genomic DNA. They observed a complete change in DNA profiles when there was only a single nucleotide change in the sequence. Under the specific assay conditions, four regions were amplified from the genome of the rifampicin resistant isolates, CR-371rif$^+$ (lane 3 and 5) whereas only two bands were detected in the rif-sensitive wild strain (CR-371 lane 2) (Fig. 6.11). One Bt isolate showing extremely slow growth and off-color on rifampicin amended plates differed from other isolates and produced one amplification product (lane 4). The CR-371rif$^+$ strain in lane 5 had been recovered from the lettuce trial in chitin-amended non-pasteurized muck soil in Geneva, New York. The DNA band of approx. 708 bp was more intense than the corresponding band in the profile of the original rifampicin-resistant isolate (lane 2). The primer used in this study could differentiate even serovars of a Bt strain (Brousseau et al., 1993) and was probably able to differentiate the population of Bt cells selected in the soil of the lettuce microplot trial in Geneva from the population inoculated at the beginning of the trial. DNA amplification with primer 0955-03 had previously not been demonstrated to be useful for the differentiation of streptomycete strains or serovars, but it successfully discriminated CR-43 (lane 7, zero bands) from two rifampicin resistant isolates (Fig. 6.11, lanes 8, 9). CR-43rif$^+$100 (lane 8, 1 band) was the selection made for greenhouse trials (discussed above) whereas CR-43rif2$^+$ (lane 9) was an extremely folded slow growing colony on rifampicin amended plates. This differential amplification might reflect the pleiotropic changes in the genomes of these mutants.

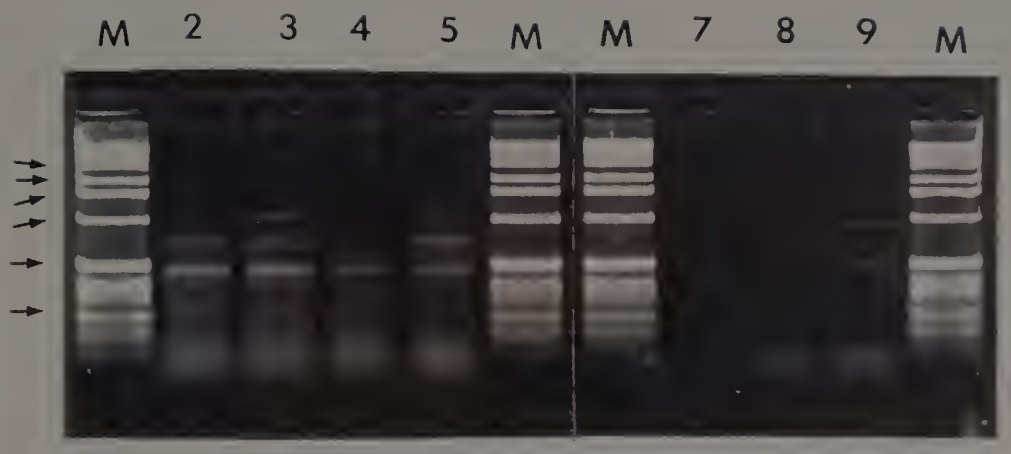


Fig. 6.11 Differences in DNA of wild and rifampicin-resistant strains of *Bacillus thuringiensis* (CR-371) and *Streptomyces costaricus* (CR-43) amplified by PCR primed with 0955-03 (T_m = 30°C) at annealing temperature of 30°C. DNA amplification products were resolved in a 1.7% agarose gel which was stained with ethidium bromide (0.1 μg/ml). Lanes M = 1 kb DNA marker, lane 2 = strain CR-371, lane 3 = CR-371rif$^+$ (bioassay isolate), lane 4 = CR-371rif2$^+$ (retarded isolate), lane 5 = CR-371rif$^+$ (isolate recovered from field trial), Lane 7 = CR-43, lane 8 = CR-43rif$^+$100, lane 9 = CR-43rif2$^+$ (a malformed isolate).

Apart from real differences that exist in the DNA of antibiotic resistant and sensitive isolates, DNA polymorphisms may have been generated by a certain degree of primer-template mismatch that would still reflect the inherent differences between these genomes. PCR amplifications and the polymorphisms that they reflect may also be a result of deletions of a priming site, insertions that place priming sites too far apart for successful amplification, or insertions that change distances between loci but not far enough to prevent amplification (Williams et al., 1990). The rif^+ mutation itself is thought to be a deletion (Sebald, 1993). Although low annealing temperatures decrease the specificity of the PCR and may produce anonymous bands, the profiles produced are reproducible even for DNA fragments amplified from complex genomes (Bowditch et al., 1993). Thus, the degree of discrimination by a primer of alternative priming sites in slightly different DNA sequences is an intrinsic reproducible feature of the particular primer for a given set of parameters and is therefore useful for differentiation.

6.5 Conclusion

The spontaneous rifampicin resistance mutation (rif^+) is useful for marking the novel antinematodal Bt strain CR-371 and *Streptomyces costaricus sp. nov.* type strain Cr-43. Bacterial survival in greenhouse and field soil could be assessed. Several bioassays revealed that the mutation was negatively pleiotropic in the streptomycete whereas a slightly positive pleiotropic effect was observed in the Bt strain. The Bt and streptomycete generally did not grow (increase) in soil. They persist but do not maintain high population densities. DNA amplification with a random arbitrary

primer, previously shown to discriminate serovars of Bt, unveiled the molecular genetic differences between the mutants and wild types.

6.6 References

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APPENDIX

CHROMATOGRAMS AND MASS SPECTRA OF UNDETERMINED COMPOUNDS

Box pairs **1 - 8**, Total ion chromatograms (TIC) and corresponding mass spectra of certain undetermined compounds extracted from the *Bacillus thuringiensis* strains (Table 2.1, chapter 2):

1 Peak 19 (t_R = 32.473 min), 16:1

2 Peak 22 (t_R = 33.764 min), 17:1

3 Peak 27 (t_R = 35.062 min), cy17:0(9)

4 Peak 30 (t_R = 36.966 min), 18:2

5 Peak 31 (t_R = 37.121 min), 18:1

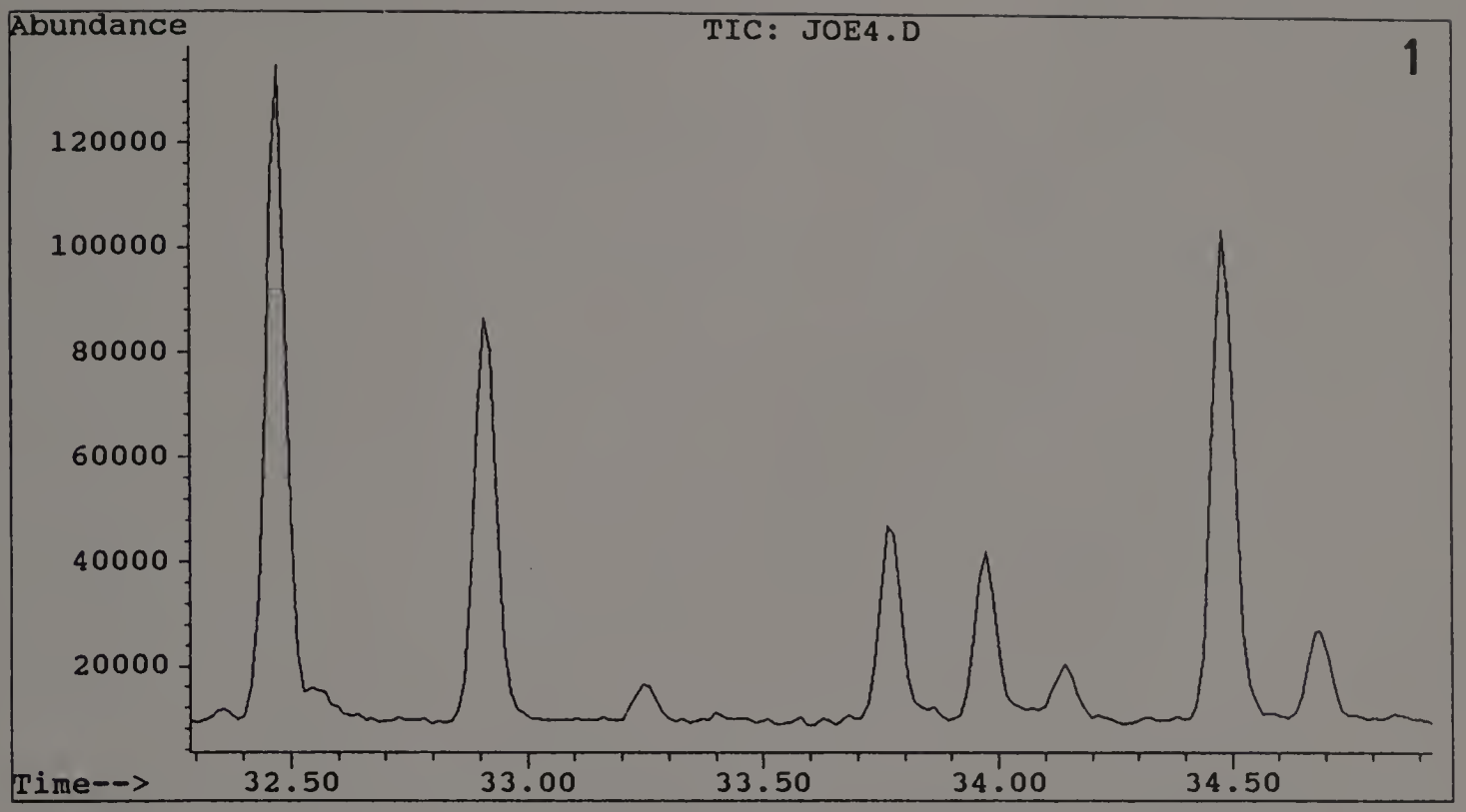
and from the streptomycete species (Table 3.2, chapter 3):

6 Peak 28 (Rt = 37.600 min), MW = 296

7 Peak 30 (Rt = 37.827 min), MW = 298

8 Peak 28 (Rt = 41.076 min), MW = 290

where t_R = relative retention time, Rt = retention time, MW = molecular weight.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60000
40000
20000
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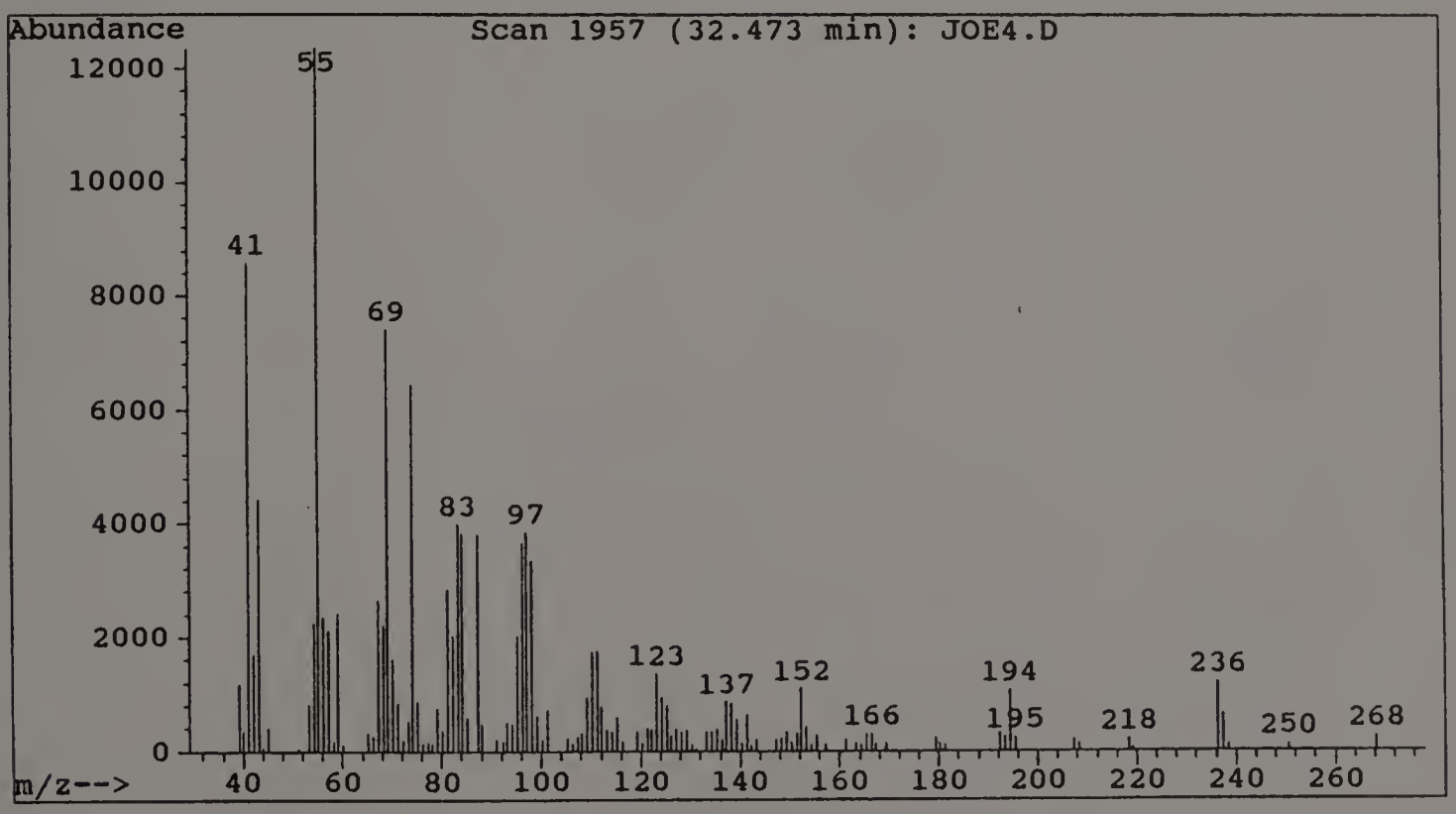
33.50
34.00
34.50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120
140
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137
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236
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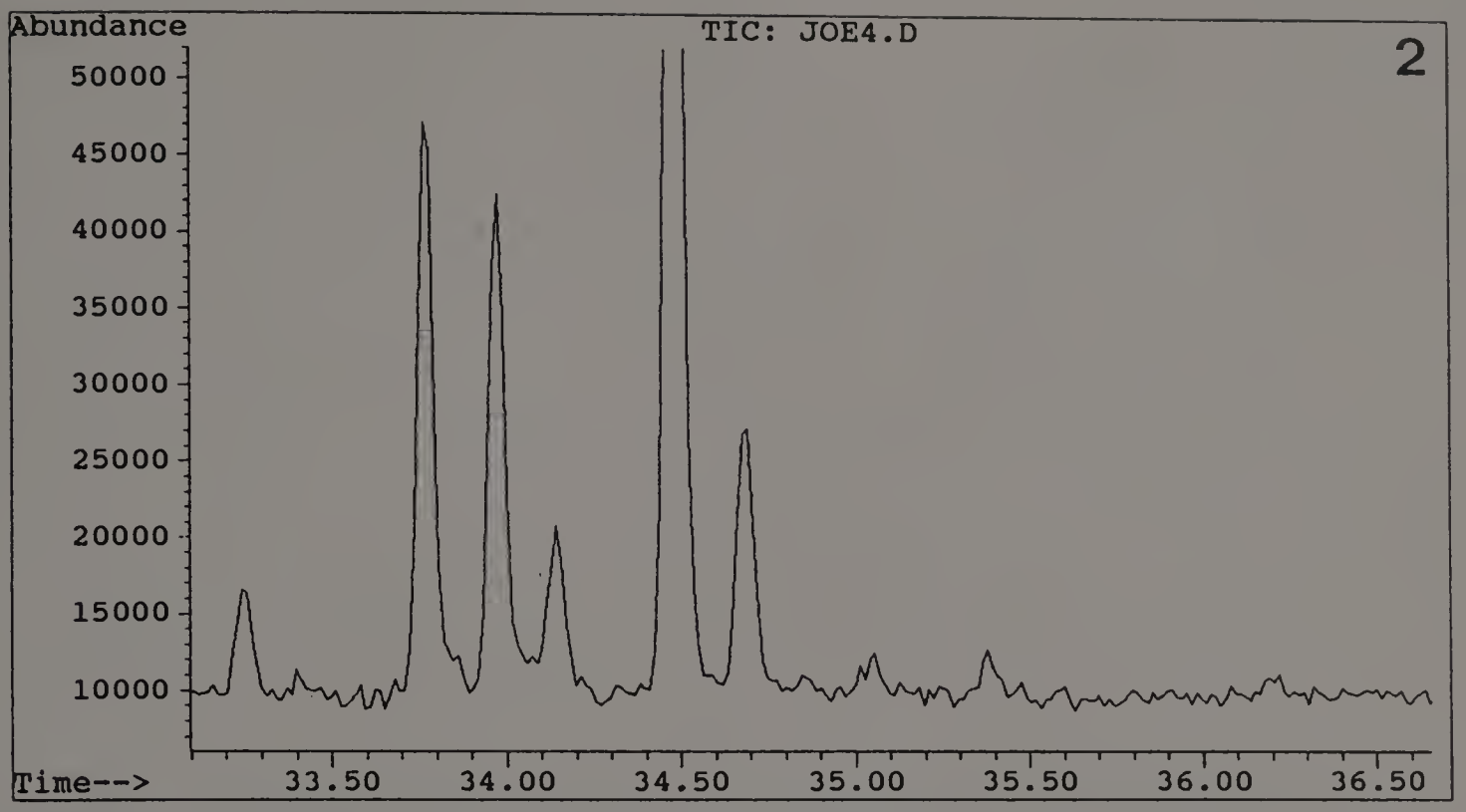
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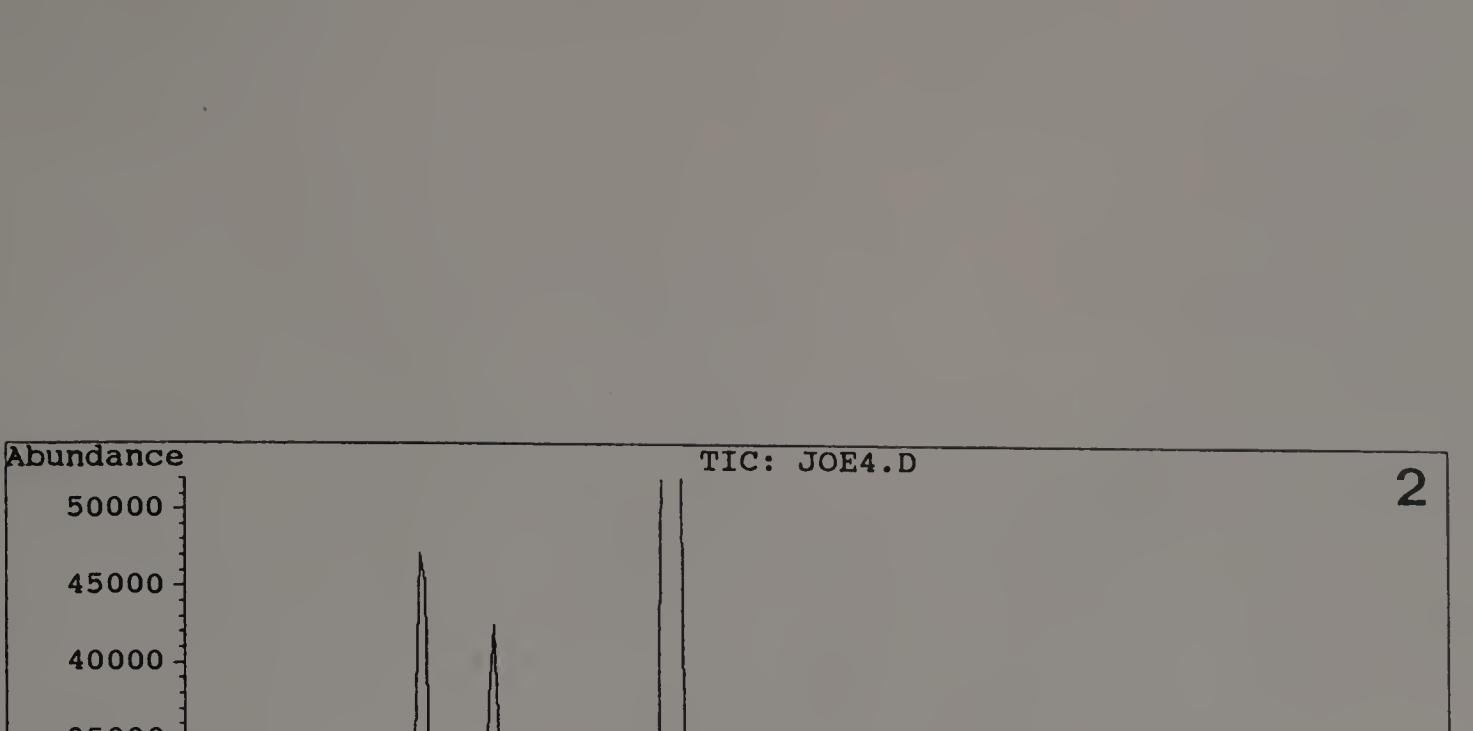
36.50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2000
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43
74
41
69
96
110
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227
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m/z-->
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80
100
120
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180
200
220
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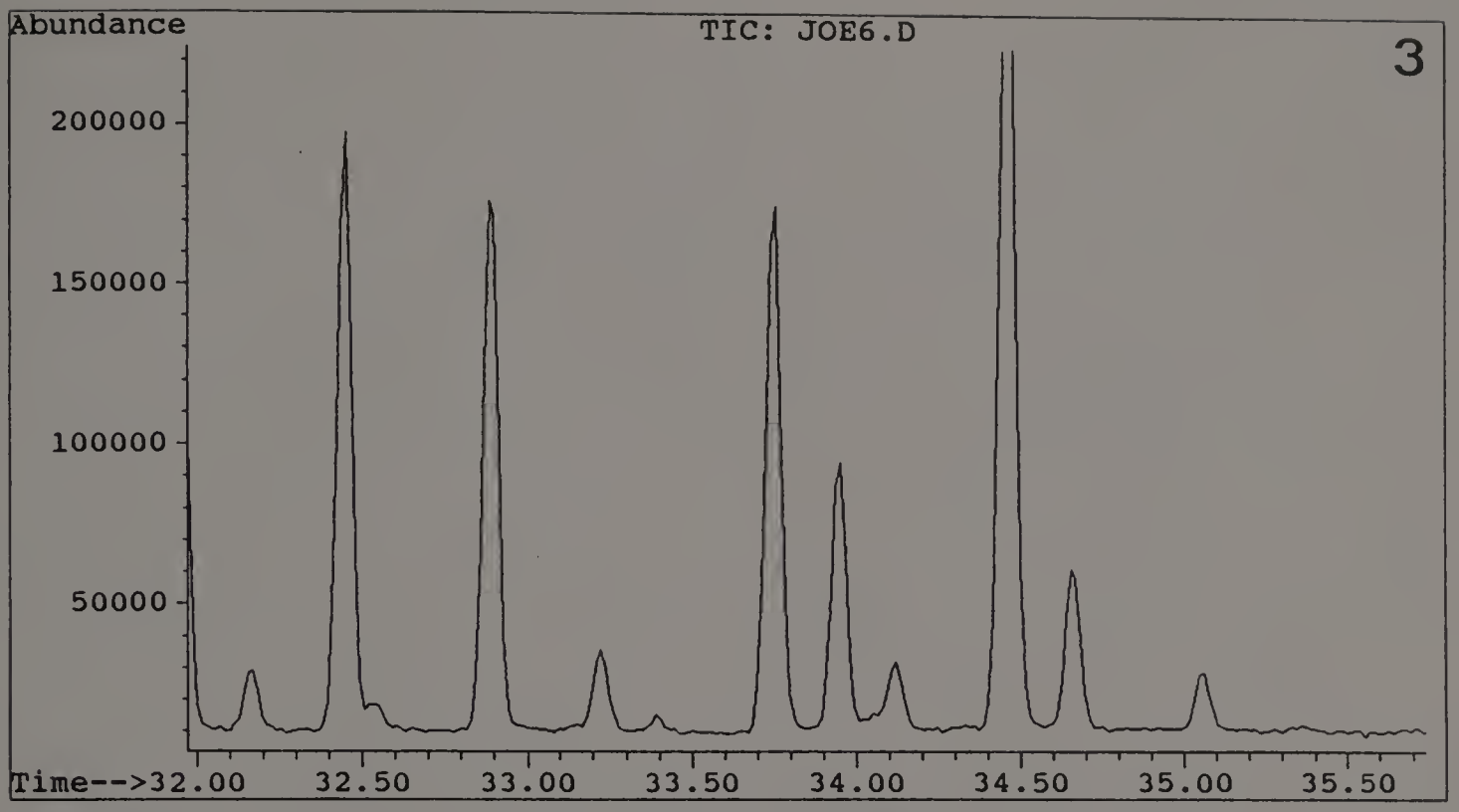
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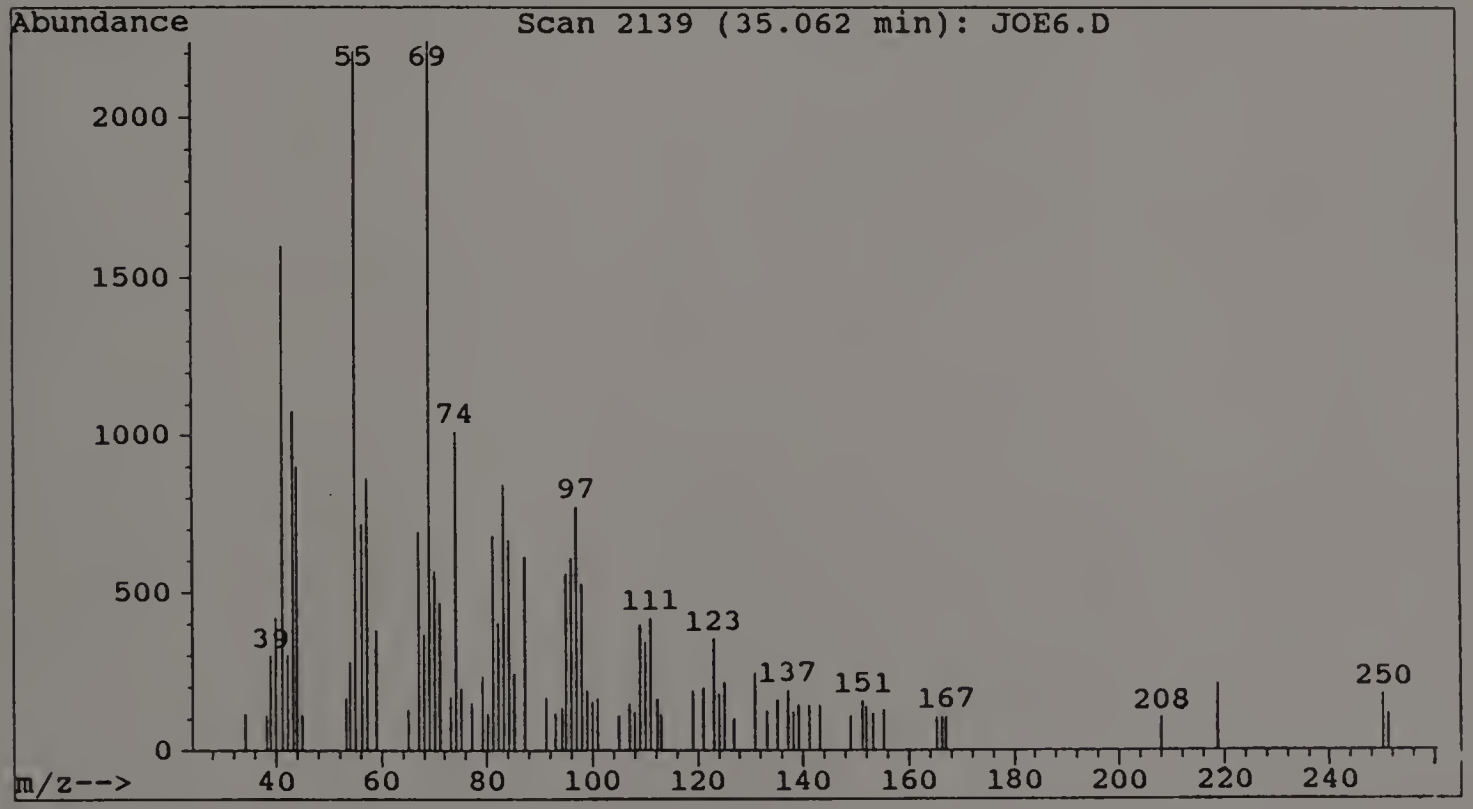
35.50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m/z-->
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100
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200
220
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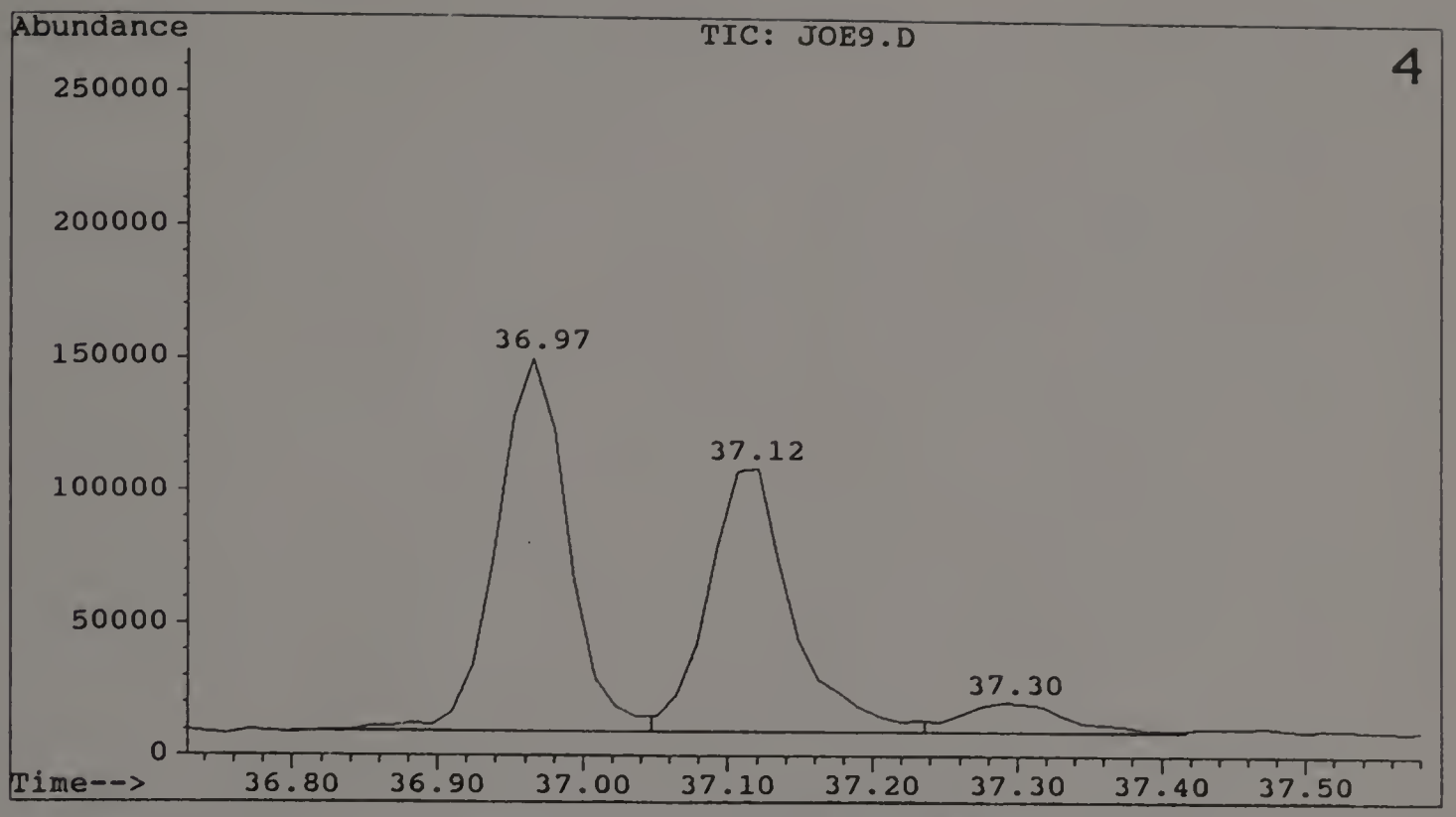
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150000
100000
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37.30
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37.10
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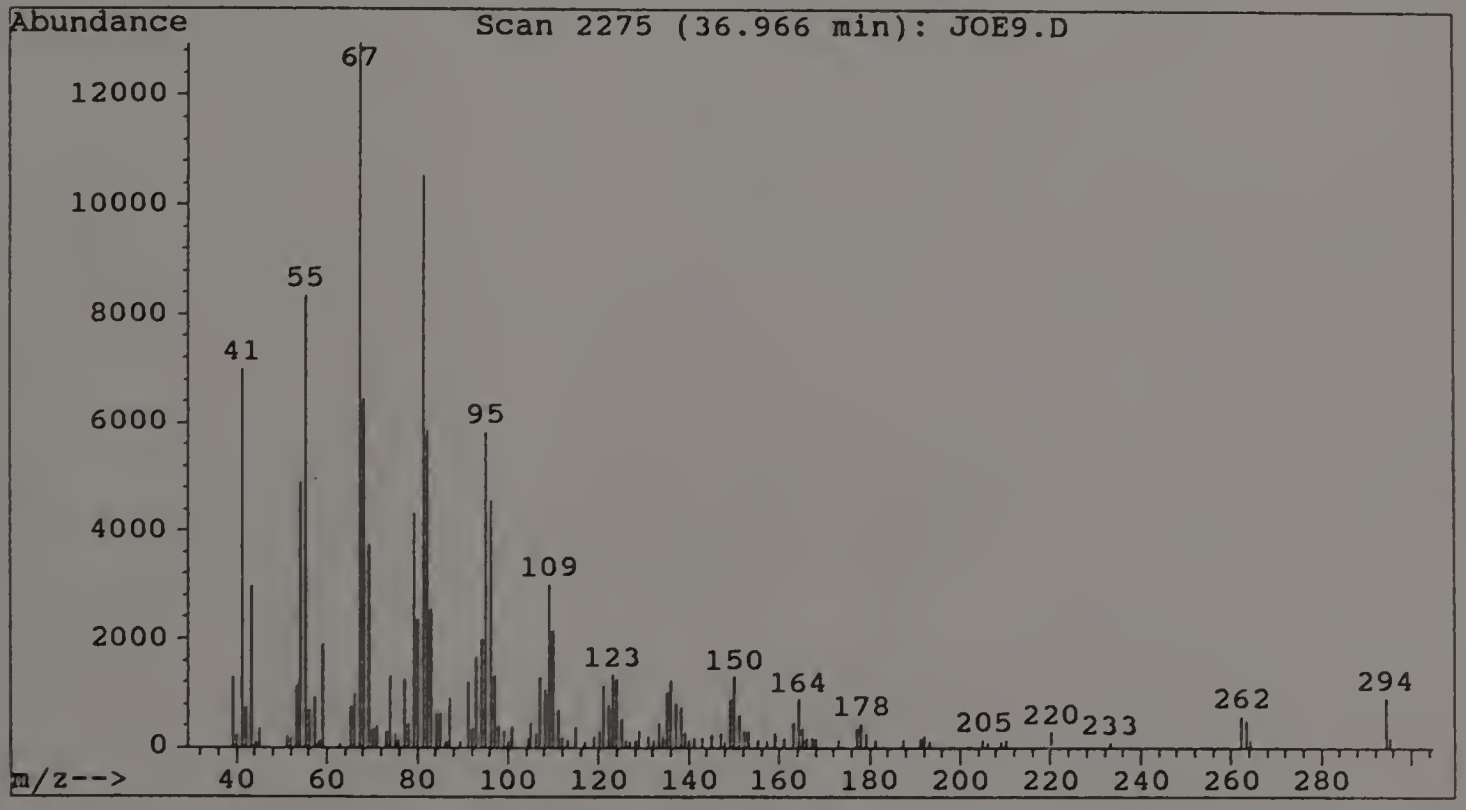
37.30
37.40
37.50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123
150
164
178
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262
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120
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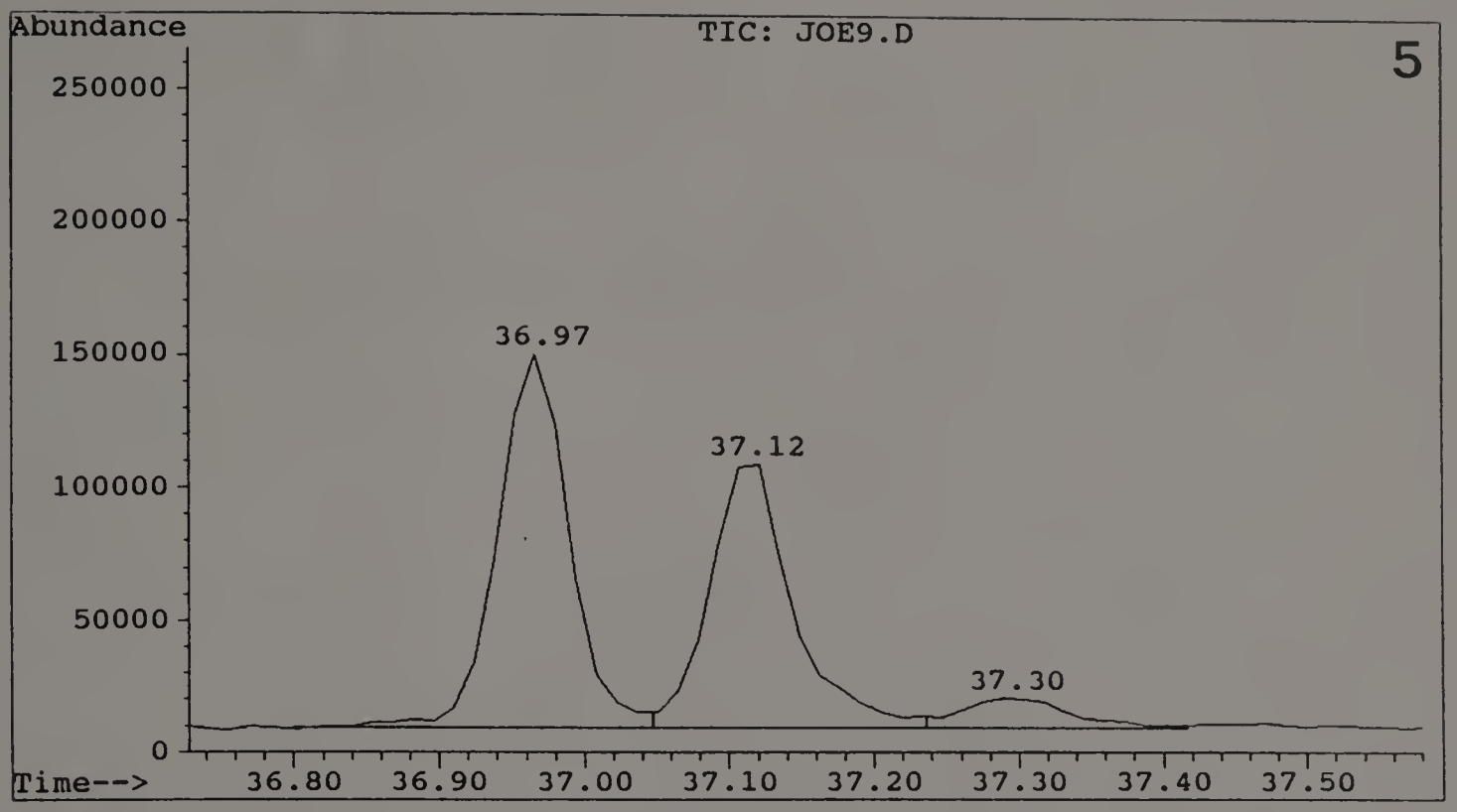
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37.10
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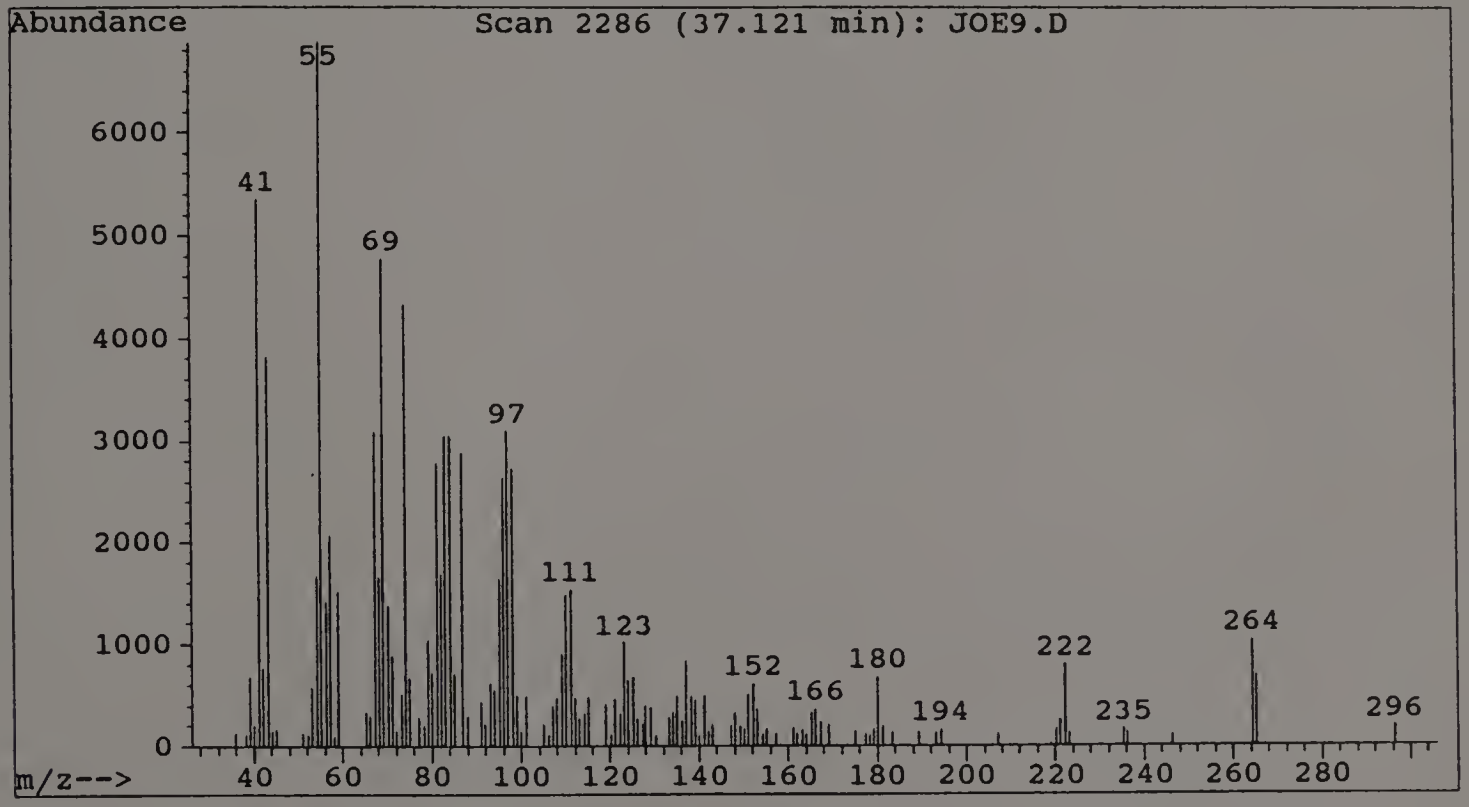
37.30
37.40
37.50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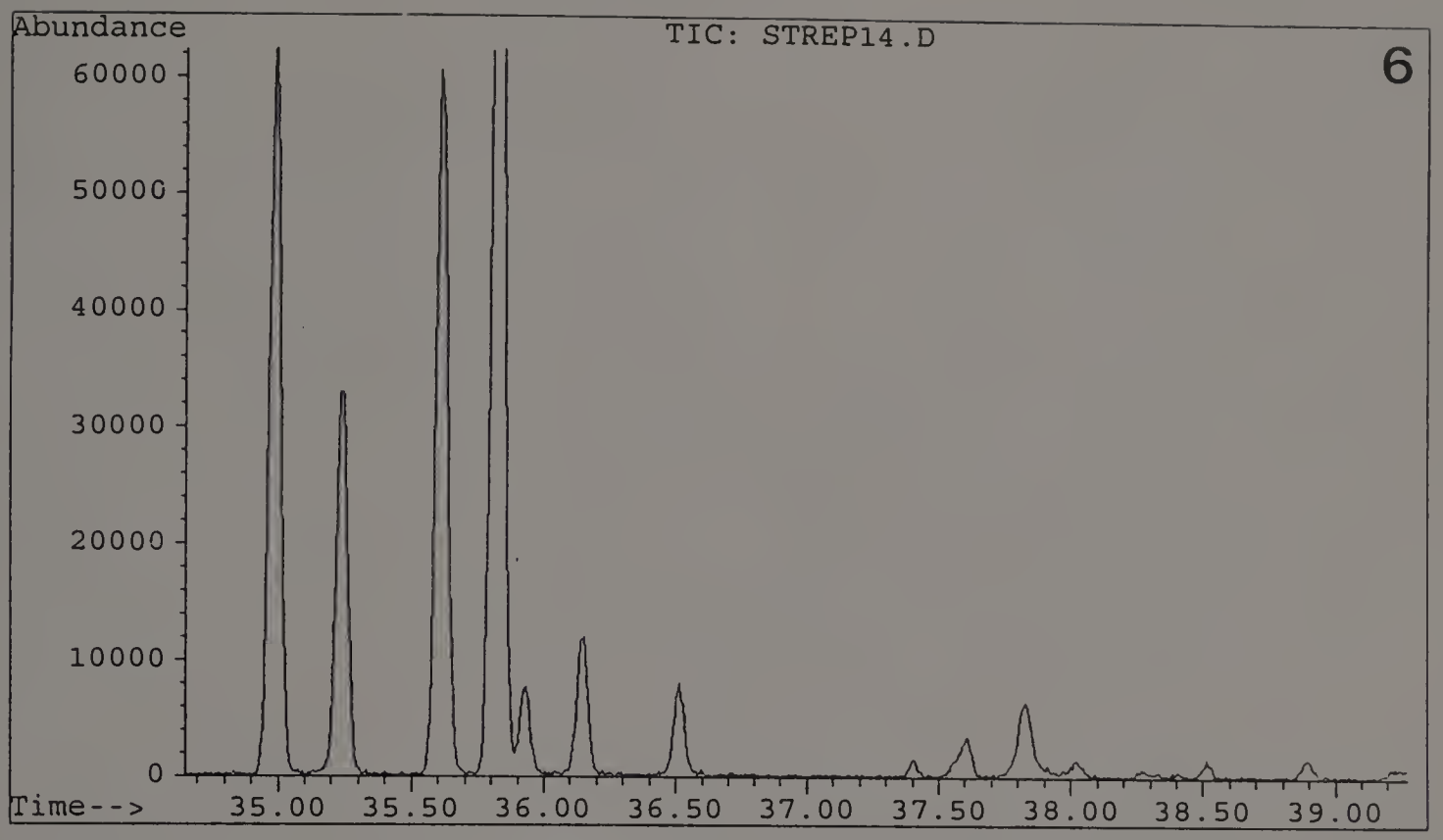
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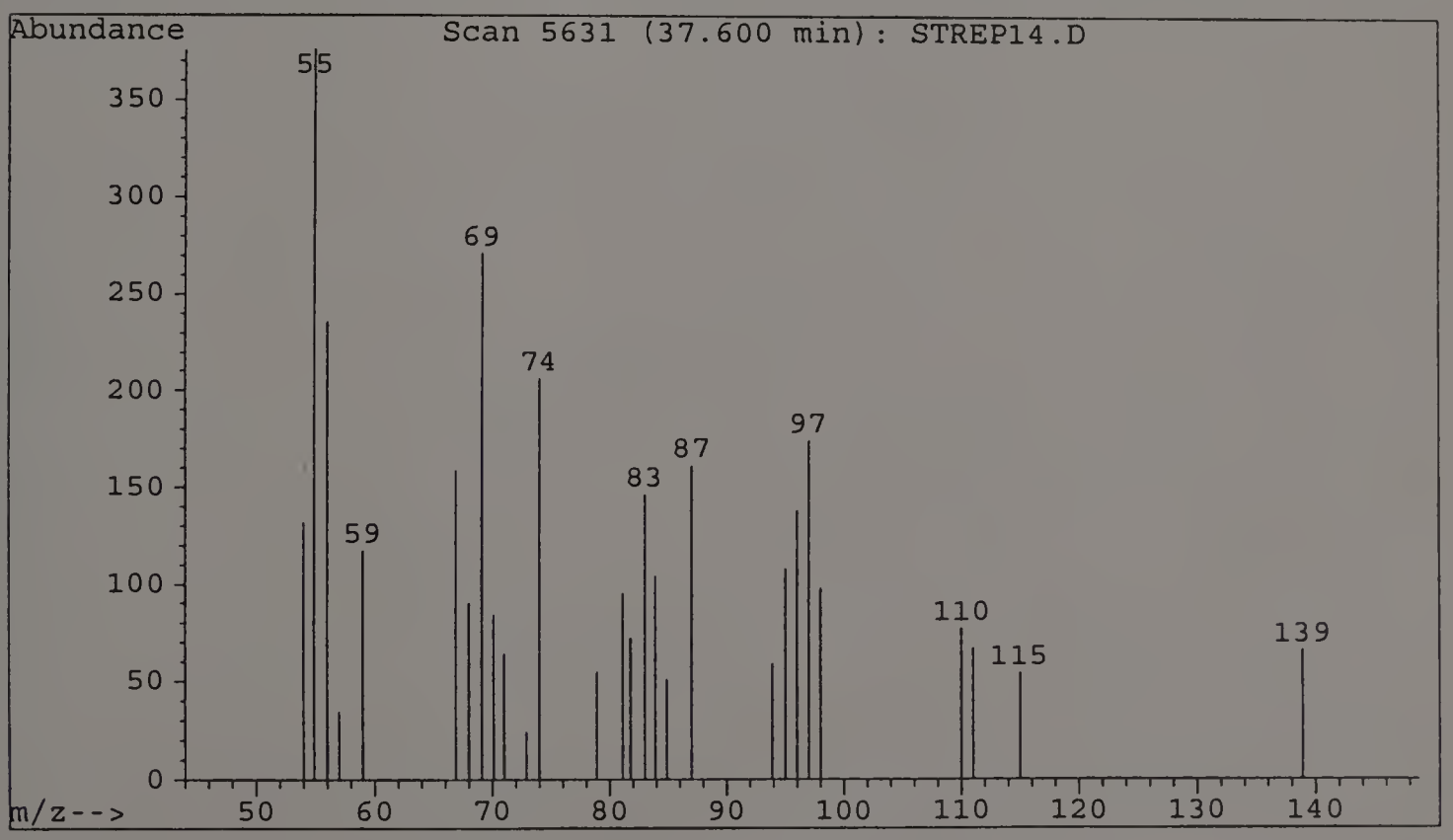
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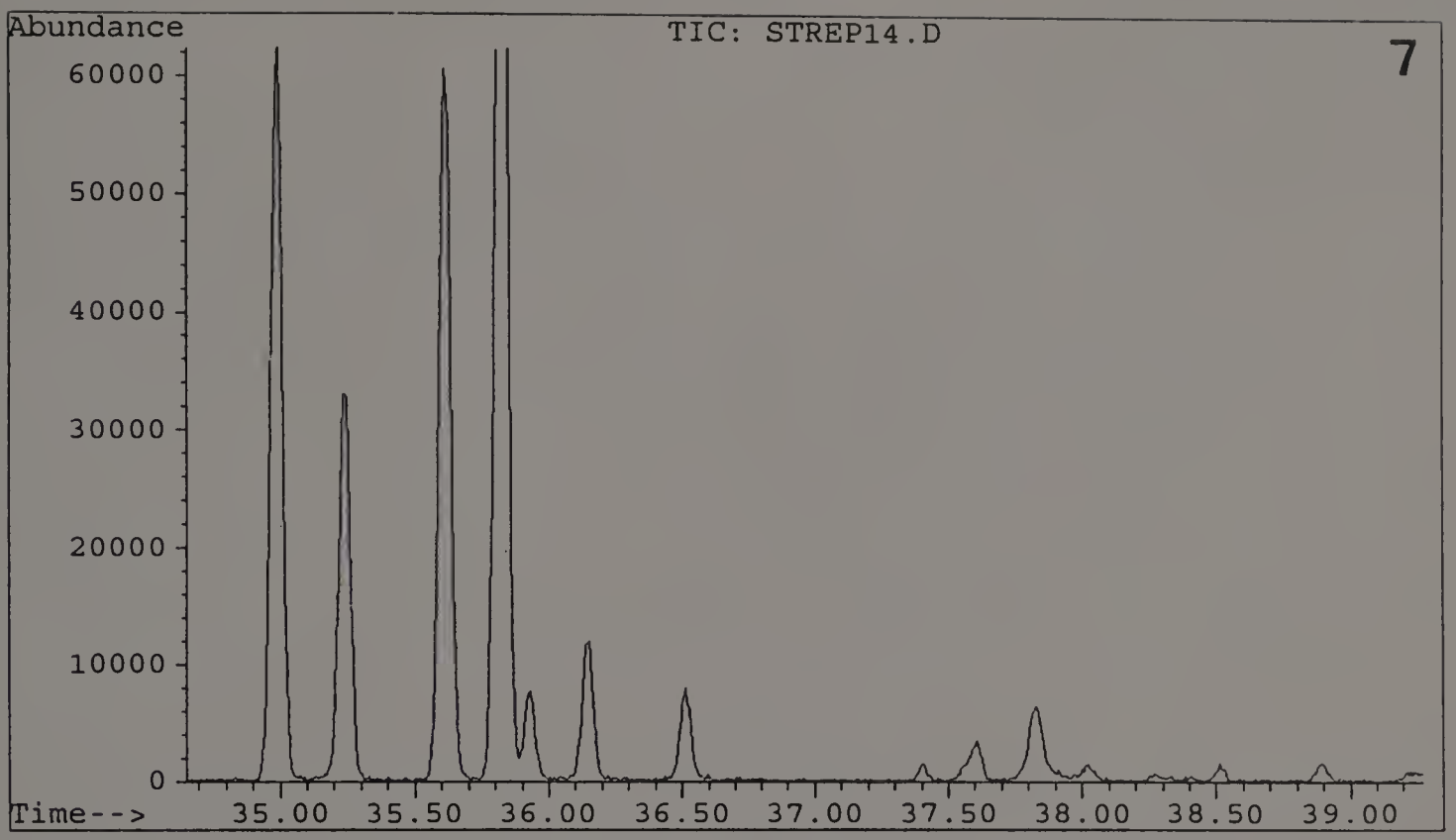
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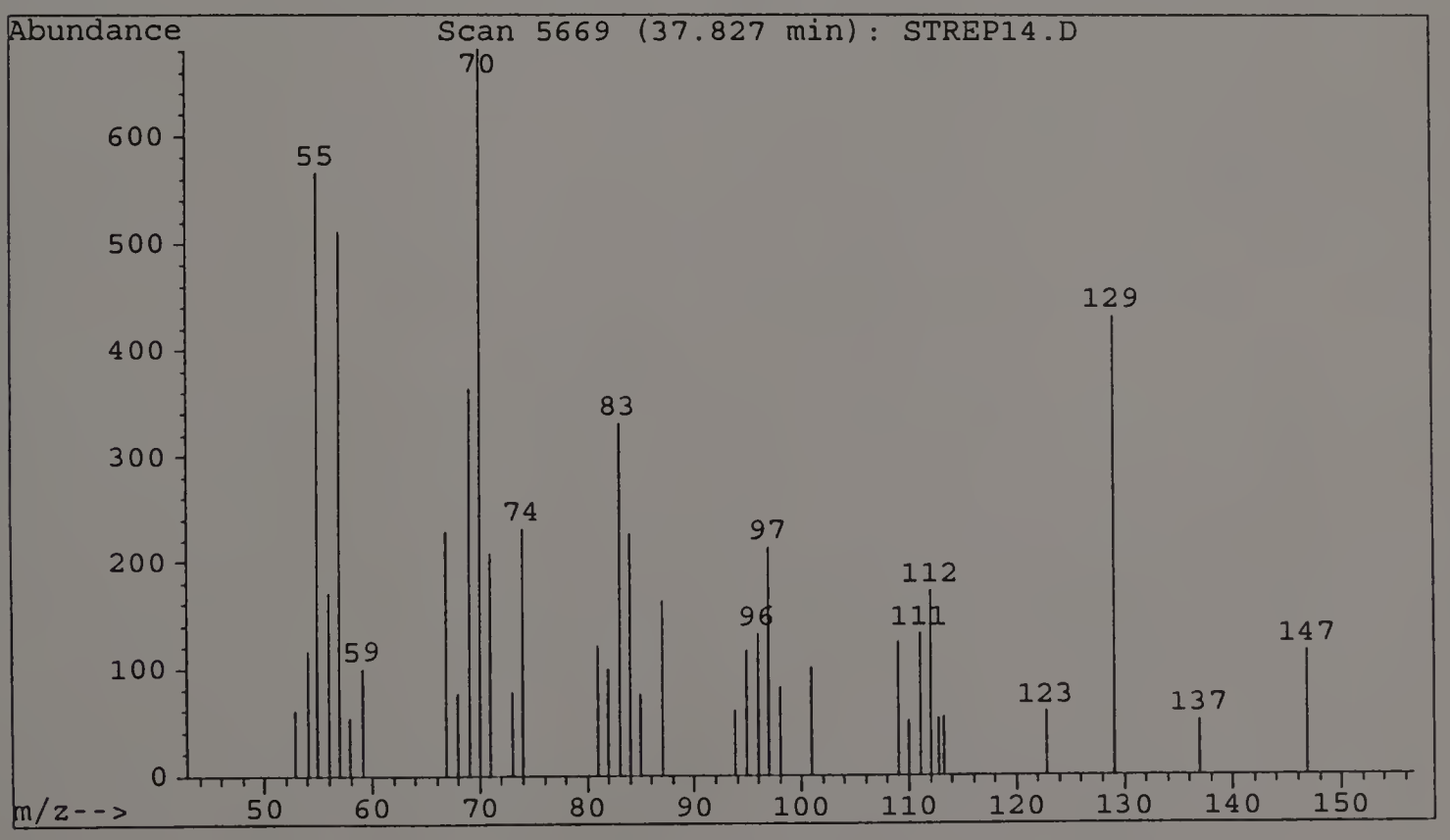
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74
97
112
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123
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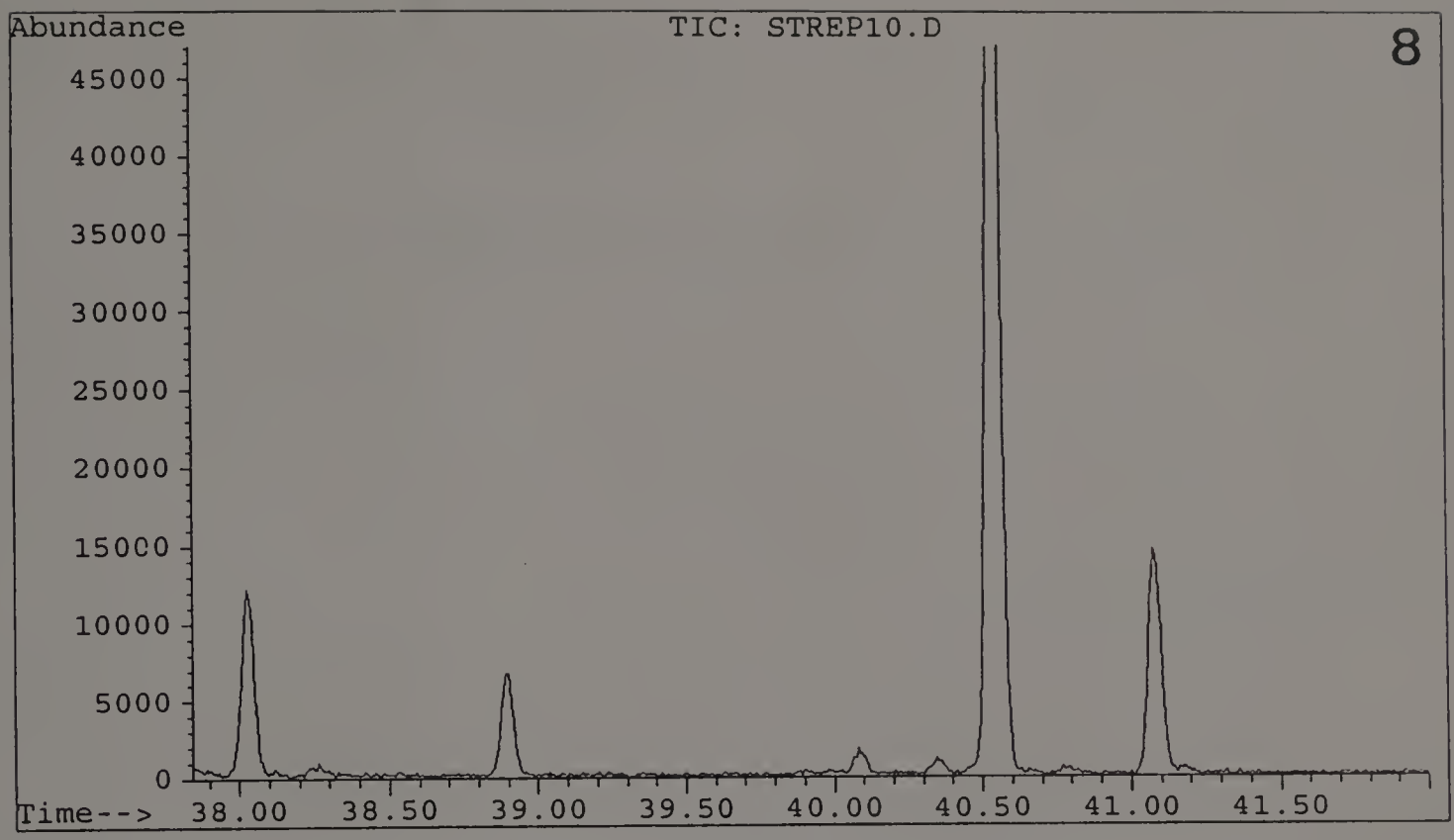
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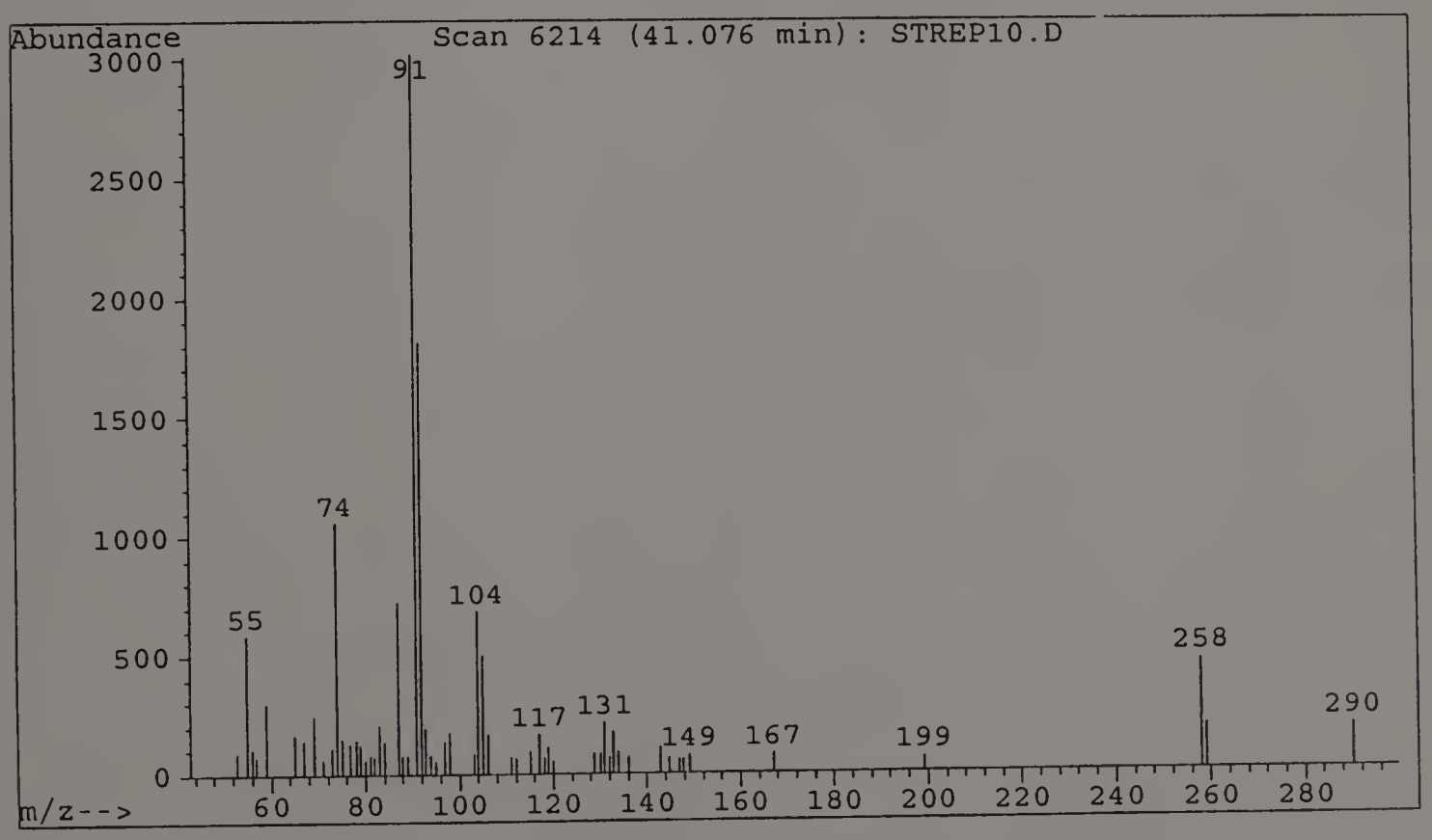
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