PHOTOACTIVATED DNA CLEAVAGE, ENZYME INACTIVATION, BACTERIAL INHIBITION, AND VIRAL INACTIVATION BY THE COTTON PHYTOALEXIN 2,7-DIHYDROXYCADALENE, ISOLATION OF PHYTOALEXIN-RESISTANT MUTANTS OF THE COTTON PATHOGEN *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM*, AND CHARACTERIZATION OF THE PATHOGEN'S MUTABILITY

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PREFACE

This thesis is divided into three sections: I. "PHOTOACTIVATED DNA CLEAVAGE, ENZYME INACTIVATION, AND BACTERIAL INHIBITION BY SESQUITERPENOID PHYTOALEXINS FROM COTTON" (Pgs 1-24); II. "INACTIVATION OF CAULIFLOWER MOSAIC VIRUS BY A PHOTOACTIVATABLE COTTON PHYTOALEXIN" (Pgs 25-43); and III. "ISOLATION OF DHC^R MUTANTS AND CHARACTERIZATION OF THE MUTABILITY OF *XCM* PHYSIOLOGICALLY AND MOLECULAR GENETICALLY" (Pgs 44-145). The work presented in this thesis provides understanding of a phytopathogen and the defence action of the cotton via phytoalexin.

I wish to thank my HEAVENLY FATHER for His UNCONDITIONAL LOVE, PATIENT and GUIDANCE. The only regret during this period was that I failed to keep my promise to Him. I also want to thanks my parents without them I won't be here and I won't be me. Thanks also to my husband, Chein-Tai Chen, for his generous and open attitude toward my career.

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Special thanks also to Dr. Ulrich K. Melcher for performing the DNA sequencing experiments in Section I, preparing the manuscript of Section II, and allowing me to include some of the unpublished data.

"I DON'T KNOW WHAT THE FUTURE WILL BE, BUT I DO KNOW WHO HOLD THE TOMORROW."

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ALPHABETICAL LISTING OF ABBREVIATIONS USED IN SECTION III

ANOVA	Analysis of variance
APS	Ammonium persulfate
CC	Closed circular form of plasmid
cfu	Colony forming unit
DHC	2,7-Dihydroxycadalene
DTT	Dithiothreitol
E	Eosin
EDDHA	Ethylenediamine di-(O-hydroxyphenyl) acetate
E_m	Electrical potential
EMB	Eosin plus methylene blue
EPS	Extracellular polysaccharide
IS	Insertion sequence
LB	Luria broth
LC	Lacinilene C
LCME	Lacinilene C 7-methyl ether
LPS	Lipopolysaccharide
MB	Methylene blue
NA	Nutrient agar
NB	Nutrient broth
OC	Open circular form of plasmid
O-type	Opaque colony
PA	Phytoalexin

RS	Repetitive sequence
SAS	Statistical Analysis System
SOD	Superoxide dismutase
TAE	Tris Acetate (0.04 M), EDTA (2 mM)
TLC	Thin Layer Chromatography
Tn	Transposon
T-type	Transparent colony
Xcm	*Xanthomonas campestris* pv. *malvacearum*
XS	Xanthan gum plus sucrose

SECTION I

PHOTOACTIVATED DNA CLEAVAGE, ENZYME INACTIVATION, AND BACTERIAL INHIBITION BY SESQUITERPENOID PHYTOALEXINS FROM COTTON

Running title: Photoactivation of phytoalexins

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Key words: *Gossypium hirsutum* / *Xanthomonas campestris* pv. *malvacearum* / 2,7-Dihydroxycadalene / lacinilene C / sesquiterpenoid phytoalexins / photosensitizer / DNA cleavage / active oxygen / deoxyribonuclease I / malate dehydrogenase

Footnote
Abbreviations used: 2,7-Dihydroxycadalene(DHC); lacinilene C (LC); lacinilene C 7-methyl ether (LCME); ethidium bromide (EtBr); malate dehydrogenase (MDH); cauliflower mosaic virus (CaMV); closed circular form (CC), open circular form(OC) of plasmid;10mM Tris·HCl, 1mM EDTA, pH7.4 (TE buffer); superoxide dismutase (SOD); s-(2-amino-ethyl)isothiouronium bromide hydrobromide (AET);

ABSTRACT

In the presence of light, the phytoalexin 2,7-dihydroxycadalene (DHC) and the structurally related phytoalexin lacinilene C (LC) induced nicks in plasmid DNA. This was concluded from observing a time-dependent decrease in closed circular and increase in open circular forms of the plasmids by agarose gel electrophoresis after exposure to DHC plus light. Light of wavelengths 239nm and 300nm (the absorbance maxima of DHC) was active, whereas 400nm, 500nm, and 600nm light was inactive in nicking DNA. When the DNA was denatured by either boiling or alkali treatment following the DHC plus light treatment, fragments smaller than intact single strands were observed. Cleavage of double-stranded DNA by light in the presence of DHC was neither nucleotide sequence-specific nor base-specific as revealed in a DNA sequencing gel. Some preferential cleavage was observed when photoactivated DHC acted on single-stranded DNA. Both chemically synthesized DHC and DHC isolated from cotton leaves required light and oxygen for full DNA-nicking ability. Scavengers of reactive oxygen species and of free radicals inhibited the nicking of plasmid by photoactivated DHC. These results suggest that light-activated DHC acts via reactions involving reactive oxygen species. The catalytic activities of DNase I and malate dehydrogenase were greatly reduced after incubation with DHC plus light. In the dark 0.1 mM DHC only partially inhibited multiplication of *Xanthomonas campestris* pv. *malvacearum*, but in the light this concentration was bactericidal. Light alone was only bacteriostatic.

INTRODUCTION

2,7-Dihydroxycadalene (DHC), lacinilene C (LC) and lacinilene C 7-methyl ether (LCME) are phytoalexins that accumulate in cotton leaves after inoculation with incompatible races of *Xanthomonas campestris* pv. *malvacearum* (Essenberg, Doherty, Hamilton, Henning, Cover, McFaul & Johnson 1982, Essenberg, 1985). DHC is the most inhibitory toward bacterial growth. LC is formed from DHC spontaneously by air oxidation. After inoculation of cotton leaves yellow-green fluorescence that is spectrally similar to the fluorescence of LC develops in the mesophyll cells closest to colonies of *X. campestris* pv. *malvacearum* (Essenberg, Pierce, Shevell, Sun & Richardson 1985). Fluorescence-activated sorting of cells isolated from inoculated leaves showed that DHC and LC are localized in the fluorescent cells (Pierce & Essenberg 1987). Local concentrations of the phytoalexins in these cells have been estimated from quantitative extraction and fluorescence microscopy. At the time that bacterial multiplication stops in leaves of highly resistant cotton lines, DHC and LC reach levels that completely inhibit growth of *X. campestris* pv. *malvacearum* when tested *in vitro* (Essenberg, Pierce, Shevell, Sun & Richardson 1985). Because DHC, LC and LCME are susceptible to photooxidation, previous bioassays of their antibacterial activities were performed in the dark (Essenberg, Doherty, Hamilton, Henning, Cover, McFaul & Johnson 1982, Essenberg 1985). We report here that in light DHC induces nicking in DNA, inactivates enzymes and has enhanced antibacterial activity. LC is also photoactivated to nick DNA.

MATERIALS AND METHODS

DHC and LC

DHC and LC were isolated from inoculated cotton plants as previously described (Essenberg, Doherty, Hamilton, Henning, Cover, McFaul & Johnson 1982). Chemically synthesized DHC was a gift of Dr. R. D. Stipanovic. Except where otherwise specified,

all work with DHC and LC was carried out under ultraviolet-deficient illumination from General Electric F06T12/GO gold fluorescent lamps to avoid photodecomposition. For assays of their effects, aliquots of DHC or LC in HPLC-grade methanol were transferred to 0.5-ml conical polypropylene centrifuge tubes and evaporated to dryness with a nitrogen gas stream.

Plasmid DNA Preparation

Escherichia coli HB101 (pBR322) was cultured overnight in 1L of Luria broth (Maniatis, Fritsch & Sambrook 1982) at 200 rpm and 37°C. Plasmid DNA was isolated by the alkaline lysis method (Maniatis, Fritsch & Sambrook 1982), and purified on NACS-37 (Bethesda Research Laboratories) in order to avoid exposure to ethidium bromide (EtBr) which can lead to single stranded nicks due to the interaction of the dye and light (Martens & Clayton 1977). Efforts to remove EtBr from plasmid DNA purified by CsCl-EtBr density gradient centrifugation by phenol extraction were not complete. Plasmid DNA was stored and used in TE buffer.

End-labeled DNA

A singly 3' end-labeled double-stranded DNA fragment was obtained from the cauliflower mosaic virus DNA-containing plasmid pCa6 (Lebeurier et al 1982) by Xho I (New England BioLabs) digestion, end labeling with α^{32}P-dCTP (New England Nuclear) and reverse transcriptase (Biorad), followed by Pvu II (IBI) digestion. The resulting 261 bp fragment encompassed nucleotides 1643 to 3990 in the sequence of CaMV (Franck et al, 1980) with nucleotides between nonanucleotide direct repeats at 1676 to 1684 and 3862 to 3870 deleted. A 3' end-labeled single-stranded DNA fragment (CaMV nucleotides 2152 to 1927) was obtained from pUM130 (Melcher et al. 1986) by digestion with BamHI (Bethesda Research Laboratories) followed by reverse transcriptase as above. The strands were separated by gel electrophoresis (Maxam & Gilbert, 1980). Aliquots of each labeled fragment were subjected to sequencing reactions (Maxam and Gilbert, 1980) or to DHC plus light treatment (see below) and analysed on 8% polyacrylamide sequencing gels.

Bacteria Used in Bioassay.

X. campestris pv. *malvacearum* race 1 and strain EMB7 stock cultures were stored in nutrient broth (Difco) containing 16% glycerol at -70°C. Strain EMB7 was a spontaneous mutant resistant to light on EMB (Difco) agar from strain RS4 (Sun & Essenberg 1986), a very DHC-sensitive mutant of race 3.

General Treatment

Plasmid DNA(0.1 to 0.2µg) in 1-2µl of TE buffer was transferred into 0.5-ml conical tubes to dissolve the dried phytoalexin aliquots to 0.7mM DHC or 1.7mM LC when applicable. The tubes were sealed, laid on their sides, and exposed to a mixture of fluorescent and incandescent lights (2 x 10^4 lux) in a Conviron E15 plant growth chamber or, where specified, to sunlight for 2 to 3 h.

End-labeled CaMV DNA fragments, 1µl of 0.11µg ml^{-1} DNase I (Sigma) or 1µl of 62.5µg ml^{-1} malate dehydrogenase (MDH) (Sigma) was treated with DHC as described above, except that with MDH 0.025mM DHC and 1.5 h illumination were used. At the end of this period, MDH was diluted 1000-fold for assay of its enzymatic activity as described below.

Irradiation with Specific Wavelengths

A piece of styrofoam was cut to fit the cuvette holder of an Aminco-Bowman spectrofluorometer. An indentation was made that would secure the lower portion of a cut-off 0.5-ml conical tube. A cut-off tube containing 2µl of test sample was placed in the holder facing the light source. The chamber containing the cuvette holder was maintained at 15°C with a circulating water bath. The sample was exposed to monochromatic light from the spectrofluorometer's xenon lamp for 1 h.

Scavengers and Oxygen-deficient Treatment

One µl of scavenger solution was introduced into the 0.5-ml conical tube containing or not containing DHC along with 1µl of plasmid DNA solution. The final concentrations of the scavengers were: 12.3 mM NaN_3, 67 mM L-histidine, 24.4 mM L-methionine, 0.22 M

D-mannitol, 6 mM ethanol, 12.1 mM AET (Sigma), 69.4 mM sodium benzoate, 31 units ml^{-1} SOD (Sigma) and 185 units ml^{-1} catalase (Sigma). Where specified, the sample-containing conical tubes were flushed with argon for 2 min and were closed while flushing with argon prior to exposure to light.

Plasmid DNA Nicking Assays

TE buffer (15µl) and 5µl of loading buffer (Maniatis, Fritsch & Sambrook 1982) were added to each tube of treated plasmid DNA prior to electrophoresis in 0.8% agarose gels in E buffer (Loening, 1969) at 70mA for 90 min. DNA bands were visualized by soaking the gel in running buffer containing EtBr (0.5 µg ml^{-1}) for 30 min, and illuminating with 365nm UV light. Photographs were taken through a No. 23 Wratten filter (Kodak). Double-stranded DNA was denatured either by immersion in boiling water for 1 min or by addition of 1µl of 1N KOH before the addition of loading buffer.

Enzyme Assays

Plasmid DNA (0.15 µg), was added as a substrate to the DNase I-containing vials after the light treatment of the enzyme. After incubation for 4 min at room temperature, the reaction was stopped by immersion in boiling water for 1 min. The samples were then electrophoresed in 1.1% agarose gels as above.

MDH was assayed using 0.2 mM NADH, 0.05 mM oxaloacetic acid and 0.1 M potassium phosphate buffer, pH 7.5, monitoring the reaction by observing absorbance at 340 nm at room temperature. One unit of enzyme activity caused the oxidation of 1 µmole NADH per minute at 25°C, assuming a molar absorptivity of 6.22 $M^{-1}cm^{-1}$ for NADH.

Bacterial Bioassay

Twenty microliters of a logarithmically growing bacterial culture (10^6 to 10^7 cfu ml^{-1}) in nutrient broth was introduced aseptically into a 1.5-ml conical tube in a laminar-flow hood. The tubes were incubated in a jacketed water bath at 25-33°C, rotated at 180 rpm and illuminated with white light (fluorescent and incandescent lights, 2 x 10^4 lux). The dark control tubes were wrapped with aluminum foil and incubated in the same bath.

RESULTS

Cleavage of Plasmid DNA by Photoactivated Phytoalexins.

Irradiation of plasmid pBR322 DNA in the presence of DHC led to a time-dependent decrease in the supercoiled, closed circular form (CC) and an increase in the nicked, open circular form (OC) of the plasmid (Fig. 1). Neither irradiaton alone or treatment of the plasmid DNA with DHC or LC in the dark caused noticeable conversion of CC to OC (Fig. 2). Irradiation of plasmid DNA in the presence of LC also resulted in considerable conversion of CC to OC (Fig. 2), although the conversion was less efficient than with DHC. Sometimes DNA products remained at the origin (Figs. 2 & 4), possibly due to crosslinking.

The pattern of migration of heated DNA was not altered by pretreatment with DHC or LC in the dark. Irradiation alone of plasmid DNA resulted in the production of smaller DNA fragments revealed after heat denaturation. Irradiation of plasmid DNA in the presence of DHC or LC caused the complete conversion of the CC to small fragments. The fragments resulting from DHC-light treatment were, on average, smaller than those from LC-light treatment (Fig. 2).

Wavelength Specificity for Activation of DHC

Irradiation of plasmid DNA with monochromatic light at the absorbance maxima (239nm, 300nm) of DHC led to conversion of CC to OC (Fig. 3). Light of nonabsorbed wavelengths (400nm, 500nm and 600nm) of DHC was inactive in the conversion (data not shown). At 239nm, the effect of DHC was not as prominant as at 300nm since that 239nm alone causes more nicking than 300nm alone (Fig. 3).

Photoactivation of DHC in Nicking DNA is Not Due to Heat.

When a thermocouple (Omega Co.) was used to measure the temperature inside the 0.5-ml conical tube under the light treatment condition the temperature never exceeded 42°C during the 2 h of monitoring. Whether the nicking of DNA by DHC could be attributed to a

temperature effect rather than to photoactivation was tested as follows. The plasmid DNA was incubated with or without DHC in the dark in 40°C, 50°C, 60°C and 70°C for 2 h. At 40°C and 50°C the gel electrophoretic patterns were indistinguishable from that of the unincubated DNA. At 60°C and 70°C bands of lower mobility, probably plasmid multimers, appeared. This pattern differed from that of conversion of CC to OC by photoactivated DHC.

Involvement of Oxygen

The cleavage of DNA by photoactivated DHC was less extensive when the oxygen concentration was decreased by flushing the sample-containing tube with argon prior to incubation in light (Fig. 4). The argon-flushed sample retained some of the CC. In contrast, the air-equilibrated sample not only showed a complete disappearance of CC but also a substantial increase in the linear form of the plasmid which is the product of introduce nick to OC at or near to a previously present nick but in the opposite strand. In this experiment, both chemically synthesized DHC and DHC isolated from cotton tissue showed similar dependence upon light and air for full DNA-cleaving activity.

Inhibition of DNA Cleavage by Scavengers of Reactive Oxygen Species and Free Radicals.

Because of the involvement of light and oxygen in phytoalexin-mediated DNA cleavage, the effects on photoactivated DHC-mediated DNA cleavage of scavengers of singlet oxygen (NaN_3 and histidine), of hydroxyl radical or singlet oxygen (methionine, mannitol, Na-benzoate and ethanol), of H_2O_2 (catalase), of superoxide (SOD) and of free radicals (AET) were tested. All of the scavengers tested partially protected the CC from cleavage by photoactivated DHC (Fig. 5). The CC bands in scavenger-treated samples (lanes 2-10) were intermediate in intensity between those of untreated DNA (lane 14) and of DNA treated with photoactivated DHC without scavenger (lane 11) in which no CC DNA remained. Fewer lower molecular weight fragments were generated in photoactivated DHC-treated samples to which scavengers had been added than in that without scavenger. Denaturation of OC was incomplete in some samples. The plasmid DNAs irradiated in the

presence of scavengers alone were indistinquishable from plasmid DNA irradiated in their absence (data not shown).

Lack of Base or Sequence Specificity in DNA Cleavage

The products of light plus DHC-induced cleavage of a singly end-labeled double-stranded DNA fragment are displayed in an autoradiograph of a DNA sequencing gel in Fig. 6a. Bands of approximately equal intensity appeared at each nucleotide position, as determined from the standard Maxam and Gilbert sequencing samples run in neighboring lanes. No bands were detected in the lane containing the untreated fragment. Thus, each nucleotide position of the labeled strand was equally susceptible to cleavage by photoactivated DHC. The pattern of photoactivated DHC-induced cleavage of α ^{32}P-end-labeled single-stranded DNA fragment was also analyzed (Fig. 6b). Some of the regularly spaced bands on the autoradiograph were of significantly higher intensity than other bands. The positions of darker bands did not correlate with areas of possible secondary structure. The darker bands appeared at positions consistent with cleavage at guanylate residues and positions one to three positions 5' of guanylate residues.

Enzyme Inactivation by DHC plus Light.

Active DNase I cleaves intact plasmid DNA (Fig. 7, lanes 2 & 7) to small fragments (Fig. 7, lane 3). Preincubation of DNase I with light alone (Fig. 7, lane 5) had no effect on this activity. When the enzyme was preincubated with DHC plus light for 3 hours before assay the activity toward plasmid DNA was greatly reduced (Fig. 7, lane 6). The presence of DHC only during the 4 minute incubation of the plasmid with DNase I (Fig. 7, lane 1) gave the same degree of interference as 3 h preincubation of the enzyme with DHC in the dark (Fig. 7, lane 4).

Inactivation of MDH by DHC plus light was also observed (Table 1). Preincubation of MDH with either DHC or light alone had only a slight effect on the enzyme activity (Table 1) that was not significant at the 1% level. MDH assayed in the presence of 1.0 μm DHC had 97 $\pm$ 5 % the activity of MDH alone. Thus the effect of preincubation with DHC

plus light was not due to inhibition by the 0.025 μM DHC carried through the 1000-fold dilution that was made for the MDH assay.

Effect of Light Upon the Antibacterial Activity of DHC.

At 0.05 mM and 0.1 mM, DHC partially inhibited growth of two strains of *X. campestris* pv. *malvacearum* (Fig . 8). Exposure of strain EMB7 to light alone was bacteriostatic while exposure of race 1 to light partially inhibited growth. Light plus 0.05mM DHC was slightly more inhibitory to EMB7 than light alone. However, light plus 0.1 mM DHC was bactericidal to both strains.

DISCUSSION

A major finding of this and the accompanying study (Sun et al 1987) is that the phytoalexin DHC (and probably LC also) *in vitro* only becomes a potent agent in the destruction of DNA and the inactivation of CaMV and enzymes in the presence of light. The slight inhibition of DNase I by non-irradiated DHC may reflect an effect of DHC on the DNA substrate rather than on the enzyme. The wavelengths at which DHC absorbs maximally, 239 and 300 nm, were also the wavelengths most effective in inducing DNA cleavage. DNA absorbs at 239 nm, but very little at 300 nm. However, the finding that DHC-induced inactivation of two enzymes was also light-dependent strongly suggests that in all of these reactions the active agent was a product of the absorption of photons by DHC.

The decreased DNA cleavage following argon flushing suggests the involvement of oxygen in the action of light-activated DHC. Many compounds of biological interest damage biological systems by photodynamic actions, requring light and oxygen(Spikes 1977, Towers 1980). Two mechanisms of photodynamic action are thought to occur. Type I reactions involve the direct reaction of the excited light receptor molecule (X) with a biological substrate. The resulting radicals react with oxygen to produce superoxide or with other molecules. Type II reactions involve physical activation of ground state dioxygen by the excited light receptor molecule, yielding either highly reactive singlet oxygen ($^1O_2$)

with high efficiency by direct energy transfer or an oxidant ($X^{+}\cdot$) and superoxide ($O_2^{-}\cdot$) with low efficiency after charge separation by electron transfer reactions (Foote 1976, Foote 1979). Gossypol, a terpenoid richly present in cotton plants and structurally related to DHC, is known to generate oxygen radicals at alkaline pH during autooxidation (dePeyster, 1984).

Flushing with argon and all the scavengers tested, which included scavengers of all known reactive oxygen species as well as a scavenger of free radicals, partially protected the supercoiled plasmid from cleavage by light plus DHC (Figs. 4 and 5). That the protection was only partial could have been due to incomplete effectiveness of the argon and scavenger treatments. Alternatively, more than one active species may be generated and be responsible for DNA nicking. More than one active oxygen species are known to be involved in the photodynamic action of other compounds such as the fungal toxin cercosporin (Daub & Hangarter 1983). Spontaneous dismutation of superoxide can generate hydrogen peroxide, and there is considerable evidence that traces of iron or copper ions can then catalyze formation of the highly reactive hydroxyl radical (Cohen 1985, Que et al 1980).

Certain compounds when irradiated with ultraviolet light can initiate oxygen-independent free radical reactions (Pryor 1976). The photoactivated DHC-mediated nicking of DNA that occurred after flushing with argon may either have been due to oxygen-independent free radical reactions or to reaction with remaining traces of oxygen.

Singlet oxygen seems to play a role in DNA strand breakage by the photodynamic dye rose bengal, although in the same study chemically generated singlet oxygen failed to break the DNA backbone (Nieuwint, 1985). In an enzymatic superoxide-producing system, single-strand breakage of plasmid DNA was inhibitable by SOD, catalase and a Fe^{+2}-chelating agent, implying that hydroxyl radical is the ultimate damaging species (Brawn 1981). A 1,10-phenanthroline-copper complex cleaves DNA in a reaction that is inhibited by catalase and hydroxyl radical scavenger (Que, Downey & So 1981). The low

specificity observed for the cleavage of DNA by photoactivated DHC (Fig 6a) is consistent with a role for the highly-reactive and nonselective hydroxyl radical in the process. Supercoiled circular, relaxed circular, double-stranded linear and single-stranded linear DNAs were all substrates for nicking by photoactivated DHC. The heterogeneous distribution of lower molecular weight single strands in samples of plasmid DNA nicked by photoactivated DHC and denatured prior to electrophoresis (Figs. 2 and 3) demonstrates that nicks occurred at random distances from one another. At the nucleotide level of resolution, the observed preference for cleavage of single-stranded DNA in the vicinity of guanylate residues is consistent with the involvement of short-lived species, such as hydroxyl radical in the cleavage. If DHC was preferentially bound to guanine residues, the irradiation of the DNA-DHC complex would have generated hydroxyl radicals which then cleaved the DNA backbone in the immediate vicinity of the guanines. This hypothesis predicts that cleavage of the backbone at more distant positions is limited because the half-life of the active species is shorter than the time needed to diffuse to those positions. Fe(II) chelators that are covalently bound to DNA cause strand breaks within 3-4 nucleotides of the probable site of hydroxyl radical generation (Dreyer & Dervan, 1985, Chu & Orgel, 1985) The failure to observe any nucleotide specificity in photoactivated DHC cleavage of double-stranded DNA may be due to intercalation of the planar DHC molecule into the double helix. Alternatively, the presence of guanines on both strands may obscure a preference for guanines.

Hydroxyl radical-generating systems have been observed to inactivate enzymes(Adams et al 1977, Roberts et al, 1974). Photoactivation of isoflavonoid phytoalexins to inactivate glucose-6-phosphate dehydrogenase in an *in vitro* assay system has been reported (Bakker, Gommers, Smits, Fuchs & deVries 1983). The characteristics of the DHC-mediated inhibition of MDH and DNase I are consistent with enzyme inactivation by photodynamic action. It is clear that during the incubation of DNase I with DHC in the presence of light the catalytic activity of the enzyme was greatly reduced (Fig .

7). MDH under similar conditions was also partially inactivated (Table 1). This loss of MDH activity was not reversed by the 1000-fold dilution made prior to assay of the treated enzyme.

In this report we showed that the antimicrobial activity of DHC observed in the dark (Essenberg, Doherty, Hamilton, Henning, Cover, McFaul & Johnson 1982) was enhanced by light (Fig . 8). The more toxic effect of DHC toward *X. campestris* pv. *malvacearum* under light is not surprising in view of the light-dependent *in vitro* activities of DHC. The nicking of DNA, the inactivation of enzymes and the cross-linking of protein with DNA (Sun, Melcher & Essenberg 1987) could all contribute to the enhanced toxicity of DHC in light. The enhancement of antimicrobial activity of DHC by light may also be due, at least in part, to as yet undescribed activities of photoactivated DHC. The inhibition of bacterial growth in the dark must be due either to mechanisms other than those described here or to the existence in bacteria of a pathway alternate to photoactivation for the activation of DHC.

The phytoalexin kievitone has been suggested to induce mutations in *Pseudomonas phaseolicola* (Gnanamanickam & Patil 1977). However, attempts to confirm this mutagenic activity of the phytoalexin were unsuccessful (cited in Smith, D. A. 1982). In *E. coli* , DNA damage or interruption of DNA replication by a variety of treatments results in induction of the SOS response, which includes increased mutagenesis due to the enhancement of error-prone DNA repair (Walker, 1984; Ossanna, Peterson & Mount 1986). The activity of photoactivated DHC and LC in nicking DNA raises the possibility that these phytoalexins may also function as mutagens toward *X. campestris* pv. *malvacearum in planta.* Virulent mutants were readily recovered from cotton lines possessing single resistance genes following inoculation with incompatible races of *X. campestris* pv. *malvacearum* (Brinkerhoff, 1970 and our unpublished work). A correlation of phytoalexin accumulation with incompatible cotton / *X. campestris* pv. *malvacearum* interactions has been observed (Shevell 1984). Phytoalexins may act as

mutagens to enhance the conversion of avirulent to virulent races of *X. campestris* pv. *malvacearum in planta.*

Some pathogenically important pectolytic enzymes of *Erwinia carotovora* subsp. *carotovora* and *E. carotovora* subsp. *chrysanthemi* are controlled by the SOS stress response system (Panopoulos, N. J. & Peet, R. C. 1985). SOS control of pectin lyase in *E. carotovora* subsp. *carotovora* by DNA-damaging compounds such as mitomycin C (Itoh, Y., Sugiura, J., Izaki, K. & Takahashi, H. 1980) was confirmed by comparing enzyme induction in isogenic $recA^+$ and $recA^-$ strains by A. K. Chatterjee (cited in Panopoulos, N. J. & Peet, R. C. 1985). Whether there are compounds present in plants at the time of infection that could trigger the SOS response in bacteria is currently unknown. Rishitin (Lyon et al 1975), one of the most important phytoalexins produced upon infection by *Erwinia* in potato, the host plant used in the pectin lyase studies, is, like DHC, a dihydroxy-sesquiterpenoid. If photoactivated phytoalexins damage DNA *in planta* then phytoalexins may induce pathogens to express their pathogenicity via the SOS mechanism. Photoactivated DHC also destroyed the infectivity of cauliflower mosaic virus (Sun, Melcher & Essenberg 1987). This inactivation required the presence of coat protein and appears to be due to the cross-linking of viral DNA and coat protein (Sun, Melcher & Essenberg 1987). The diversity of photoactivated reactions reported here and in the accompanying study suggests that where DHC accumulates, it may serve to protect the plant from virtually any invading microorganism.

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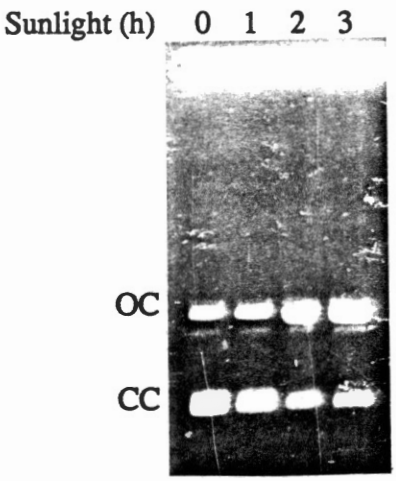

Fig. 1. Time-dependence of DNA cleavage by light plus DHC. Plasmid pBR322 DNA was treated for the indicated times with DHC and sunlight prior to agarose gel electrophoresis.

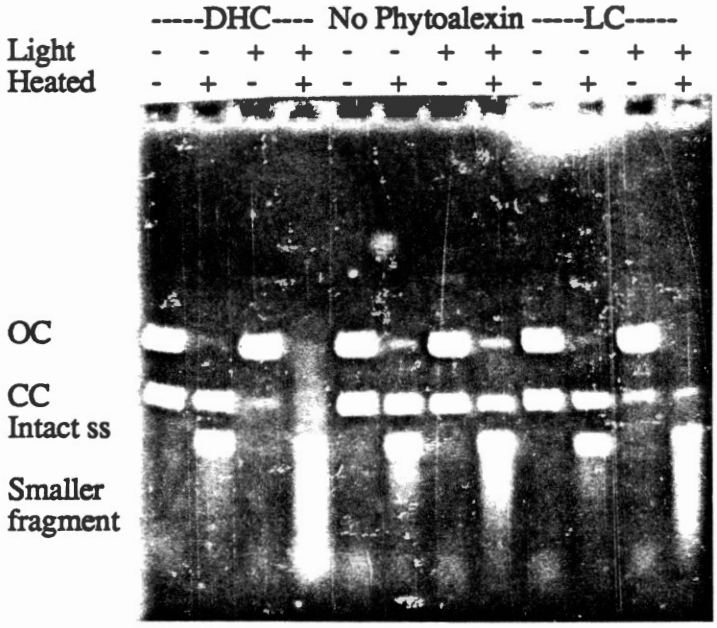

Fig. 2 Light and phytoalexin-dependence of DNA cleavage.
Duplicate samples of plasmid pBR322 DNA were treated with or without the indicated pytoalexins in the presence or absence of light for 3 h. One of each duplicate was heat-denatured prior to agarose gel electrophoresis of all samples.

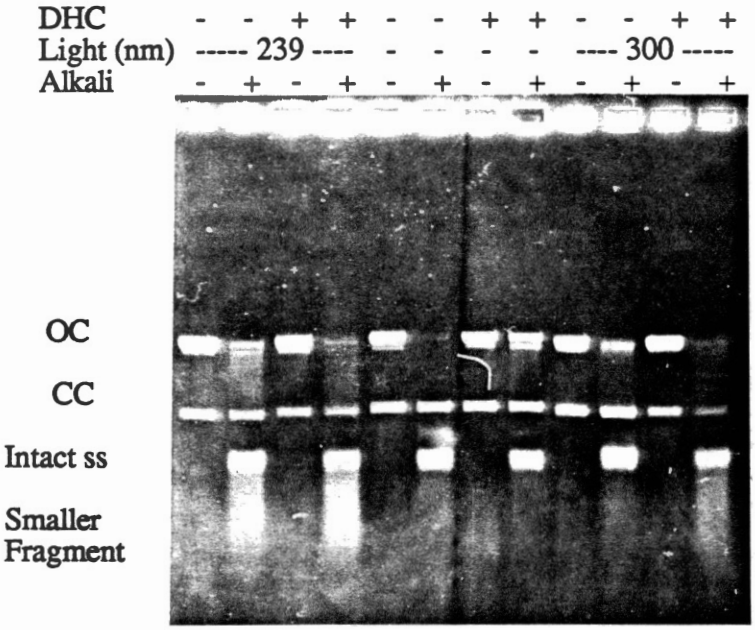

Fig. 3 Activity of light of 239 and 300 nm in DHC-induced cleavage of DNA. Samples of plasmid pBR322 DNA with or without DHC were illuminated with light of the indicated wavelengths or kept in the dark for 1 h. Half of the samples was alkali-denatured prior to agarose gel electrophoresis.

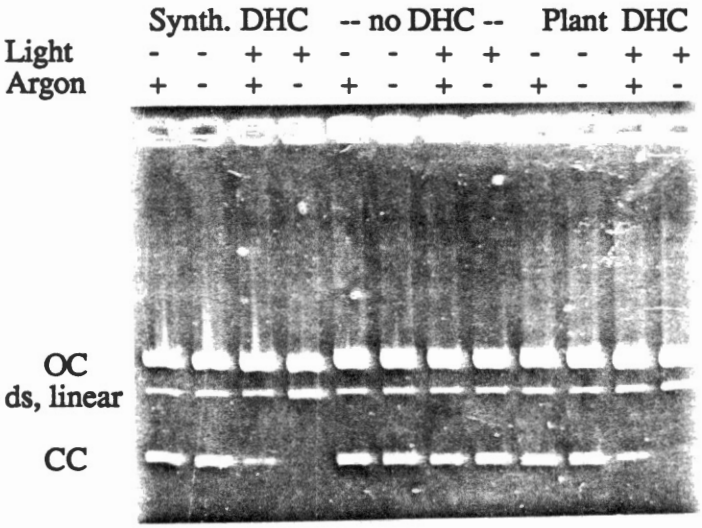

Fig. 4. Inhibition of photoactivated cleavage of DNA under a low-oxygen atmosphere with either plant-derived or synthetic DHC. Indicated samples were purged with argon before 3 h treatment in light or dark.

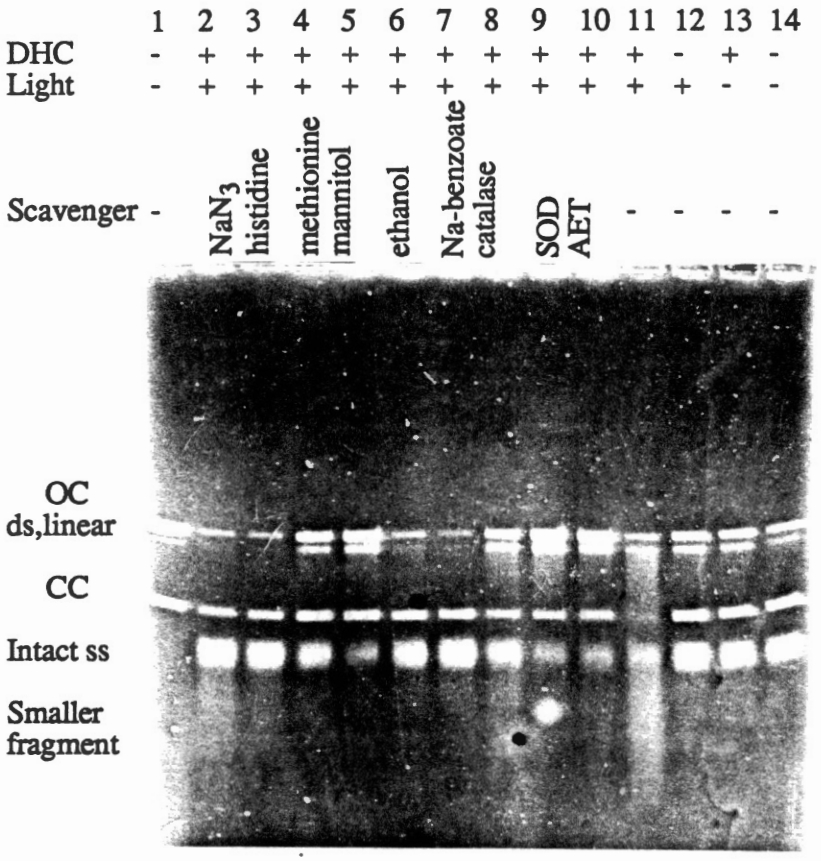

Fig. 5. Effects of scavengers of reactive oxygen species and of free radicals on the nicking of plasmid pBR322 DNA by DHC plus light. All samples except that in lane 1 were subjected to alkali denaturation prior to electrophoresis.

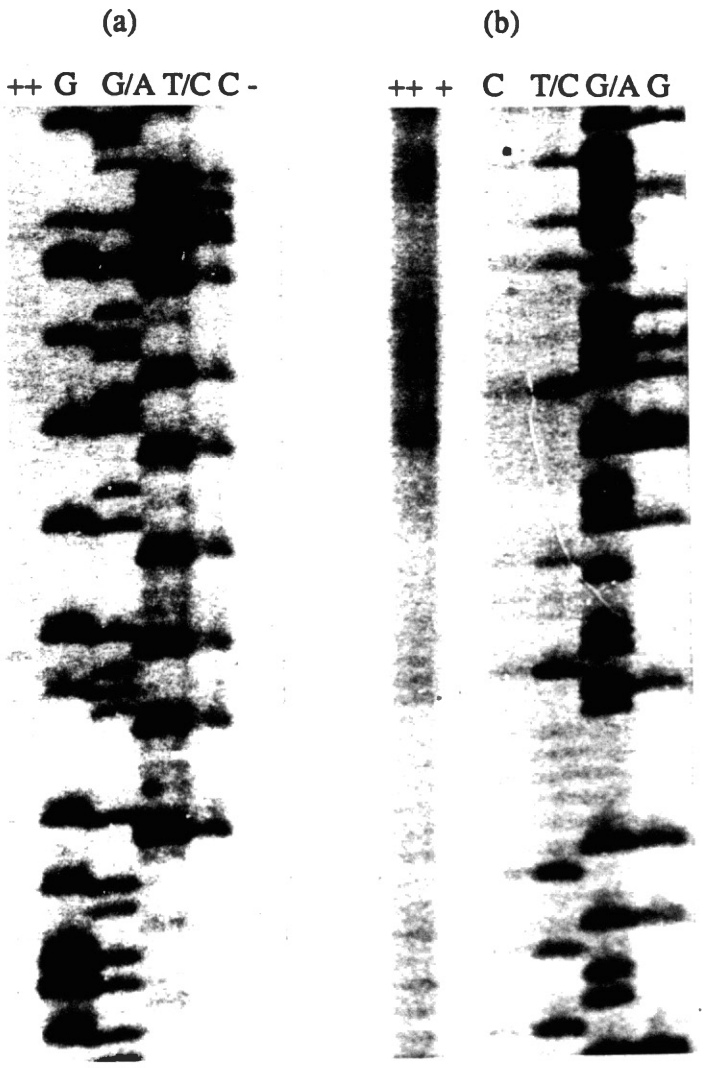

Fig. 6. DHC and light-induced cleavage of 3'-end-labeled double-stranded (a) and single-stranded (b) DNA restriction fragments analyzed on 8% polyacrylamide nucleotide-sequencing gels. Lanes G, G/A, T/C, C represent Maxam and Gilbert sequencing reactions; "++", fragment treated with DHC and light for 3 h, "+", fragment treated with light for 3 h, and "-", no treatment.

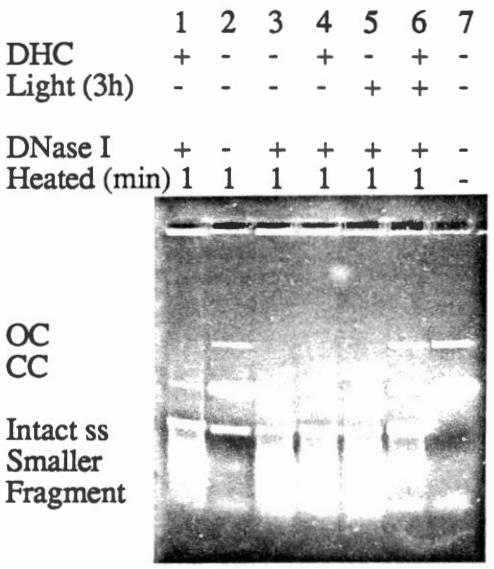

Fig. 7. Effect of DHC and light upon DNase I activity. DNase I was incubated with or without DHC and/or illuminated for 3 h (lanes 3 to 6). Plasmid DNA was then added for assay of DNase activity. In one assay (lane 1) DHC was added at the same time as DNA. Samples were heat-denatured prior to analysis by agarose gel electrophoresis. Non-denatured (lane 7) and heat-denatured (lane 2) plasmid DNA were included for comparison.

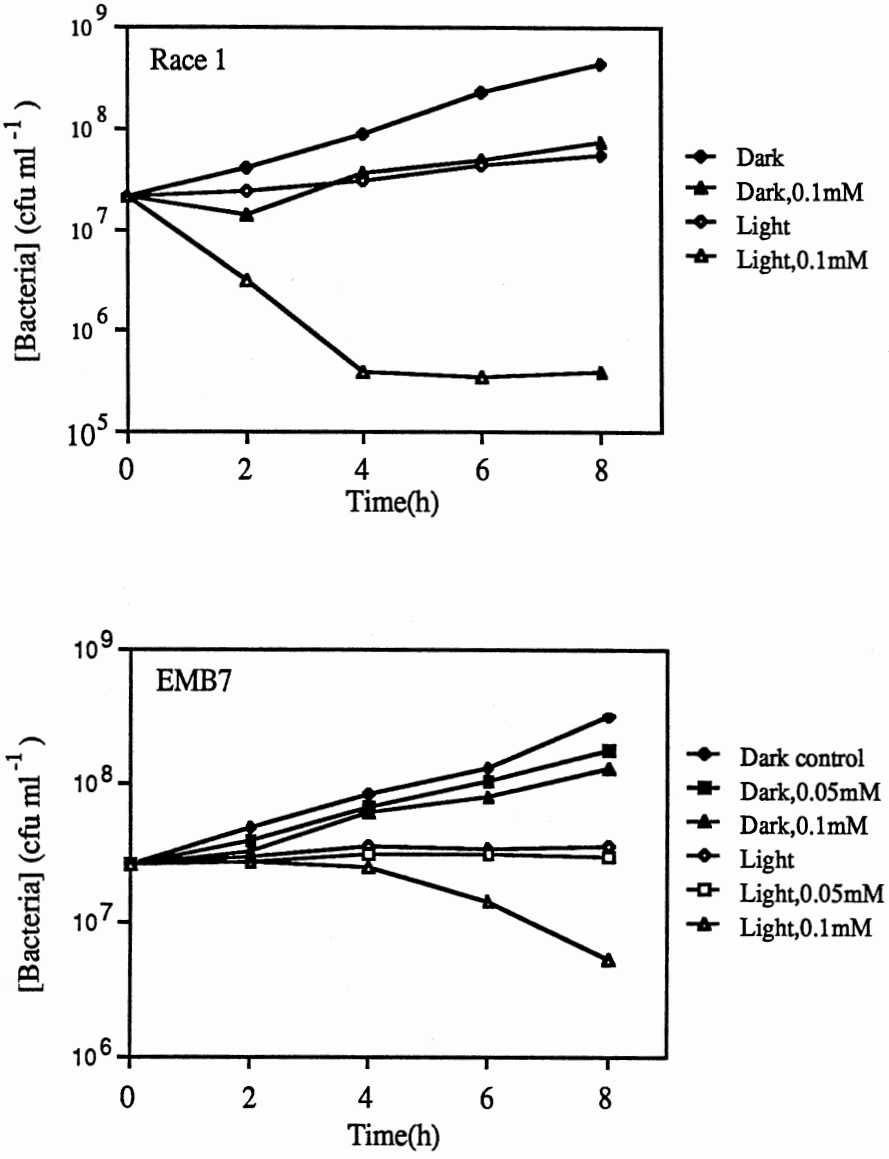

Fig. 8 Effects of light and DHC on multiplication of race 1 and strain EMB7 of *X. campestris* pv. *malvacearum in vitro* .

Table 1. Inactivation of MDH by DHC plus light *in vitro*.

	Activity of malate dehydrogenase (expressed as per cent of dark control)	
Treatment	Experiment 1	Experiment 2
Dark control	100a	100a
Light	91a	96a
DHC	88a	96a
Light + DHC	41b	53b

Control contained 0.025 units ml^{-1} of enzyme in experiment 1 and 0.040 units ml^{-1} in experiment 2. Values are means of five and six replicates for experiment 1 and 2 respectively. Values with different letters are significantly different at the 1% level within each experiment by Duncan's multiple-range test.

SECTION II

Inactivation of Cauliflower Mosaic Virus by a Photoactivatable Cotton Phytoalexin

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ABSTRACT

Upon irradiation, 2,7-dihydroxycadalene (DHC), a phytoalexin produced by resistant cotton lines in response to bacterial infection, inactivated virions of cauliflower mosaic virus (CaMV). The treatment destroyed infectivity observed either as local lesions on inoculated turnip leaves or as systemic infection of turnip plants. Neither irradiation alone nor DHC alone destroyed infectivity. Irradiation of a DHC-CaMV DNA solution resulted in numerous nicks in the CaMV DNA. The nicked DNA was, however, as infectious as the untreated DNA. Irradiation of a suspension of CaMV virions in DHC greatly decreased the electrophoretic mobility of 39 and 43 kilodalton coat protein fragments, but not of a 37 kilodalton fragment. The treatment also resulted in a reduction of the extractability of the DNA. We suggest that the inactivation of CaMV virions by DHC is due to the cross-linking of the nucleic acid binding domain of the coat protein to the viral DNA.

Key words: *Gossypium hirsutum*/cauliflower mosaic virus/*Brassica rapa*/2,7-dihydroxycadalene/photoactivation/DNA-protein cross-linking/infectivity/CysHis box/nucleic acid binding domain/phytoalexin/antiviral

Footnote
Abbreviations used: CaMV, cauliflower mosaic virus; DHC, 2,7-dihydroxycadalene; kb, kilobase; kbp, kilobase pair; PAGE, polyacrylamide gel electrophoresis; SSC, saline sodium citrate (0.15 M NaCl, 0.015 M sodium citrate).

INTRODUCTION

Phytoalexins, whose roles in limiting bacterial and fungal infections of plants have been extensively studied, are also produced by plants in response to viral infections [3-8, 15, 27]. The role of phytoalexins in the limitation of viral infectivity is, however, in doubt. Phytoalexins have been observed to accumulate in leaves of plants inoculated with local-lesion inducing viruses [3-8, 15, 27]. Phytoalexins are localized to lesions, and their accumulation coincides with the development and browning of lesions [4, 6]. Phaseollin accumulates in bean (*Phaseolus vulgaris*) in response to tobacco necrosis virus, but substantially after the primary events limiting infection have taken place [4]. Limited attempts to determine whether phytoalexins have antiviral activity have been made [14]. Vacuum infiltration of cucumber mosaic virus-inoculated cowpea (*Viana sinensis*)leaves, a procedure thought to dilute phytoalexins, increased viral multiplication in those leaves [15, 16]. Compounds, similar or identical to phenolic phytoalexins, were found in extracts of tobacco (*Nicotiana tabacum*) leaves induced to resistance by prior inoculation of plants with tobacco mosaic virus. A reduction of the number of virus-induced lesions was observed when extracts containing the compounds were mixed with virus prior to inoculation [38]. On the other hand, the infectivity of tobacco necrosis virus was unaffected by incubation in extracts of soybean (*Glycine max*) leaves containing hydroxyphaseollin [27].

We report here evidence substantiating a preliminary communication [41] that the sesquiterpenoid phytoalexin, DHC, has potent antiviral activity against CaMV. DHC is one of several sesquiterpenoid phytoalexins that accumulate in leaves of highly resistant cotton (*Gossypium hirsutum*) plants during their response to inoculation with *Xanthomonas campestris*, pv. *malvacearum* [18]. Their production is induced locally, at the site of infection [36], and is associated with the death of a limited number of cells in the affected area [19]. The sesquiterpenoid phytoalexins also accumulate in cotton in response to mechanical injury [37]. The phytoalexins, when assayed *in vitro* at

concentrations at which they are found locally in cotton tissue, effectively limit bacterial growth [17]. DHC, upon illumination *in vitro*, nicked DNA and inactivated enzymes, in a reaction thought to involve active oxygen species, suggesting that the anti-bacterial properties of DHC may be due to its photodynamic action [40].

CaMV (reviewed in [11]) is a 50 nm icosahedral virus whose capsid consists of 43 kD polypeptide subunits [1, 39]. The genetic information in CaMV virions is contained in a circular, double-stranded DNA of approximately 8 kbp. The DNA is found in the virion immediately interior to the protein capsid shell [9]. CaMV infects crucifers and a few solanaceous plants systemically and is transmitted in nature by aphids in a semi-persistent manner. In the laboratory it is easily transmitted by mechanical inoculation. Cotton cells are hosts for CaMV multiplication and allow the spread of virus to neighboring cells, producing local lesions [26]. Yet, cotton plants are not hosts for CaMV, since systemic spread of infection is limited. Factors limiting the systemic spread of CaMV in cotton are not known.

MATERIALS AND METHODS

Plant and virus materials

The third, fourth and fifth leaves of 3 1/2 week old turnip (*Brassica rapa*, L., cv. Just Right) plants maintained in a growth chamber with 12 h light 21°C and 12 h dark, 19°C were inoculated with 1.5 μg ml^{-1} (unless otherwise specified) CaMV virions of the W isolate [10] in 1% K_2HPO_4 or with 6.4 ug ml^{-1} CaMV DNA extracted from virions of the CabbS isolate [20] in 2 X SSC by spreading a 20 μl drop of inoculum on the leaves with a gloved finger as previously described [10]. CaMV virions were isolated from systemically infected turnip plants as described by Hull *et al.* (1977). CaMV DNA was prepared from virions by proteinase K-SDS digestion, phenol extraction, and ethanol precipitation [21]. Chlorotic local lesions on inoculated leaves were counted 10 to 14

days post-inoculation. Plants were scored for systemic symptoms (chlorotic spots, chlorotic mottling, and vein clearing) over a 30 day post-inoculation period.

Analytical methods

For polypeptide analysis CaMV virions were briefly incubated in 2% SDS, 0.1 M 2% mercaptoethanol at 100°C prior to electrophoresis in 10% polyacrylamide gels [28]. Molecular weights of the Coomassie Brilliant Blue stained bands were estimated by comparison with protein standards (Bio-Rad) myosin, phosphorylase, β-galactosidase, bovine serum albumin and ovalbumin. For analysis of DNA, samples were electrophoresed in horizontal 1% agarose gels in either a neutral buffer (Tris-phosphate-EDTA) [29] or 30 mM NaOH, 1mM EDTA. DNA in these gels was transferred to nitrocellulose by Southern blotting. CaMV DNA was detected by hybridization with nick-translated CaMV DNA as previously described [31]. In some experiments DNA was detected directly in alkaline agarose gels by staining with ethidium bromide in tris-phosphate-EDTA [29]. The same gels were then stained for protein with Coomassie Brilliant Blue.

DHC treatment

Chemically synthesized DHC (a gift of Dr. R. D. Stipanovic) stored in methanol was transferred to 0.5 ml polypropylene tubes and evaporated to dryness with a nitrogen stream. Volumes of 3 to 15 µl of an aqueous suspension of 0.3 mg ml^{-1} CaMV virions or 2 mg ml^{-1} CaMV DNA were added to the dried DHC to produce 0.46 mM DHC. Irradiation of samples was as described in the accompanying paper [40].

RESULTS

Effect of DHC on CaMV Infectivity

The third and fourth leaves of turnip plants were not useful in measuring virion infectivity since the number of chlorotic lesions observed varied widely. Lesion

numbers on the fifth leaves were more reproducible and were proportional to virus concentration (Fig. 1). Irradiation of CaMV virions prior to inoculation of turnip leaves reduced the number of lesions relative to the non-irradiated control (Table 1, expt. 1), but the reduction was not statistically significant nor reproducible (Table 1, expt. 2). Incubation of virions with DHC did not reduce the infectivity of the virions. Irradiation of a CaMV virion preparation in the presence of DHC completely destroyed the ability of the virions to induce local lesions (Table 1). Plants inoculated with irradiated or DHC-treated, non-irradiated CaMV virions developed visible symptoms of systemic CaMV infection three weeks after inoculation. Plants inoculated with virions irradiated in the presence of DHC remained healthy.

Consistent with previous reports [30], inoculation of leaves with CaMV DNA, rather than virions, did not produce reproducible numbers of lesions. Thus, the effects of treatment of CaMV DNA with DHC on the infectivity of the DNA were assessed by comparing the proportions of inoculated plants that became systemically infected. Treatment of CaMV DNA with light, with DHC, or with both (Table 2) did not dramatically affect the infectivity of the DNA.

Effects of DHC on CaMV DNA

The 8 kbp double-stranded circular DNA of CaMV is composed of an 8kb strand and two complementary strands of 2.6 and 5.4 kb. Electrophoresis of CaMV DNA on an alkaline agarose gel (Fig. 2A) resolved the three single strands expected as well as a minor component of 4.0 kb. The lesser intensity of the 8.0 kb band may be due to cleavage of this strand during DNA preparation Consistent with the report in the accompanying paper [40], neither light-treatment nor DHC-treatment altered the pattern of CaMV DNA single strands, but the DNA treated with DHC in the presence of light was considerably fragmented. Electrophoresis of CaMV DNA on a neutral agarose gel (Fig. 2B) separated circular from linear and knotted CaMV DNAs [42]. Illumination of CaMV DNA or treatment of the DNA with DHC in the dark did not affect the pattern.

Illumination of CaMV DNA in the presence of DHC resulted in some barely detectable double-stranded fragments smaller than 8 kbp. However, most of the CaMV DNA retained its integrity, suggesting that double-strand nicks were not induced in CaMV DNA by photoactivated DHC.

CaMV DNA can be extracted from virions by proteinase K-SDS digestion, followed by phenol extraction and ethanol precipitation [21]. Similar amounts of DNA were recovered from a comparatively sized aliquot of virions and virions treated with DHC (Fig. 3). Light treatment of virions resulted in an apparent decrease in yield of DNA. There were no qualitative changes in CaMV DNA as result of DHC or light treatment. Illumination of virions during DHC treatment resulted in a large decrease in recovery of CaMV DNA upon subsequent extraction. That DNA that was recovered was slightly more degraded than DNA recovered from the other treatments (Fig. 3A).

Effects of DHC on CaMV Polypeptides

Virions of the W isolate prepared by the method of Hull *et al.* [25] had three major polypeptides of 43, 39, and 37 kD (Fig. 4). A band of medium intensity at 72 kD and minor bands at 59, 48 and 84 kD were also detected. The polypeptide pattern was not noticeably altered by incubation of the virions with DHC in darkness. When virions were irradiated prior to SDS-PAGE, a reduced staining of the three major polypeptides, and an increase of staining at the top of each the separating and stacking gels was noted. When virions were irradiated during incubation with DHC and then subjected to SDS-PAGE, the major polypeptide bands, especially the 43 and 39 kD polypeptides, were reduced in quantity. The material at the top of the separating gel was more intensely stained. Coomassie blue-staining material at the top of the stacking gel and two high molecular weight bands appeared.

Alkaline gel analysis of cross-linked CaMV

CaMV virions are unstable in alkali [1]. Fig. 5A shows that alkaline treatment of CaMV virions released CaMV DNA single strands of mobility identical to those of DNA isolated by proteinase K-SDS treatment of virions and phenol extraction. CaMV DNAs of anomalous mobilities reported by Al Ani *et al.* [1] were not detected. Pretreatment of virions with DHC did not change the profile of DNA single strands, while light pretreatment caused a fraction of the DNA to migrate slowly in the gel. The combination of light and DHC treatment of virions completely prevented the release of free DNA strands from virions by alkali. Instead the DNA was heterodispersely distributed from the origin to the region of free DNA. The distribution of protein in the gel of Fig. 5A is shown in Fig. 5B. CaMV coat protein when released from virions (untreated, DHC-treated or light-treated) by alkali treatment migrated as a diffuse spot well in advance of the CaMV DNA. A small fraction of the protein from light-treated virions remained near the origin. After DHC plus light treatment of virions, however, all of the protein failed to migrate rapidly and instead was distributed in the same region as CaMV DNA.

DISCUSSION

Inactivation of CaMV virions by photoactivated DHC was complete whether assayed by local lesion numbers or by the number of systemically infected plants. The complete destruction of infectivity provides the first clear demonstration of antiviral activity for a highly purified phytoalexin. The observation supports the view that plant defense compounds are non-specific antibiotics, toxic to bacteria, fungi, plant cells, and viruses.

The antiviral activity of photoactivated DHC is probably not related to the known ability of plant phenolic compounds to inactivate plant viruses. The products of polyphenol oxidase oxidation of polyphenols, *o*-quinones, inactivate some viruses *in vitro* probably by cross-linking of coat protein subunits through the formation of disulfide bridges [24, 34]. Other viruses are resistant to *o*-quinone action [35]. The protein aggregates formed by the action of photoactivated DHC on CaMV were resistant

to mercaptoethanol treatment. Disulfide bridge formation is thus probably not responsible for the inactivation of CaMV.

Two effects of photoactivated DHC on CaMV virions could be discerned: scission of DNA strands and cross-linking of protein. Consistent with the report in the accompanying paper [40], photoactivated DHC nicked isolated CaMV DNA. Whether extensive nicking of DNA also occurred when DHC-virion mixtures were irradiated could not be determined because most of the CaMV DNA was not recovered in a protein-free form after treatment. What DNA was recovered nonetheless showed signs of degradation. Apparently, DHC, like other small molecules (such as ethidium bromide), can diffuse into viral particles. Naturally occurring double-stranded virion DNA contains three single-strand interruptions. These are ligated when virion DNA enters the nucleus on infecting a new cell. Viral DNA, although susceptible to induction of single strand nicks by photoactivated DHC, did not lose its infectivity after this treatment. It is likely that the DHC-damaged DNA was repaired readily *in planta.*

Photoactivated DHC probably cross-linked CaMV DNA to the coat protein of virions. The attachment of peptide material to the DNA is consistent with the failure to recover DNA from treated virions since the DNA would be lost to the interface during phenol extraction. Alkali, capable of releasing viral DNA from untreated virions [1], failed to release DNA strands of the expected electrophoretic mobility from CaMV treated with DHC and light. The reduced mobility of these DNA strands suggests cross-linking of the DNA strands to another molecule. The most likely molecule for the DNA to be cross-linked to is the coat protein.

Photoactivated DHC cross-linked polypeptides, probably to DNA. Polypeptide cross-linking correlated with a loss of extractability of DNA from virions. No bands corresponding to a series of protein multimers were detected, suggesting that the polypeptides had become attached to virion DNA rather than to one another. That DNA and protein in alkali-disrupted particles of CaMV cross-linked by photoactivated DHC

comigrated during electrophoresis in alkaline agarose gels further supports a protein-DNA cross-link. The present data do not allow us to rule out the possibility that photoactivated DHC cross-linked the coat protein polypeptides to one another creating a cage that trapped the DNA inside.

The probable cross-linking induced by photoactivated DHC of CaMV DNA to coat protein has a parallel in the UV light-induced inactivation of Rous sarcoma virus. The p12 polypeptide product of the gag gene binds strongly to the viral RNA. UV light cross-links p12 to specific sequences in the RNA [13]. A sequence conserved in nucleic acid-binding proteins from retroviruses and retroviral-like elements, $Cys.X_2.Cys.X_4.His.X_2.Cys$ (a Cys His box), is responsible for the RNA binding activity of p12 [32, 33]. RNA-protein cross-linking by UV light requires close proximity of protein to RNA due to the short life times of light-activated intermediates [13]. Observations presented in the accompanying paper [40] suggested that DNA nicking by photoactivated DHC is mediated by short-lived active oxygen species. CaMV coat protein is probably as closely bound to CaMV DNA as p12 is to Rous sarcoma virus RNA. Neutron diffraction studies showed that CaMV DNA makes close contact with the protein shell [9]. The CaMV coat protein binds double-stranded DNA [22]. A Cys His box is present in CaMV coat protein [12].

The differential loss of viral polypeptides after DHC-light treatment of virions suggests that the Cys His box of the CaMV coat protein may be bound to CaMV DNA in virions. A p56 polypeptide is the primary translation product of the coat protein open reading frame of CaMV [23]. It must be processed at both N and C termini to yield a polypeptide of about 43 kD, the major polypeptide in rapidly isolated CaMV virions [2, 39]. The locations of the N and C termini of p43 were estimated [20] based on an optimization of fit of the amino acid composition predicted by the nucleotide sequence with published amino acid compositions of CaMV virions assuming a molecular weight of 41 to 43 kD for the coat protein (Fig. 6A, second bar). An examination of the

predicted amino acid sequence revealed (Fig. 6B) homologous regions near the predicted N and C termini which could represent a target sequence for proteolytic maturation of the coat protein precursor. The region of p56 that would give rise to a 41.4 kD polypeptide (p41.4) as a result of cleavage within the homologous regions is shown in Fig. 6A (third bar). Polypeptides p39, and p37 are proteolytic products of p43 produced during virion isolation [1, 39]. The nucleic acid binding domain (Cys His box) lies between 37 and 39 kd from the N-terminal end of both p41.9 and p41.4. The persistence of p37 in free form under conditions that link p39 and p43 to DNA is consistent with the possible absence of the nucleic acid binding domain in p37.

Although photoactivated DHC is clearly antiviral when tested *in vitro*, its role in the limitation of viral infection *in vivo* is not clear. Cotton is not a host for CaMV, but allows local replication and spread of the virus [26]. Under some conditions virions that are produced in cotton are unusually permeable to exogenously added enzymes and contain fragmented DNA [26]. Further experimentation is needed to decide whether the fate of CaMV in cotton plants is to any extent determined by the induction and action of DHC in response to CaMV infection.

DHC has been shown to play a significant role in the limitation of infection of cotton by *X. campestris* pv. *malvacearum* [17]. It remains to be seen whether either protein cross-linking or DNA nicking are involved in the inhibitory effect of DHC on *X. campestris* pv. *malvacearum*, or whether other actions of the sesquiterpenoid phytoalexins of cotton are more significant for the inhibition.

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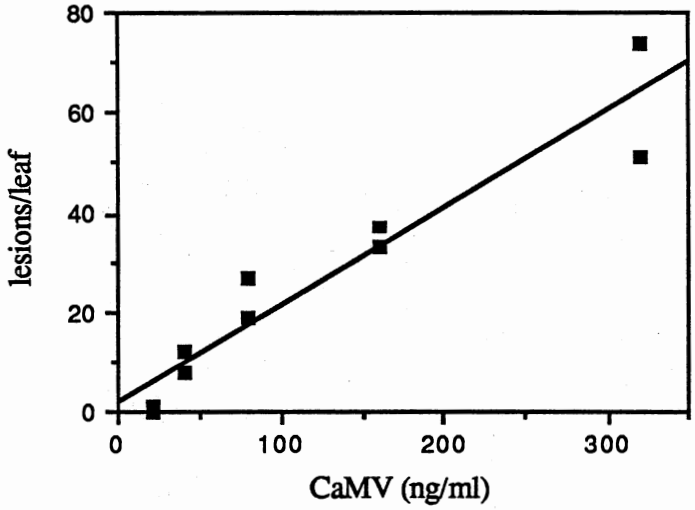


Fig. 1. Relation of number of lesions to virus concentration in inoculum. Chlorotic lesions on the fifth leaves of turnip plants were counted 2 weeks after inoculation of that leaf with the indicated concentration of CaMV.

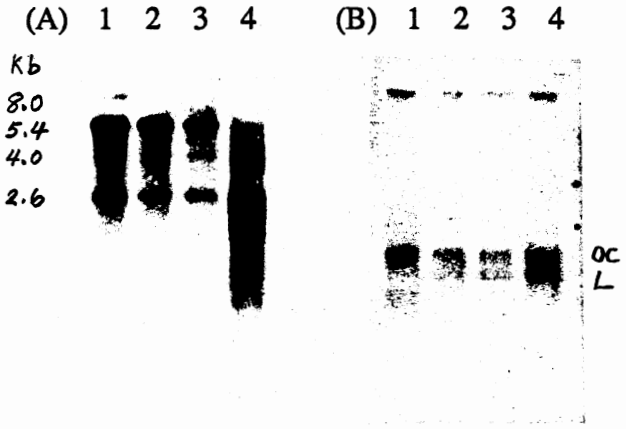


Fig. 2. Effect of DHC and light treatment of CaMV DNA on the electrophoretic behavior of DNA. A. Alkaline agarose gel analysis of CaMV DNA treated as follows: dark (lane 1); DHC (lane 2); light (lane 3); DHC and light (lane 4). Sizes of relevant bands are indicated in kb. B. Non-denaturing gel. Samples were the same as in A. Detection was by nucleic acid hybridization with ^{32}P-CaMV DNA. Positions of open circular (OC) and linear (L) forms of CaMV DNA are indicated.

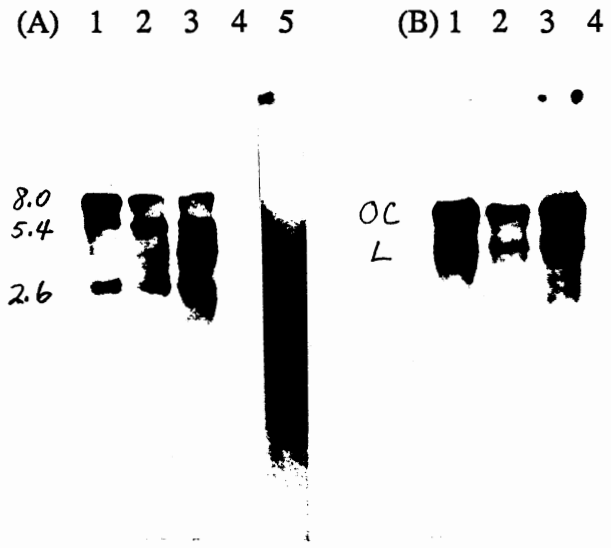


Fig. 3. Effect of DHC and light treatment of CaMV virions on the extractability and gel migration of CaMV DNA. A. alkaline gel: no treatment (lane 1); light (lane 2); DHC (lane 3); DHC and light (lane 4); a ten-fold longer exposure of lane 4 (lane 5). Sizes of relevant bands are indicated in kb. B. non-denaturing gel: lanes (1 to 4) were the same as in A. Detection was by nucleic acid hybridization with ^{32}P-CaMV DNA. Positions of open circular (OC) and linear (L) forms of CaMV DNA are indicated.

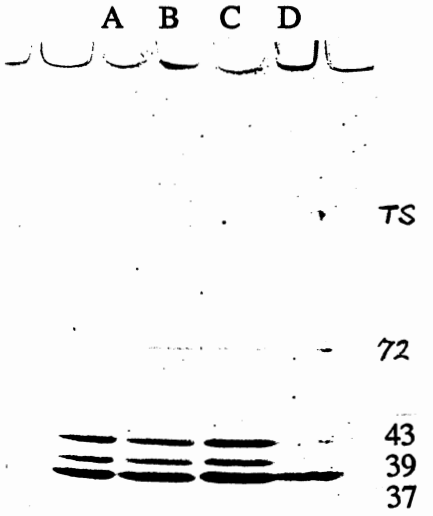


Fig. 4. Effect of DHC and light treatment on the SDS-PAGE profile of CaMV polypeptides. A. no treatment; B. light; C. DHC; D. DHC and light. Sizes of relevant bands are indicated in kD. TS indicates the top of the separating gel.

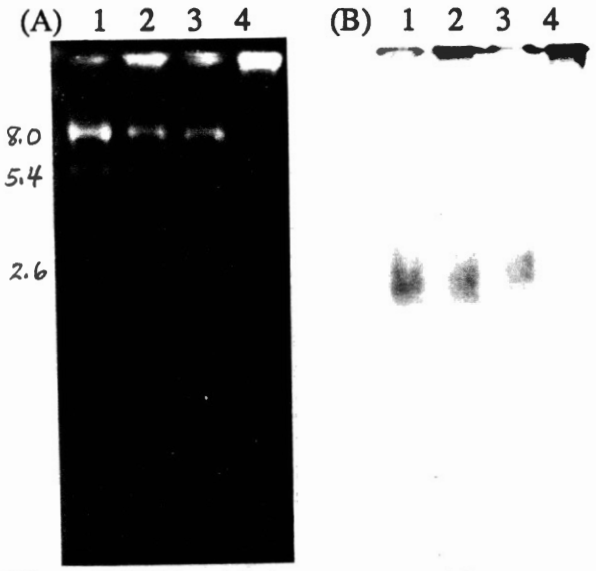
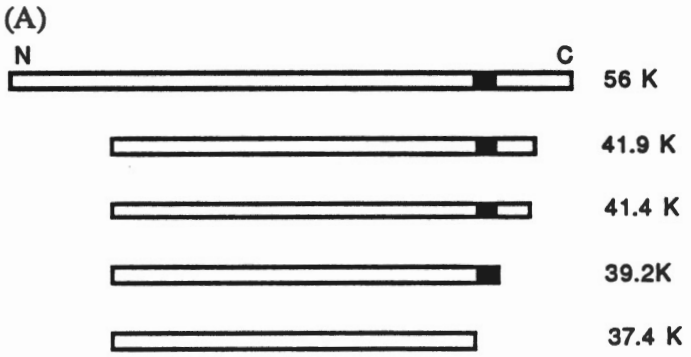


Fig. 5. Effect of DHC and light treatment of CaMV virions on the electrophoretic mobility of DNA and polypeptides in an alkaline agarose gel. A. DNA as detected by ethidium bromide staining. Sizes of relevant bands are indicated in kb. B. Protein as detected by Coomassie blue staining. CaMV virions were treated as follows: no treatment (lane 1); light (lane 2); DHC (lane 3); DHC and light (lane 4).



(B)

N-terminus	glu.glu.pro.glu.phe.glu.gln.val	
	*** *** *** * *** ***	
C-terminus	glu.glu.pro.	tyr.glu.gly.val

Fig. 6. CaMV coat protein fragments and a nucleic acid-binding domain. A. Location of the nucleic acid binding domain (Cys His box) in the CaMV coat protein polypeptide precursor and some possible proteolytic products. The dark bar identifies the domain. For details of the proteolytic products, see text. B. Homologous sequences near the N and C termini of mature coat protein that could represent sites for proteolytic processing.

Table 1. Inhibition of CaMV Virion Infectivity by DHC and Light

	Lesions/Leaf[a]	
Treatment	Expt. 1	Expt. 2
none	117 ± 53	108 ± 16
light[b]	66 ± 36	122 ± 33
DHC	ND[c]	108 ± 16
DHC + light	0	1 ± 1

[a]average number of chlorotic lesions for 6 (Expt. 1) or 4 (Expt. 2) leaves, with mean deviation from mean.
[b]3 h irradiation with growth chamber lights.
[c]ND: not determined.

Table 2. Infectivity of CaMV virions and DNA after DHC or light treatment

Treatment of Inocula	Virion Inocula	DNA Inocula
none	6/6[a]	9/10
light[b]	6/6	6/10
DHC	ND[c]	5/10
DHC + light	0/6	10/14

[a]number of diseased plants/number of plants inoculated.
[b]3 h irradiation with growth chamber lights.
[c]ND: not determined.

CHAPTER I

INTRODUCTION

Xanthomonas campestris pv. *malvacearum* (Xcm) is the causal agent of bacterial blight in the cotton plant. It is a destructive disease which exists in most areas where cotton is grown (Brinkerhoff, 1970; Knight et al., 1950). Upon infection the susceptible plants develop water-soaked lesions and the resistant plants produce a hypersensitive response. The interaction between the plant and the bacteria is called compatible when the water-soaked symptom is observed, and the pathogen is said to be a virulent strain; when the hypersensitive response is observed it is called an incompatible interaction by an avirulent strain.

Phytoalexins (PA) are low-molecular-weight, antimicrobial compounds that are both synthesized by and accumulated in plants after exposure to microorganisms (Paxton, 1981). Although they have been studied intensively, definite proof that they play a major role in inhibiting growth of the pathogen that elicits their production has not yet been obtained for any host-pathogen system. Their role in resistance remains controversial (Ellingboe, 1982; Mansfield, 1982; Misaghi, 1982). It has been demonstrated in this laboratory that at least four structurally related sesquiterpenoid phytoalexins (PA) accumulate in cotton leaves in the incompatible reaction (Essenberg et al., 1982; 1986). The PA most toxic to Xcm in this system is 2,7- dihydroxycadalene (DHC) (Essenberg et al., 1982).

One way to test the importance of the PAs in resistance to Xcm would be to isolate mutants with resistance to the PAs. Resistant mutants should show better growth than the parent strains in incompatible cotton lines. My initial objective was to isolate stable

mutant(s) which show DHC-resistance *in vitro* and *in planta*. A limited supply of synthetic DHC was available from Dr. R. D. Stipanovic. Since the mechanism of the toxicity of DHC toward Xcm was unknown, it was not possible to choose commercially available compounds which have a similar toxic effect toward Xcm, to aid in the selection of DHC-resistant mutant(s), circumventing the limited supply of DHC. Part of the work described here was directed toward isolation of DHC^R mutants. In order to develop a strategy of mutant selection, work was also directed toward understanding the toxic effects of DHC and the mutability of the pathogen.

CHAPTER II

REVIEW OF SELECTED LITERATURE

Plant defense in general

Plants use many different biochemical and morphological characteristics to defend themselves against invaders. Some characteristics physically retard penetration or limit growth of these invaders. The cuticle is a good example of this kind of protection. Cutin is an effective physical barrier to some, but not all pathogens (Maiti et al., 1979).

Biochemically there are more than 10,000 known low molecular weight secondary metabolites found in higher plants and fungi, and there is increasing evidence that the plants' secondary metabolites are the backbone of its resistance (Swain, 1977). Secondary metabolites are not distributed evenly throughout the plant, either qualitatively or quantitatively, in time and space (Swain, 1977). Chemically they are classified into groups: flavonoids, coumarins, terpenes, quinones etc. Usually, amounts are greatest in epidermal tissue. This is the best location if these compounds function as a defense (McKey, 1974). When a plant is attacked by pathogens, it is sometimes induced to synthesize additional secondary metabolites. If these induced metabolites are unique to the diseased tissue and exhibit an antibiotic function they are called phytoalexins. Genera within a plant family usually produce PAs that belong to the same chemical group. Slight structural differences in the metabolites are frequently found within a family (Stoessel et al., 1976).

The defense antibiotics of the plant cell are composed of constitutive antibiotics and PAs. Constitutive antibiotics may be completely synthesized, then transported and stored in vacuoles, glands, ducts and the periderm. The pigment glands of cotton plants are one

example of this. There are more than 20 different antibiotic terpenoid aldehydes and quinones which have been identified from the pigment glands of the Gossypium spp. (Bell et al., 1978). Alternatively, less toxic precursors of the antibiotics may be stored in vacuoles, and the final antibiotics are formed rapidly when a wound brings them into contact with hydrolases and oxidases located in other compartments of the cells (Schlosser, 1980). The surfaces of many plant leaves are covered by glandular trichomes that exude a variety of constitutive antibiotics. Surface washing from the cotton plant contains catechins and condensed proanthocyanidins that originate from the glandular trichomes (Bell, 1981).

Resistance of cotton to bacterial blight

Xcm incites a leaf-spot disease in which infected, susceptible tissues become occluded with bacteria-filled polysaccharide slime. The tissue suffers damage first to chloroplast membranes, then to other membranes, which results in eventual tissue death (Al-Mousawi et al., 1982; Novacky et al., 1982). Essenberg (1979) has demonstrated that single bacterial cells can establish colonies in inoculated susceptible or resistant leaves and that growth of each colony is inhibited in resistant cotton lines by a local resistance response involving initial envelopment of the bacteria on the outer surfaces of plant cell walls by what appears to be loosened plant cell wall material (Cason et al., 1978; Al-Mousawi et al., 1983), rapid death of the leaf mesophyll cells closest to the bacterial colony, and form a small bacteriostatic zone around the colony (Essenberg et al., 1979).

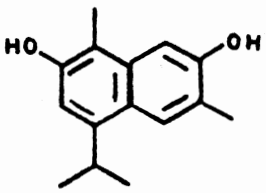
Electrical potentials (E_m) across plasma membranes of cotton cotyledons following inoculation with compatible and incompatible bacteria have been determined (Novacky et al., 1982). During the compatible interaction, the passive diffusion potential was impaired, and the damage seemed limited to the energy-dependent component of E_m in the dark. During the incompatible interactions, on the other hand, both the diffusion potential and the energy-dependent component of Em decreased (Pavlovkin et al., 1986). Since the diffusion potential is due to concentration gradients maintained by plasma membrane

semipermeability, its decline suggests membrane leakiness. Predictably, electrolyte leakage was observed from the incompatibly responding tissues (Pavlovkin et al., 1986).

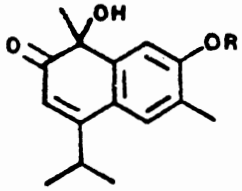
Ultrastructural studies have indicated that although bacterial envelopment and rapid plant cell necrosis always accompany an effective resistance response in cotton/Xcm interactions, it is unlikely that either of these events directly inhibits bacterial multiplication, since in plants with only one or two resistance genes bacteria are inhibited only after they have multiplied and ruptured the enveloping structures (Al-Mousawi et al., 1982) and since genetically resistant plants maintained in darkness postinoculation permit as much bacterial multiplication as susceptible plants even though necrosis occurs (Morgham et al., 1987). Phytoalexin production, however, appears to account quantitatively for the observed bacterial inhibition during incompatible interactions (see below).

Phytoalexins in the cotton/Xcm system

Three sesquiterpenoid PAs have been isolated from inoculated resistant cotton plants and identified: DHC, lacinilene C (LC) and lacinilene C 7-methyl ether (LCME) (Essenberg et al., 1982; 1985).



DHC



R = H, LC
R = CH_3, LCME

Of the three compounds, DHC is the most inhibitory to Xcm. In cotton leaves or cotyledons inoculated with incompatible races of Xcm, mesophyll cells develop yellow-green autofluorescence that has an emission spectrum similar to those of LC and LCME. There is a good correspondence of yellow-green fluorescent cells with the brown cells that

are adjacent to the extracellular bacterial colonies (Essenberg et al., 1985). Mesophyll cells from inoculated, resistant cotyledons were isolated and a fluorescence-activated cell sorter was used to separate brightly fluorescent cells from other mesophyll cells. Analysis of the two cell fractions indicated that DHC, as well as the yellow-green fluorescent LC and LCME, are predominantly localized in the brightly fluorescent cells (Pierce & Essenberg, 1987). With this finding the local concentrations of the PAs at infection centers could be calculated. Results indicated that in highly resistant lines WbM(0.0), OK1.2, and Im216, the day on which inhibitory concentrations were attained in fluorescent cells adjacent to essentially every bacterial colony coincided with the day on which bacterial multiplication stopped (Essenberg et al., 1985). The fluorescent cells have damaged membranes that constitute no permeability barrier between the PAs and the extracellular bacterial colonies. These results imply that the PAs contribute significantly to bacterial blight resistance in cotton.

Genetic determinants of incompatibility

In cotton, resistance to specific races of Xcm can be conferred by any of 18 known resistance genes (Brinkerhoff, 1970; Brinkerhoff et al., 1985). A set of cotton lines developed by L. A. Brinkerhoff by crossing each of nine different resistance genes into the Ac 44 cotton line, which is susceptible to all known races, is available for studying the basis of race/cultivar specificity. Using differential strains and the congenic cotton lines containing resistance genes B_2, B_3, b_7, and B_N, it was found that bacterial multiplication was less and phytoalexin accumulation was greater in all 23 incompatible interactions studied than in any of the 13 compatible interactions (Shevell, 1985). A similar association of the isoflavonoid phytoalexin glyceollin with incompatible interactions has been observed in a survey of many strains of *Pseudomonas syringae pv. glycinea* in differential soybean cultivars (Long et al., 1985). Thus elicitation of PA accumulation is a consequence of incompatible interactions in these two host/pathogen systems. Compatibility or

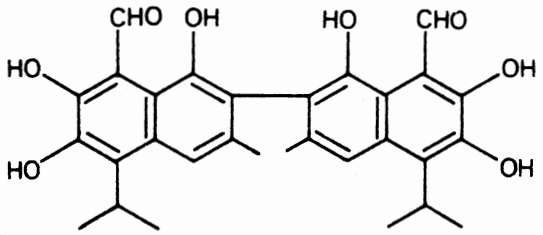
incompatibility toward a given resistance gene appears to be determined by single genes in Xcm. Gabriel et al. (1986) have cloned Xcm DNA segments that when conjugated into virulent strains confer avirulence toward individual resistance genes.

Dye-DNA fluorescence

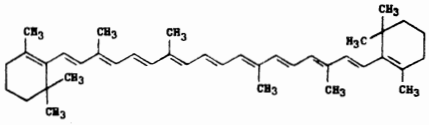
The ability of a number of intercalative drugs to fluoresce allows the study of initial steps in the dye sensitization processes by fluorescence spectroscopy. Fluorescent ligands can be classified into two groups : (1) dyes enhancing fluorescence regardless of DNA base composition, e.g. acridine orange, ethidium bromide and (2) dyes whose fluorescence is quenched upon complexing with nucleic acids, e.g. acriflavine, proflavin, methylene blue, quinacrine, and aromatic hydrocarbons (Kittler et al., 1977). Guanine possesses a unique quenching ability compared to other bases of DNA. A charge transfer process taking place between the excited dye molecule and guanine, which is known to have good electron-donating properties, is discussed as being responsible for the quenching effect. There is evidence that guanine moieties are involved in the quenching of the fluorescence of acriflavine (Tubbs et al., 1964), proflavin (Weisblum et al., 1972) and quinacrine (Michelson et al., 1972; Weisblum et al., 1972). It is interesting that polynucleotides containing no guanine i.e., poly(dA · dT), poly(dA) · poly(dT), and poly(A) · poly(U), enhance the fluorescence (Michelson et al., 1972; Weisblum et al., 1972). In the specific case of natural DNAs, enhancement is only in regions rich in AT base pairs. The ability of GC base pairs to quench quinacrine fluorescence predominates over the apparent tendency of AT base pairs to enhance quinacrine fluorescence, and the overall effect by most natural DNAs is fluorescence quenching. This mixed effect suggests a possible explanation for the occurrence of fluorescence banding patterns in chromosomes which have been stained with dyes (Kittler et al., 1977).

Photooxidation and protection

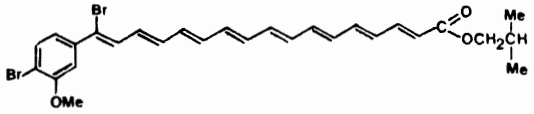
It is known that polycyclic aromatic hydrocarbons, conjugated cyclic dienes, and many heterocycles are susceptible to photooxygenation to yield cyclic peroxides. It is the reactive double bonds in these compounds that readily react with singlet oxygen (Adams, 1971). Singlet oxygen is extraordinarily reactive and has been shown to be involved in many photochemical oxidations. The phytoalexins identified by our laboratory (Essenberg et al., 1982 and unpublished work) fit the above description well and are known to be unstable when exposed to light and air, but the involvement of free radicals of these compounds is unknown. However gossypol, a dimeric sesquiterpene of the cadalane class, is a normal metabolite of cotton and is known to generate oxygen radicals at high pH during autooxidation (dePeyster, 1984).



Gossypol



Carotene



Xanthomonadin1

Carotene is an effective singlet oxygen quencher which deexcitates $^1O_2$ to $^3O_2$. The resulting triplet carotene is deactivated by heat transfer (Knox et al.,1985a). All carotenoid pigments with nine or more conjugated double bonds are almost equally effective, but below nine, quenching becomes increasingly ineffective (Krinsky, 1979). Xanthomonadins are membrane-bound yellow pigments found in *Xanthomonas* (Stephens et al., 1963). They are brominated aryl-polyene compounds (Starr et al., 1977). The yellow pigments found in members of the herbicola group of plant-associated *Erwinia* are carotenoids. In *E. herbicola*, the production of these pigments is plasmid encoded

(Gantott et al., 1982) and it is not known if xanthomonadins are also plasmid encoded. The function(s) of these pigments in plant pathogens is unknown. In the photodynamic killing of the nonphotosynthetic organism *Sarcina lutea*, high intensities of sunlight were lethal to a carotenoid-less mutant while the carotenoid-containing wild-type survived this treatment (Mathews et al.,1960). This suggests that the role of bacterial endogenous carotenoid is protection against photodynamic action. With respect to the number of conjugated double bonds, xanthomonadins are identical to β-carotene--both have 11 double bonds. It is possible that xanthomonadins could serve as a resistance factor in *Xanthomonas* against toxic singlet oxygen species that may come into contact with the pathogen.

Photosensitization

Photochemical oxidations of biologically important molecules via sensitized dyes can be distinguished as photodynamic action with involvement of oxygen or as photosensitized reaction without involvement of oxygen. In the latter case other appropriate electron and/or hydrogen donors are present. For example, riboflavin illuminated under anaerobic conditions is known to undergo reduction, with water serving as the ultimate hydrogen donor. Another example is the furocoumarins (Musajo et al.,1970), such as 8-methoxypsoralen, which sensitize bacteria to killing by visible light under anaerobic conditions. The presence of oxygen decreases the efficiency of this dye-sensitized reaction (Oginsky et al., 1959).

In photodynamic action, oxygen and an oxidizable substrate are consumed, but the dye is reused. The discovery of photodynamic action can be attributed to Raab, who in 1900 observed that low concentrations of acridine orange (and other dyes) had no effect on *Paramecia* in the dark but rapidly killed them in the light. It was soon determined that oxygen was required for this reaction (Smith et al., 1969).

Many compounds of biological interest damage biological systems by photodynamic action, requiring light and oxygen (Spikes, 1977; Towers, 1980). Two mechanisms of photodynamic action are thought to occur. Type I reactions involve the direct reaction of the excited light receptor molecule (X) with a biological substrate. The resulting radicals react further with oxygen to produce superoxide or with other molecules. Type II reactions involve physical activation of ground state dioxygen by the excited light receptor molecule, yielding either highly reactive singlet oxygen ($^1O_2$) with high efficiency by direct energy transfer or an oxidant ($X^{+}\cdot$) and superoxide ($O_2^{-}\cdot$) with low efficiency after charge separation by electron transfer reactions (Foote, 1976; Foote, 1979).

A large solvent effect on singlet oxygen makes the lifetime of singlet oxygen ten times longer in D_2O than in H_2O (Merkel et al., 1972). Azide ion (N_3^-) will quench singlet oxygen with high specificity (Hasty et al., 1972). The enhancement effect of D_2O and the protective effect of azide ions are valuable diagnostic tools for studying photodynamic action both at the chemical as well as the cellular level (Krinsky, 1977; Ito, 1978). In the photodynamic killing of *Stentor coeruleus*, singlet oxygen plays an important role as the oxidant by the Type II mechanism. However, a free radical mechanism of Type I photosensitization appears to play a supplementary role in the photodynamic killing of this protozoan (Yang et al., 1986).

Singlet oxygen seems to play a role in DNA strand breakage by the photodynamic dye rose bengal, but chemically-generated singlet oxygen failed to break the DNA backbone (Nieuwint et al., 1985). Photoactivated methylene blue can bind to DNA and damage guanine *in vitro* (Simon et al., 1962), damage DNA in bacteria but not in yeast (Jocob, 1975), and induce mutations in bacteria (Imray et al., 1975; Gutter et al., 1977; Webb, 1979). A substantial amount of work has been done to characterize the photodynamic sensitizers acting upon linear molecules of DNA. These studies have shown that deoxyguanosine is specifically attacked in such a way that alkali-labile sites are created which become manifest as strands break upon exposure to alkaline (pH > 12) conditions

(Simon et al., 1962, Spikes, 1977). When porphyrins were exposed to light of 360-390 nm in the presence of plasmid DNA, single-strand DNA breakage was observed at pH 8.2; this reaction was inhibited by the singlet oxygen quencher sodium azide (Fiel et al., 1981).

It has been suggested that a significant part of the mutagenicity of 8-methoxypsoralen plus light in *Escherichia coli* is due to the $^1O_2$-generating capacity of the dye (deMol et al., 1981a). 8-Methoxypsoralen covalently bound to DNA, was more effective in generating $^1O_2$ than free dye (deMol et al., 1981b). The photodynamic DNA-damaging action of acridine orange in *Saccharomyces cerevisiae* was grouped into two types: one is produced via $^1O_2$ and leads to mitotic gene conversion and mitotic crossing over, and the other is produced via a non-$^1O_2$ reaction pathway which leads to mutation (Kobayashi, 1978).

The precise role of singlet oxygen in the photodynamic destruction of DNA is difficult to assess, because (1) in addition to $^1O_2$, other active oxygen species such as $O_2^{-\cdot}$ may be produced, and (2) active oxygen species including $^1O_2$ may react with sensitizer, generating product(s) that may inflict the damage (Nieuwint et al,1985). After examining the superoxide-related literature, Fridovich concluded that the superoxide radical does exert deleterious effects independent of the participation of hydrogen peroxide in the production of the hydroxyl radical (Fridovich, 1986). (see discussion of hydroxyl radical generation below.)

Of the 20 commonly occurring amino acids, only histidine, methionine, tryptophan, tyrosine and cysteine are generally susceptible to sensitized photooxidation (Spikes et al., 1970). Histidine is the most susceptible among these five when methylene blue is used as the sensitizer (Smith et al., 1969). The rate of photooxidation of free amino acids depends on the sensitizing dye, the solvent, the pH, and the concentrations of reactants, including oxygen (Spikes et al., 1970). Histidine is photooxidized only above pH 6, when the imidazole nitrogen is ionized (Spikes et al., 1970). Tyrosine is most rapidly photooxidized above pH 10, when the phenolic group is ionized (Spikes et al., 1970).

Disulfide and peptide bonds in proteins are usually not susceptible to photodynamic action; chemical alteration involves the side chain of the five susceptible amino acids mentioned above (Spikes et al., 1970). The rate of photooxidation of a protein depends on the sensitizer and the reaction conditions as well as with free amino acids, except that the "exposed" amino acid of a protein is more rapidly photooxidized than the "buried" one (Spikes et al., 1970). Photodynamic reactions typically alter the biological properties of proteins, such as loss of the catalytic activity of an enzyme. The enzymes diastase, invertase and papain can be photodynamically inactivated by eosin (Spikes et al., 1969). Photodynamic inactivation can occur in two ways: directly, by the destruction of amino acid residues in the active site region, or indirectly, by altering the necessary active conformation of the enzyme.

Evidence indicates that a reaction between free amino groups of proteins and a photooxidation product of histidine residues is involved in the cross-linking reaction by photodynamic protoporphyrin (Dubbelman et al., 1978). The components of the respiratory chain associated with cell membranes were found to be sensitized by methylene blue (Prebble et al., 1973). The cross-linking of DNA and protein in the presence of methylene blue has been observed *in vivo* and *in vitro* (Smith et al., 1969). A good correlation has been observed between the enhanced killing of *E. coli* in the presence of methylene blue and the enhanced cross-linking of DNA with protein in the presence of methylene blue (Smith et al., 1969). When erythrocyte ghosts were used as targets for photodynamic damage, two types of damage other than catastrophic breakdown were observed: the leakage of small ions and the leakage of larger molecules (M.W. 10 000), corresponding to damage in proteins and lipids, respectively (Deziel et al., 1981).

Eosin was reported to photogenerate $^1O_2$, which inhibited photosynthesis in pea leaves by inactivating certain photosynthetic enzymes and by promoting the disorganization of chloroplast thylakoids (Knox et al., 1985b). Eosin acts on the membrane in the inactivation of yeast cells and does not permeate the cell membrane (Cohn et al., 1977).

With methylene blue, whose primary site of attack on the cell is the cell membrane, the osmotic fragility was found to be rapidly repaired within five minutes after treatment in *Proteus* (Jocob et al., 1975), the repair occuring faster than DNA repair in this organism (Jocob, 1975).

In summary, many types of photodynamic damage have been observed in cells: (a) damage to DNA, evidenced by base damage and mutation; (b) damage to protein, evidenced by the inactivation of enzymes; (c) damage to cell membranes, evidenced by altered permeability; and (d) killing of cells.

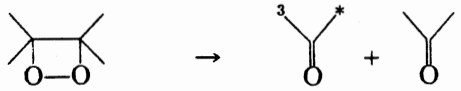
Extrapolation of chemical knowledge obtained from a homogenous system does not ensure a proper view of cellular photosensitization. Numerous difficulties exist: the pH inside the cell is hard to control through outside conditions; distribution and local concentration of the given sensitizers is difficult to assess; steady oxygen concentration is rare; and the behavior of quenchers and scavengers as diagnostic aids in cellular environment can only be guessed (Ito et al., 1983). When one plans to study the cellular photodynamic action of a sensitizer, knowledge of the permeability and the specific interaction of the sensitizer with the cell components is helpful. The hydrophobicity and the molecular weight of a compound affects the degree of membrane penetration. The photophysical properties, such as yield and lifetime of the triplet state, and oxygen quenching efficiency, are other important factors. Since the bacterial membrane, comprised of proteins, steroids, and unsaturated fatty acids, and the DNA are in close proximity, all of them are likely to be vulnerable to photodynamic action of a sensitizer.

Activity of photodynamic compounds in the dark

Most photodynamically active compounds are also subject to the redox system of the cell. Access to intracellular redox systems, such as the electron transport chain, is an important element to be considered (Ito et al., 1983). In this situation, only sensitizers that survive such an interaction in the dark could cause photodynamic action in the light. The

biological electrons that pass through carriers of the electron transport chain accomplish the tetravalent reduction of dioxygen to water without the release of intermediates (Chance,1952, Antonini et al., 1970). If a redox active compound could enter cells and compete with one of the electron carriers for electrons and give rise to an autooxidizable form, diverting a portion of this electron flow from the normal water-producing pathway to superoxide and hydrogen peroxide-producing pathways, this may acccount for the lethal effect of some compounds like paraquat, menadione, and methylene blue (Hassan et al., 1979). Depending on the redox potential of a compound, it is possible that it will be activated by the cell's electron transport system in the dark as well as being photodynamically active *in vivo*.

1, 2-Dioxetanes are among the best chemical generators of excited species.



1, 2-Dioxetanes Carbonyl groups

Upon cleavage (e.g., by heat), one of the carbonyl fragments is in an electronically excited state, principally the triplet state, if no groups of extensive conjugation and low ionization potential are attached to the dioxetane ring (Cilento, 1980). Many photochemical reactions have been duplicated in the dark by this excited species, e.g., conversion of ergosterol to vitamin D_2 (Ullman, 1972), and formation of pyrimidine dimers in DNA (Lamola, 1971). Several biological systems that could potentially produce this excited state have been discussed by Cilento (Cilento, 1980). Basically, if a compound has part of the structure similar to dioxetanes, it may upon cleavage produce a triplet carbonyl compound. This excited carbonyl compound can then transfer its energy and excite efficient acceptors to the excited singlet state, thereby producing $^1O_2$ in the dark by a reaction analogous to a photodynamic reaction. Another very important way of generating an excited species is via

electron transfer (Freed et al., 1972). Peroxidases (oxidases) and related enzymes are the candidates for catalysing the formation of electronically excited species as mentioned by Cilento (Cilento, 1980). What the possible role of these enzymes is in relation to DHC, LC and LCME has not been explored. However, hemigossypol, a cotton sesquiterpene phytoalexin, is reported to form gossypol via the action of peroxidase (Veech et al., 1976). Peroxidase was used *in vitro* as the catalyst in the oxidation of catechin, an important factor in the resistance of cotton to fungi, to produce products which were toxic toward *X. campestris*. pv. *malvacearum* (Venere, 1980).

Deleterious roles of iron

The nonenzymatic cleavage of proteins by reactive oxygen species generated by dithiothreitol and iron was reported by Kim et al.(1985). Dithiothreitol (DTT) reduced Fe(III) to Fe(II), which then reduced O_2 to produce superoxide, which upon dismutation could generate hydrogen peroxide. Hydrogen peroxide can then react with Fe(II) to give the hydroxyl radical (HO·) and OH^-, this is called the Fenton reaction. Dithiothreitol is not the only compound which could generate a reactive oxygen species via such a series of reactions. Theoretically any compound whose redox potential is more negative than that of Fe(III) should be able to generate those reactive oxygen species via the Fenton reaction. If DHC has redox potential more negative than Fe(III), then:

$$\text{3DHCred.} + 3Fe^{+++} \text{ ---> } \text{3DHCox.} + 3Fe^{++}$$
$$2Fe^{++} + 2O_2 \text{ ---> } 2Fe^{+++} + 2O_2^{-}\cdot$$
$$O_2^{-}\cdot + H^+ \text{ <---> } HO_2\cdot$$
$$O_2^{-}\cdot + HO_2\cdot + H^+ \text{ ---> } H_2O_2 + O_2$$
$$Fe^{++} + H_2O_2 \text{ ---> } Fe^{+++} + \cdot OH + OH^-$$
$$H^+ + OH^- \text{ ---> } H_2O$$

$$\text{3DHCred.} + O_2 + 3H^+ \text{ ---> } \text{3DHCox.} + \cdot OH + H_2O$$

Of course the available Fe(III) and oxygen are also crucial for the nonenzymatic generation of a reactive oxygen species. In an enzymatic superoxide producing system, single strand breakage of plasmid DNA was inhibitable by superoxide dismutase (SOD), catalase and a Fe^{+2}-chelating agent, implying that the hydroxyl radical is the ultimate damaging species (Brawm et al., 1981). Both gossypol and catechin are reported to generate reactive oxygen species or free radicals at alkaline pH upon autooxidation (dePeyster et al., 1984; Rosin, 1984; Jensen et al., 1983). It seems possible that these compounds may simply act as reducing agents in the presence of oxygen, and with trace amounts of Fe(III) contaminating the experimental solution to produce the hydroxyl radical and increase the pH.

The availability of Fe(III) can be blocked by certain chemically synthesized chelating agents such as ethylenediamine di-(O-hydroxyphenyl) acetate (EDDHA), 2,2'-bipyridyl (Neilands, 1984), or by naturally occurring siderophores. Diethylenetriaminepentaacetic acid (Halliwell, 1978a; Buettner et al., 1978), bathophenanthroline sulfonate (Halliwell, 1978b), and desferrioxamine (Gutteridge et al., 1979) were also used as iron chelators to block the OH· generating reaction and the last is the most effective inhibitor of the three.

Siderophores are defined as low molecular weight, Fe(III)-specific ligands produced by microorganisms as scavenging agents in order to combat low iron stress (Lankford, 1973). The majority of the aerobic and facultative anaerobic bacterial species form siderophores. They are by now firmly established as one very important determinant of virulence for bacterial pathogens of animals (Neilands et al., 1986). The same conclusion has not yet been extended to plants. Several Gram-negative bacterial phytopathogens, e.g. *Agrobacterium*, *Erwinia* and *Pseudomonas* have been reported to produce siderophores or siderophore-like compounds (Leong et al., 1982), but there is no similar report on *Xanthomonas* as yet. The possible roles of siderophores in relation to plant growth and disease have been discussed (Neilands et al., 1986; Swinburne, 1986).

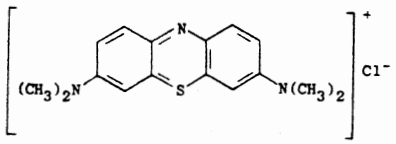
Upon irradiation under anaerobic conditions with light of wavelengths greater than 310 nm, uridine, cytidine, thymidine and guanosine are destroyed in the presence of ferric ions as a photosensitizer (Cernohorsky et al., 1971); tobacco mosaic virus RNA is also inactivated on irradiation in the presence of iron salts (Singer et al., 1965).

Possible mutagenic effects of phytoalexins

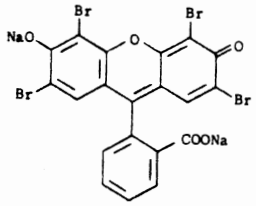
The induction of sister chromatid exchanges in peripheral human lymphocytes by gossypol has been reported (Best et al., 1985). This induction of sister chromatid exchanges and chromosome damage by gossypol suggests that it is a potentially mutagenic and clastogenic agent in murine bone marrow cells (Nayak et al., 1986). Yet gossypol failed to show a mutagenic effect with the Ames test (Majumdar et al., 1982; Lee et al., 1985). The Ames test uses a series of histidine auxotrophs of *Salmonella typhimurium* and measures the induction of reversion caused by the tested compound (Maron et al., 1983). Two possibilities may be responsible for the negative results with gossypol: 1) the strains of *S. typhimurium* used by the previous author were insensitive to the action of gossypol; 2) the addition of histidine in the assay procedure may have masked the action of gossypol. If the mutagenic effect of gossypol involved reactive oxygen species then the following strains are better choices. Strain TA 102 is used to detect oxidative mutagens (Levin et al., 1982, Levin et al., 1984). Strain TA 104 is used to detect the mutagenicity of quinones (Chesis et al., 1984) and carbonyl compounds (Marnett et al., 1985).

Resistance and new proteins

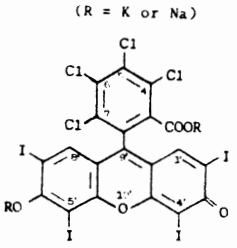
A new membrane protein (21 kD) was rapidly synthesized in *Arthrobacter* cells during photodynamic action of rose bengal (which is structually related to eosin) and methylene blue (Hoober, 1977,1978).



Methylene blue



Eosin



Rose bengal

Generation of singlet oxygen was later found unnecessary for the photodynamic induction of the 21 kD protein (Hoober et al., 1980). However, the binding of sensitizers to DNA at a site that is unusually susceptible to photooxidation was suggested to be responsible for the expression of this gene (Franziet et al., 1985).

The susceptibility of *Pseudomonas aeruginosa* to polymyxin, aminoglycosides, and EDTA can be reduced by growth in Mg(II)-limited culture (Nicas et al., 1980). The effects of cation limitation seem mediated through changes in the cell envelope. It has been shown that Mg-deprived *P. aeruginosa* cells have a significantly increased amount of outer membrane protein H1 of 17 to 18 kD molecular weight. It has been suggested that this protein replaced Mg(II) at a site of a lipopolysaccharide which could otherwise bind cationic antibiotics (Nicas et al., 1980).

Parallelism of antibiotic-animal and phytoalexin-plant relationships

The response of infectious bacteria to antibiotic therapy in human or animal systems depends upon the susceptibility (or resistance) of bacteria to the drug used. From a medical point of view susceptibility of a bacterium to an antibiotic can be defined by any of the following criteria : clinical efficacy, serum antibiotic levels that are effective, population distribution of bacterial susceptibility, or resistance mechanisms. A thorough review is available on this subject (Bryan, 1982).

Some aspects of the relation between antibiotics and bacteria which are relevant to my study will be reviewed. One way of defining susceptibility, as pointed out by Bryan (1982), is based on a comparison of the susceptibility level of an isolate to that of a large population group of the same species or similar species. One could designate population members which are less susceptible as relatively or absolutely resistant. An example is *Neisseria gonorrhoeae* and its response to penicillin. This organism responded well to pencillin over many years. During this period population members with reduced susceptibility to pencillin appeared. These organisms had resistance levels several times those of the most sensitive members of the group and were associated with an increased incidence of treatment failures. However, increasing the dose of penicillin effectively controlled such gonococci. Eventually, a third population group appeared which had resistance levels several times those of the moderately resistant group and could be regarded as a fully resistant group (Bryan, 1982).

Methods for testing bacterial susceptibility to antibiotics fall into three general groups : a) diffusion methods in which antibiotics were applied on the center of a plate which has a lawn of bacteria to be tested and the inhibition zone determined; b) dilution methods in which minimum bactericidal concentrations are determined; and c) detection of resistance mechanisms, e.g. measurement of β-lactamase activity to determine the resistance to penicillin. Since there is a very limited supply of DHC, and the resistance mechanism to DHC is unknown, the dilution method was chosen. Other reasons exist for using the dilution method in the system studied. The major advantages of dilution testing as pointed out by Bryan (1982) are the much reduced effect of bacterial growth rate, a reduced influence of inoculum size, the ability to determine bactericidal concentrations and more readily observed endpoints. Susceptibility of slowly growing organisms or organisms requiring unusual growth conditions are best determined by a dilution test. The components of the growth medium (e.g. pH, high glucose) and incubation conditions could influence test results. In general, an inoculum of 10^5 to 10^6 cfu/ml is widely used.

The minimum inhibitory concentration differences observed between agar and broth with the same medium are most influenced by the higher concentration of cations in the agar. Addition of cation solutions to 60°C agar could reduce the binding of aminoglycoside with agar (Bryan, 1982).

New or additional exopolysaccharides may be formed and the structure of the bacterial cell envelope may be altered. Susceptibility to antimicrobial agents may vary between *in vitro* and *in vivo* growth conditions mainly because of altered rates of antibiotic diffusion through the cell envelope or by the reduced availability of antibiotic transport sites or targets (Bryan, 1982). *In vivo* growth conditions are unlikely to be associated with a single specific change in bacterial structure or metabolic activity. Rather, effects are a complex interaction of changes in the permeability of the cell-envelope and in the activity of energy-dependent transport and the target structures (Bryan,1982).

The three major sets of conditions which influence the *in vivo* susceptibility of bacteria to antibiotics in animal systems are 1) the concentration of the active drug within the infection site; 2) the capability of host defenses to prevent spread and to eradicate the infecting organism; 3) the bacterial phenotype resulting from growth in the host. The parallel conditions which may influence the *in vivo* susceptibility in the cotton system are 1) production of the active antibiotics (phytoalexins) by plant cells at the infection site in incompatible interactions to effective concentrations (Essenberg et al., 1986); 2) correlation of the presence of phytoalexins with the inhibition of bacterial growth (Shevell, 1985); 3) unknown bacterial phenotype in terms of DHC resistance resulting from growth in cotton in an incompatible interaction.

EPS & LPS of phytopathogen

Erwinia stewartii which causes leaf blight and vascular wilt of corn, is known to produce an enormous amount of extracellular polysaccharide (EPS) both as slime and as tightly bound capsule. Some mutants deficient in EPS production cause water soaking but

not systemic wilting. Two other mutants produced only occasional small lesions that were not water soaked (Panopoulos et al., 1985). These mutants were found to be *gal* E (UDP-Gal-4-epimerase deficient) and had greatly reduced galactose content in their lipopolysaccharide (LPS) (Panopoulos et al., 1985).

Another phytopathological bacterium *Pseudomonas solanacearum*, has also received much attention in terms of the EPS and LPS production in infected plants with respect to pathogenicity (Panopoulos et al., 1985). Evidence suggests that the LPS of *P. solanacearum* is not an inducer of HR but may serve as an attachment molecule to plant cell surfaces and thus indirectly contribute to the elicitation of HR. Mutants of *P. solanacearum* that were avirulent and have rough (incomplete) LPS that lacks all or part of its O-antigenic sidechains were also noticed (Panopoulos et al., 1985). Many avirulent mutants being studied are often extremely pleiotropic and these pleiotropic phenotypes are not due to a loss of plasmid (Panopoulos et al., 1985). EPS was found to inhibit attachment of *P. solanacearum.* mutant tobacco. The normal attachment in this system occurs apparently in two stages (Young et al., 1986).

Virulent mutants-plasmid, Insertion Element (IS) involvement

The theory of plasmid macroevolution was first described and defined by Cohen et al. (1978). They suggested that plasmids undergo evolution by large DNA rearrangements and further that some of these rearrangements might be mediated by transposable elements. DNA rearrangement in drug resistance plasmid was accounted for by the action of transposable elements (Rubens et al., 1979). During nine years, descendents of the index plasmid, which was responsible for an epidemic of gentamicin resistance, had suffered DNA rearrangement with a persistent molecular polymorphism (Lee et al., 1984).

Three different repetitive sequences (RS-I, II and III) have been described in *P. syringae* pv. *phaseolicola*, with multiple copies of each sequence on a single plasmid, pMC 7105 (Quant et al., 1984, Szabo et al., 1984a, b). At least 20 copies of RS-II, 13 copies of

RS-III but no copies of RS-I were also present on the chromosome of various strains. RS-II apparently serves as the primary site of plasmid integration and excision with pMC 7105 (Curiale et al., 1982), either through a general (rec-dependent) recombination or by specialized recombination mechanisms (Panopoulos et al., 1985). These episomal properties (integration and excision) have long been known for the F' plasmid in *E. coli* and have also been documented in *Erwinia* spp. (Panopoulos et al., 1985). Plasmids involved in resistance to toxic chemicals e.g. antibiotics are well known. However, for bacterial phytopathogens only copper resistance in *X. campestris* pv. *vesicatoria* (Stall et al., 1984) and arsenite/arsenate/antimony resistance in *Corynebacterium flaccumfaciens* subsp. *oortii* (Hendrick et al., 1984) are known to be plasmid-associated. Plasmids involved in pathogenicity of bacterial phytopathogens were extensively studied with the Ti plasmid of *Agrobacterium ttumefaciens.*

High-frequency spontaneous mutation in the bacterio-opsin gene in *Halobacterium halobium* are mediated by transposable elements (Das Sarma et al., 1983). In the same system, the authors also observed drug-resistant mutants at a frequency of 10^{-7} and they suggested the different mutation frequencies may reflect different underlying mechanisms such as point mutations, insertions of transposable elements and other rearrangements. *X. campestris* pv.*vesicatoria* loses the hypersensitive response-inducing avirulent phenotype in pepper (*Capsicum annuum*) at a rate of 4×10^{-4} virulent mutants per cell per division (Dahlbeck et al., 1979). This high rate of mutation for change of race did not occur with the same organism in change in resistance to streptomycin (1.9×10^{-9} mutants/cell/division). It has been suggested that a 0.5 kb insertion element was involved in this race change (Staskawicz, 1986).

A cluster of genes involved in pathogenicity of *X. campestris* pv. *campestris* , spanning a region of approximately 10 kb was reported by Turner (1985). A gene cluster which controls pathogenicity of bean plants and hypersensitivity on non-host plants was also reported with *P. syringae* pv. *'phaseolicola'* (Lindgren et al., 1986). A hypermutable

locus has been identified that controls virulence (wilt induction) and several other properties in *P. solanacearum* (Kelman, 1954).

Genes that appear to be necessary for pathogenicity have been identified in many phytopathological bacteria (Panopoulos et al., 1985). Avirulent mutants studied thus far can be grouped according to the phenotype into three catogories : (a) Mutants avirulent on their host but retaining the ability to elicit the hypersensitive reaction (HR) on nonhost plants. (b) Mutants avirulent on their host which failed to elicit HR on one or more nonhost plants. (c) Mutants showing reduced levels of virulence on hosts but unable to induce HR on nonhosts (Panopoulos et al., 1985). When the differential hosts were available, the reactions of these mutants were not cultivar specific (Panopoulos et al., 1985). DNA fragments restoring the mutant phenotypes were reported for several pathogens. These genes appeared to be present in diverse bacterial pathogens and in cluster form to control the phenotype. Southern blot hybridization studies further suggested part of this cluster was conserved (Panopoulos et al., 1985). In the well-studied pathogen *X. campestris* pv. *campestris* both protease and polygalacturonate lyase appeared to be responsible for the recovery of pathogenicity (Daniels et al., 1984).

CHAPTER III

MATERIALS AND METHODS

DHC

Chemically synthesized DHC was a gift of Dr. R. D. Stipanovic. Except where otherwise specified, all work with DHC was carried out under ultraviolet-deficient illumination from General Electric F06T12/GO gold fluorescent lamps to avoid photodecomposition. Aliquots of DHC in HPLC-grade methanol were transferred to polyethylene tubes and evaporated to dryness with a nitrogen gas stream.

Inactivation of MDH by DHC

MDH was exposed to DHC at different concentrations or for different lengths of time as specified. The shortest period of incubation was obtained by addition of 1µl of MDH (62.5 µg $ml^{-1)}$ to the dry DHC and immediate transfer of this mixture into the 1.0 ml enzyme assay mixture (Section I). The estimated time of contact of MDH with DHC was less than 1 min. The light treatment was done under the same condition as described in Section I, pg 5. In a separate experiment a portion of the data was used to statistically investigate the effect of light plus DHC on MDH (Table II). The data were analyzed by analysis of variance (ANOVA) using the Statistical Analysis System (SAS). The data of 0 h incubation were omitted in the analysis due to the inherently unbalanced data (0 h in the dark = 0 h in the light).

Effects of iron on DNA nicking by DHC

A solution of 0.05 mM $Fe_2(SO_4)_3$ was used to dilute the concentrated plasmid pBR322 DNA. An anoxygenic condition was created by bubbling argon through the 1 ml iron solution for 2 minutes in an argon-filled (5 minutes deaeration) chamber and preparing the diluted plasmid solution in the same chamber. Controls were prepared as described in Section I. Approximately 0.15 μg of plasmid DNA per treatment was used. Light treatment was for 2.5 h, and dark controls were incubated under the same conditions except that the reaction vials were wrapped with aluminum foil.

Cotton lines

Acala 44 (Ac44) possesses no major genes for resistance to Xcm and is susceptible to all 18 known races of this pathogen. Acala B_1 (AcB_1), Acala B_2 (AcB_2), Acala B_3 (AcB_3), Acala B_4 (AcB_4), Acala B_5 (AcB_5), Acala B_6 (AcB_6), Acala b_7 (Acb_7), and Acala B_N (AcB_N) are congenic host lines each of which contains one major gene for resistant to Xcm in the Ac44 background (Brinkerhoff, 1970). Other differential lines Stoneville 2B-S9 (polygenic complex B_{Sm}), Stoneville 20 (b_7 and polygenes), 1-10B (B_{In} and polygenes) and Gregg (unknown) (Brinkerhoff, 1970) were also used in some experiments. OK1.2 possesses major resistance genes B_3 and b_7 (more detailed description of this line is in Pierce et al., 1987).

Plants

Three to five acid-delinted seeds were planted < 1/2 inch deep in a commercially prepared mixture of peat moss and vermiculite (Jiffy Mix Plus) in 6-inch clay pots. The plants were watered daily and fertilized weekly with aqueous Stern's Miracle Grow. Plants at six weeks of age or younger were kept in growth chamber 1 (Percival) set for a 14 h light, 10 h dark cycle with an average temperature of 30°C during the light period and 23°C during the dark period. The plants were then moved to growth chamber 2 (Conviron) set

for a 14 h light, 10 h dark cycle with ramped temperature program from maximum temperature of 30°C during the light period to minimum temperature of 23°C during the dark period. Growth chamber 3 (Percival) which is located in the Life Sciences West Building was used as an overflow chamber and the conditions were set as for chamber 1. The differential lines were moved on July 24, 1986 from chamber 2 into chamber 3. The pots in chamber 2 and chamber 3 were adjusted individually so that the youngest leaf of the tallest plant was approximately one foot from the light source. The light intensity at the experimental leaves was approximately 3 x 10^3 lux in both chambers 2 and 3. The second, third and/or fourth youngest well-expanded leaves of plants which were older than five weeks were used in this work. Plants were arranged so that the inoculated leaves were not shaded by other leaves. The apex of some of the plants were pinched off to maximize the expansion of the experimental leaves in virulent mutant selection studies, and the newly emerged lateral buds were removed constantly during the experiment.

Bacteria

Principal of the strains used in this study are presented in Table I. Mutants derived from these strains are described in the results section. Either an isolated single colony or a small piece of frozen bacterial stock from a cryovial (stored at -70°C) was used to start a culture. Bacteria were cultured in Difco nutrient broth (NB) at 30°C with agitation at 180 rpm overnight for futher usage. The standard bioassay (as described in Section I) was followed with the exception of the location of the incubation which was in a 30°C room with 180 rpm agitation.

Inoculation

Each experimental leaf was enclosed in a 4 x 6-inch plastic bag for at least 1 h to ensure that the stomates were open. The inoculum was then forced through the lower epidermis by a blunt-ended syringe without pricking the leaves (Essenberg et al, 1982) or

TABLE I

STRAINS OF XCM USED IN THIS WORK.

Strain	Origin	Drug Resistance	References
Race 1		streptomycin	Essenberg, 1982
Race 2	Race 1	streptomycin	Brinkerhoff, 1970
Race 3*		streptomycin	Essenberg, 1982
Race 4	Race 1		Brinkerhoff, 1970
Race 7			Brinkerhoff, 1970
Race 10			Brinkerhoff, 1970
H	Race 4		Gabriel et al., 1986
N	Race 2		Gabriel et al., 1986
RN1**	N	rifampicin	This work
RS4**	Race 3	rifampicin, streptomycin	This work
RS8**	Race 3	rifampicin, streptomycin	This work
JH	Race 3	streptomycin	Shevell, 1985
MP	Race 3	streptomycin	Shevell, 1985

Rifampicin: 100 ppm; Streptomycin: 200 ppm.
* cryovial dated 6-19-84
** strain recovered from rifampicin containing nutrient agar at frequency of 10^{-8}.

by a high pressure (80-90 psi) sprayer (Campbell Hausfeld). During the syringe inoculation the leaf was held against at least four layers of tissue paper to absorb the overflow inoculum. Macroscopical wounding was not tolerable nor detectable.

Bioassay *in vitro* or *in planta*

All the *in vitro* bioassays for the toxicity of DHC in this work were done in nutrient broth (NB), and logarithmically growing bacterial cultures were used. Unless specified otherwise, the treated group was exposed to 0.1 mM DHC for 8 h, and the control group was incubated for 8 h without DHC. This is referred to as the standard bioassay. Independent duplicate vials were used for both control and treated groups. In time course experiments, duplicate vials were plated at each specified time. All the preparations for the *in vitro* bioassay were done in the laminar flow hood with indirect dim yellow light (Bug.away, Westinghouse) which was reflected from the wall of the lab.

Bacterial growth *in planta* was determined by excising from the inoculated leaf three 1/3 cm^2 discs and homogenizing them together in sterile saturated $CaCO_3$ solution. Appropriate dilutions were then plated on rifampicin-containing NA.

DHC^R mutant selection in DHC-medium

Selection for DHC^R mutants was carried out for (1) approximately 8 h, (2) more than 8 h but less than 24 h, or (3) more than 24 h. Mutants derived from strain RS4 by spontaneous mutation and UV mutagenesis were isolated and tested for their DHC resistance. Spontaneous DHC^R mutants were obtained from the survivors of the DHC treatment. Both UV (Miller, 1972) and nitrosoguanidine mutagenesis were used and the survivors were then subjected to the DHC selection. Bacteria were subjected to DHC in a liquid medium during the selection and were then plated on nutrient agar (NA) without DHC. The survivors picked from these plates were subjected to further selection.

To prevent the possible reversion to DHC sensitivity due to lack of selection pressure between two selections, a second selection strategy was developed. An appropriate dilution was made at the end of each liquid culture selection and immediately subjecting these survivors to the next selection vial . Three independent selections were carried through eight transfers of this kind for approximately 10 days in an attempt to select DHC^R mutants. The resistance of bacteria to DHC is expressed as resistant population (%) which is defined as follows:

$$\text{Resistant population (\%)} = \frac{\text{Final treated population (cfu/ml)}}{2^n \times \text{Initial population (cfu/ml)}} \times 100\%$$

n : Generations of untreated bacteria during bioassay.

DHC^R mutant selection in EMB medium

RS4, RS8 and H were used as parent strains to isolate spontaneous mutants. Nutrient agar containing eosin (E) at 1 g/l, methylene blue (MB) at 0.15 g/l or both are designated E, MB, or EMB plates, respectively. Commercial EMB medium (Difco) which contained 0.4 g/l of eosin and 0.065 g/l of methylene blue was also used and designated EMBa. The selection for mutants that were resistant to these dyes was done under white fluorescent light at intensity of 2×10^2 lux. The surviving bacteria were then inoculated into NB. After overnight culture they were then stored at -70°C in NB containing 16% glycerol. The DHC resistance was tested at 0.1 mM for mutants derived from RS4 and RS8 and at higher concentrations (0.2, 0.5 mM) for the mutants derived from H. The resistant population (%) was used to express the resistance of bacteria to DHC. Xcm resistance to DHC plus light was determined under white fluorescent light at an intensity of 4×10^2 lux. Growth of one of the mutants (EMB7) in Ac44, AcB_3 and OK1.2 during a 10 day period was also determined.

One tenth of a milliliter of 2 x 10^9 cfu/ml of RS4 was spotted on the centers of the E, MB, EMBa, and NA plates. After several days incubation under light, patches of bacteria were harvested and washed with NB two to three times to get rid of the excess dyes. The washed bacteria were used directly to test for DHC resistance without subculturing in NB. The survivors of the bioassay were then picked from the NA plates and subjected to a second bioassay.

NB containing 0.1 mM $MnSO_4$ was used as the condition to enhance the SOD of Xcm. Same medium was used to start a bacterial culture and test the possible enhancement of DHC resistance of RS4, RS8 and EMB81/RS8 (a EMB^R mutant derived from RS8).

DHC^R mutant selection in an iron-chelated medium

Sequestrene138 Fe (CIBA-GEIGY), 2,2'-bipyridyl (Sigma) and $Fe_2(SO_4)_3$ (J. T. Baker Chemical Co.) were dissolved in water and filter-sterilized. These solutions were added to nutrient broth (NB) for determination of their effects on the growth rate of Xcm. Nutrient agar (NA) containing sequestrene138 Fe (2 mM to 3.5 mM) or 2,2'-bipyridyl (0.1 mM to 1.0 mM) was used to select mutants. Bacterial growth was monitored either by the change of OD_{600} with a spectrophotometer or by the standard bioassay. The absorption spectrum of 2,2'-bipyridyl was obtained with a spectrophotometer (Hitachi 100-80A). Strains RS4, EMB4 and H were used for mutant selection. Mutants derived from RS4 were assayed at 0.1 mM DHC and mutants derived from H and EMB4 were assayed at 0.2 and 0.5 mM DHC.

Does DHC cause the conversion of O to T-type Xcm?

T-type Xcm were identified as transparent (T) colonies when the plate was transilluminated with an incandescent light. Race 3 was used in these experiments. Both time course and dose response curves were obtained. In the experiment to test if dilution of DHC at various times would prevent further enhancement of the T population, 10-fold

dilutions with NB were made aseptically at each specified time. The percentage of T-type bacteria was recorded. Single colonies of both morphologies of Race 3, before and after exposure to DHC, were also tested for their DHC resistance.

T-type Xcm characterization

RS4 from a cryovial or as a single colony was used to start a culture for determination of the frequency of occurrence of T-type bacteria. Xanthan gum (Sigma) at 0.125 - 1% was used in conjunction with the bacteria to observe the possible varying of the vir/avir response of the plants by syringe inoculation. Overnight cultures of T2 and O2 from cryovials were used to obtain the bacterial growth in Ac44 and AcB_3 with 10^4 - 10^5 cfu/ml in the initial inocula. The *in planta* bacterial growth was obtained in cfu/cm^2 (Essenberg, 1979). A NA plate containing 1% skimmed milk was used to check the protease activity of O2 and T2. The total bacterial protein was obtained by freezing and thawing, then denaturing in 5 % mercaptoethanol in boiling water bath for 3 min. Protein concentration was determined as described by Bradford (1976) . Proteins were then separated in a denaturing gel with 10% polyacrylamide or a 10 to 20% gradient gel. The protein bands were visualized with Coomassie blue staining, and the relative intensities of the bands were determined with a gel scanner (Helena Lab.). The T2 and O2 overnight cultures were diluted in sterile $CaCO_3$ saturated solution to approximately 3 x 10^3 cfu/ml, then spray-inoculated into Ac44 and AcB_3. The bacteria-plant interaction was observed macroscopically with the naked eye or microscopically with transmitted white light and epifluorescence. Photomicrography were performed with a Nikon Optiphot biological microscope equipped with episcopic-fluorescence capability using a mercury lamp (12 V-100W) and B2 filter cassette (IF460-485 excitation filter, DM510 dichroic mirror, and 520W emission filter) coupled with a -550IF auxiliary red suppresion filter. Similar cultures of T2 and O2, diluted to approximately 2 x 10^7 cfu/ml, were also used to check their response in Ac44, AcB_3, OK1.2, and Im216 by syringe inoculation. Cultures of T2

and O2 in Luria broth (LB)(Miller, 1972) containing 0.5% sucrose or diluted with a saturated $CaCO_3$ solution containing 0.5% sucrose were also used to determine the possible change of Xcm/plant interaction.

Virulent mutant isolation

Logarithmically growing RS4 was diluted with sterile saturated $CaCO_3$ solution to prepare a bacterial suspension (5×10^3 cfu/ml) and sprayed into incompatible cotton lines AcB_1, B_2, B_3, B_4, B_5, B_6, b_7, B_N, Stoneville 2B-S9 and Gregg. For some of these inoculations, replicate cultures were grown from separate single colonies of RS4. Seven to fourteen days later single incompatible or compatible lesions were macroscopically visible (Essenberg et al., 1979) and were counted. Bacteria were reisolated from some well-isolated compatible lesions by removing tissue from the spot with a pair of sterile tweezers or by dissecting and homogenizing the complete compatible lesion in sterile $CaCO_3$ solution, making an appropiate dilution, and then immediately transferring it to a rifampicin-containing NA plate and streaking for single colonies. After two more sequential single colony isolations, a single colony was picked, cultured in NB overnight and stored at -70°C in NB containing 16% glycerol. The frequencies of spontaneous mutation in regard to race specificity in differential cotton lines were the average of at least two independent experiments with the exception of Acb_7, Stoneville 2B-S9 and Gregg.

Mutant phenotype check in differential cotton lines

Logarithmically growing bacteria were diluted in sterile saturated $CaCO_3$ solution to approximately 5×10^6 cfu/ml. Young, fully expanded foliage leaves were used to test the race specificity of mutants. Syringe spot inoculation was applied in these experiments. A maximum of fourteen different isolates were inoculated on the same leaf with two spots for each isolate. At least two independent plants of each cotton line were tested. The inoculated plants were kept in a growth chamber. Symptoms were scored at 6, 7, 8 and 9

days post-inoculation. Virulence reaction was determined on a scale of 0-4. Lesion scores of 4 had completely confluent water-soaking in the inoculated area. Lesion scores of 3 to 3.5 had 0.02 to 0.05 mm diameter non-water-soaked spots scattered in the water-soaked inoculated area. Lesion scores of 2 - 2.5 had 0.05 mm diameter of confluent spots within the inoculated area. Lesion scores of 0 - 1.5 had no speckles or some water-soaked speckles (< 0.02 mm in diameter) within the inoculated area. The lesion scores of 3 or greater are designated compatible interactions, and scores less than 2 are designated incompatible interactions.

Analysis of plasmids of race-changed mutants

Analytical amounts of plasmid DNAs of Xcm were isolated by a procedure described by Kado and Liu (1981). They were then electrophoresed in 0.4% agarose gels with TAE as a running buffer. The gel was then stained with EtBr for 30 mins and viewed under long wave UV light. A photograph was taken through a No. 23 Wratten filter (Kodak). The plasmids derived from the parent RS4 were called pTJS, the next letter or number indicating the resistance gene of the Acala cotton line from which the mutant was recovered and the last being a serial number for the specific isolate, e.g. pTJS401, the first isolate from AcB_4. Size-changed plasmids pTJSn03, 06, 12, 13, 17 and 19 were further characterized with restriction enzymes HindIII, SalI, BamH1, EcoRI, PstI and SstI. The fragmented plasmids were electrophoresed in 0.7% agarose gels with TAE as a running buffer. The same procedure was followed as with the intact plasmid. The size of each fragment was estimated by interpolation from standards of known size.

Molecular genetic techniques

E. coli strains with cloned avirulence genes against AcB_3 and AcB_N were provided by D. W. Gabriel, where pUFA-717 has $AvirB_3$ and pUFA-H1 has $AvirB_N$ (Gabriel et al., 1986). Plasmid DNA from *E. coli* was isolated by alkaline lysis as

described by Maniatis et al. (1982) and preparative amounts of plasmid DNA from Xcm were isolated as described (Gabriel et al., 1986). The total chromosomal DNAs of Xcm were extracted (Gabriel et al., 1986) and digested to completion by EcoRI, BamH1 or SalI. These digests were then electrophoresed in separate tracks on a 0.65% agarose gel with TAE as a running buffer and blotted onto nitrocellulose (Maniatis et al., 1982). Plasmid DNAs of pUFA-717, pUFA-H1 and RS4 were nick translated in the presence of α^{32}P-deoxycytidine triphosphate and used to probe the blots (Mackey et al., 1977). An autoradiograph was then obtained. When rehybridization was needed, the first probe was removed as described (BIOTRANS). The sizes of the fragments that hybridized to the probe were estimated in the way described in the previous paragraph.

CHAPTER IV

RESULTS

Inactivation of MDH by DHC

Instantaneous inactivation of MDH in the dark by DHC was observed and showed a concentration dependence (Figure 1). This loss of MDH activity was not reversed by the 1000-fold dilution made prior to assay of the treated enzyme. A 30% loss of activity from 0 to 0.3 mM DHC and an additional 10% loss from 0.3 mM to 1 mM DHC was observed. The treatment effects of light or DHC were both significant at the 0.01% level (Table III). Bigger difference between light and dark at 0.025 mM than at 0 and 0.05 mM DHC was observed (Table II, III). The apparent activity of MDH increased with the time of incubation (at 0.01% level). The inactivation of MDH by light (1.5 h) plus DHC (0.025 mM) was reproducible (Table 1, Section I).

Effects of iron on DNA nicking by DHC

Nicking of DNA by DHC alone in the dark has not been observed (Sections I, II). The possibility that Fe(III) can potentiate DHC to nick DNA was explored. In the dark, in the presence of both Fe(III) and DHC, there was significant nicking of DNA (lane 6, Figure 2). This nicking reaction was prevented by argon treatment. In the presence of Fe(III) alone, only a minor increase in the linear form of the plasmid was observed (lane 5, Figure 2). OC was the form that is most susceptible to Fe(III) plus DHC, where CC was the most susceptible form of the plasmid nicked by photoactivated DHC. In the light, in the presence of both Fe(III) and DHC, nicking was extremely extensive so that only very small fragments were left after the treatment (lane 8, Figure 2). Again the argon treatment

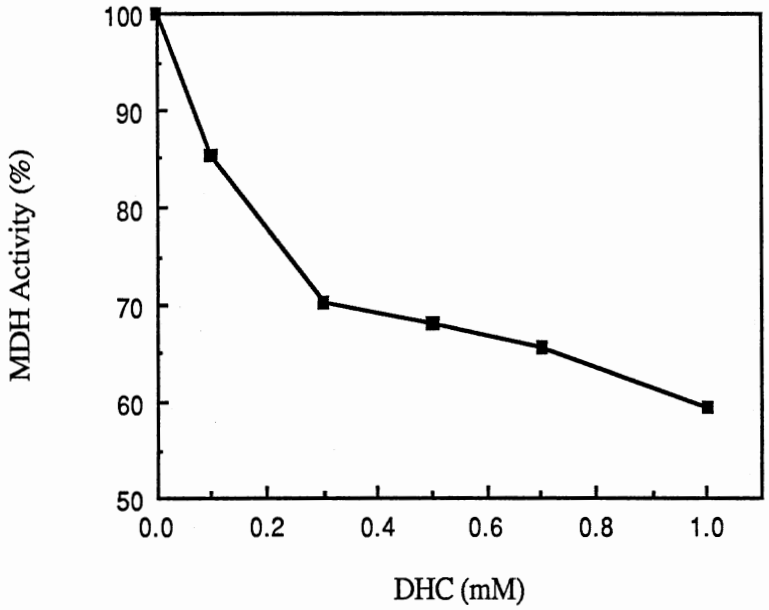


Figure 1. Instantaneous inactivation of MDH by DHC in the dark.
MDH activity at various DHC concentrations was expressed relative to MDH activity in the absence of DHC. Each point was an average of 14 (0.0 mM DHC) or six replicates (others). One µl of 62.5 $\mu g ml^{-1}$ of MDH was used in each replicate and enzyme reaction volume was 1 ml.

TABLE II

EFFECT OF LIGHT AND DHC ON ENZYMATIC ACTIVITY OF MDH.

	Dark, DHC (mM)			Light, DHC (mM)		
	0	0.025	0.05	0	0.025	0.05
Time (h)						
0.0	100	81	70	----	----	----
1.5	114	101	96	104	47	78
3.0	138	147	116	160	79	88

Control contained 0.035 units ml^{-1} of enzyme. Values are expressed as percent of dark, time 0 h, no DHC control and are means of six replicates. One µl of 62.5 $\mu g ml^{-1}$ of MDH was used in each replicate and enzyme reaction volume was 1 ml. One unit of enzyme activity caused the oxidation of 1 µmole NADH per minute at room temperature.

TABLE III

ANALYSIS OF PARTIAL DATA OF TABLE II WITH SAS (ANOVA).

12:03 TUESDAY, NOVEMBER

ANALYSIS OF VARIANCE PROCEDURE

CLASS LEVEL INFORMATION

CLASS	LEVELS	VALUES
TIME	2	3 1.5
LIGHT	2	D L
CONC	3	0 0.05 0.025

NUMBER OF OBSERVATIONS IN DATA SET = 66

==>

SAS

12:03 TUESDAY, NOVEMBER

ANALYSIS OF VARIANCE PROCEDURE

DEPENDENT VARIABLE: Y

SOURCE	DF	SUM OF SQUARES	MEAN SQUARE
MODEL	11	0.11444547	0.01040413
ERROR	54	0.04180703	0.00077420
CORRECTED TOTAL	65	0.15625250	

R-SQUARE	C.V.	ROOT MSE	Y MEAN
0.732439	19.2557	0.02782453	0.14450000

SOURCE	DF	ANOVA SS	F VALUE	PR > F
==>				
TIME	1	0.02934855	37.91	0.0001
LIGHT	1	0.01972547	25.48	0.0001
TIME*LIGHT	1	0.00003336	0.04	0.8363
CONC	2	0.03417855	22.07	0.0001
TIME*CONC	2	0.00428035	2.76	0.0719
LIGHT*CONC	2	0.02360048	15.24	0.0001
TIME*LIGHT*CONC	2	0.00327870	2.12	0.1302

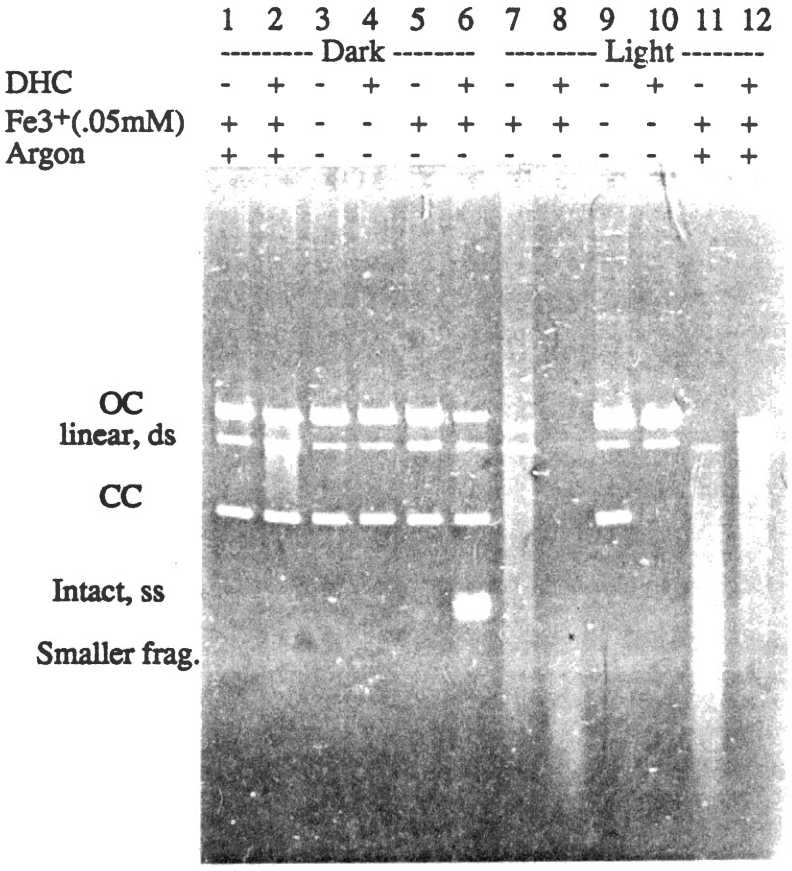


Figure 2. Ferric activation of DHC nicking of DNA.

Plasmid DNA was diluted with or without 0.05 mM $Fe_2(SO_4)_3$ then incubated with or without 0.7 mM DHC or argon or illumination prior to analysis by gel electrophoresis.

reduced the nicking but did not block it completely (lane 12, Figure 2). Fe(III) alone was capable of nicking the DNA in the light (lane 7, Figure 2) and the argon treatment enhanced this nicking (lane 11, Figure 2).

Variation of resistance of different strains

Race 1, Race 2, Race 4, strain H, strain N, strain JH, and strain MP were most resistant to DHC. After a 2 to 3 h lag period, they started to multiply logarithmically (Figure 3-a, b). Race 3 was partially resistant to DHC. During the first 2 h bacteria were killed but later the bacteria grew logarithmically (Fig 3-b). RS8 and RS4 were the most sensitive to DHC; after significant killing during first 2 h, DHC was bacteriostatic to RS8, but remained bacteriocidal to RS4 (Figure 3-b).

DHC^R mutant selection in DHC medium

Direct selection of mutants in DHC-containing NB was used as the first strategy in the selection of DHC^R mutants. In a time course of exposure to 0.1 mM DHC, a 10^4-fold killing of RS4 during the first 8 h was observed, but a multiplication of Xcm was seen from 8 h to 24 h (Figure 4). Based on the killing curve of UV treatment (Figure 5), Xcm surviving a 120 $\mu J/mm^2$ sec irradiation were used for mutant selection. Both spontaneous and UV mutagenesized putative DHC^R mutant(s) showed instability of DHC resistance (Table IV, V). The increase in the resistant population during early selections was not retained in later selections (Table IV, V). Continuous selection pressure enhanced the resistant population in a more stable fashion with a final resistant population reaching 42 - 63% (Table VI). Yet two distinct colony morphologies were observed among the survivors of this final selection: one was opaque (O) and the other was smaller and transparent (T).

Mutant A1 was more resistant to DHC than RS4 as revealed in the dose response curves (Figure 6). *S. typhimurium* (TA98) was more sensitive than *E. coli* (JC10240),

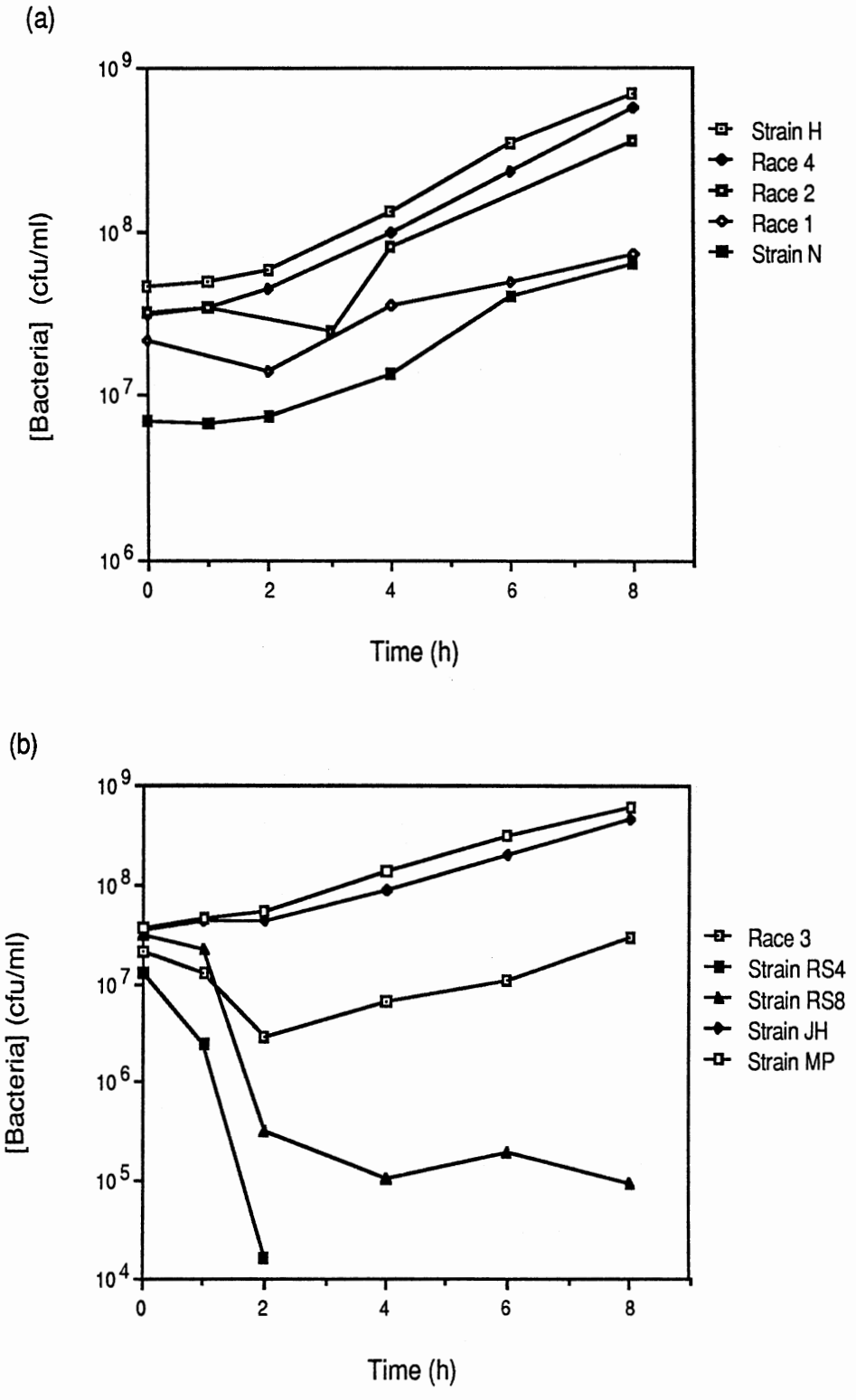


Figure 3. DHC resistance of 10 different strains of *X. c.* pv. *malvacearum.*

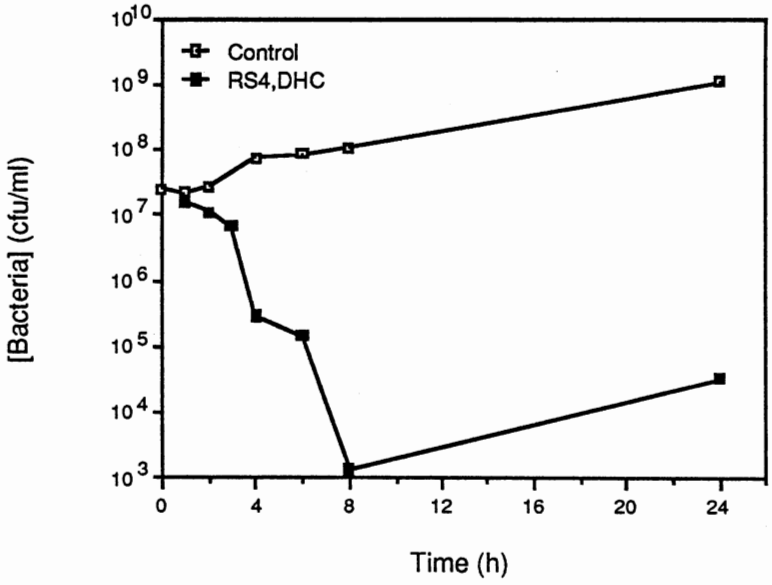


Figure 4. Resistance of RS4 to DHC during 24 h period.

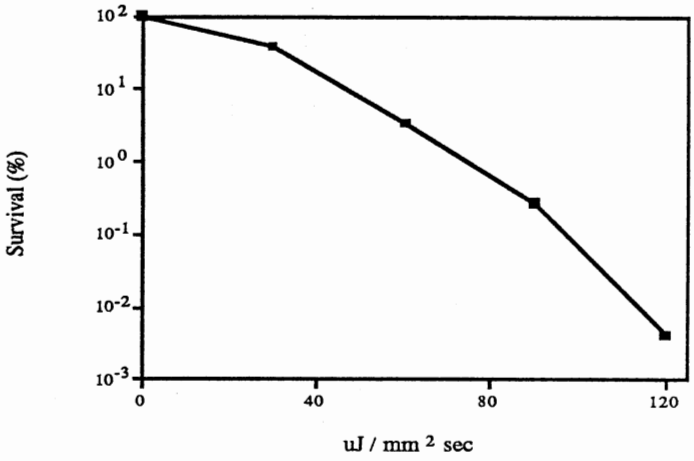


Figure 5. UV survival curve of *X. c.* pv. *malvacearum* (RS4).

TABLE IV

CONSECUTIVE BIOASSAYS OF DHC RESISTANCE OF SPONTANEOUS DHC^R MUTANTS.

	Resistant population(%) / DHC (mM) / T*			
	1st	2nd	3rd	4th
mutant				
A1	0.065/0.10/1	1.16/0.15/3	0.65/0.25/3	0.142/0.17/3
A2	0.004/0.15/2	1.6/0.15/3	0.0/0.25/3	-----
A3	0.001/0.15/3	2.1/0.15/3	0.057/0.25/3	-----
A4	0.065/0.10/1	8.5/0.15/3	0.042/0.25/3	-----

*T is bioassay time 1: 8 h; 2: between 8 and 24 h; 3: ≥ 24 h.

TABLE V

CONSECUTIVE BIOASSAYS OF DHC RESISTANCE OF UV-INDUCED MUTANTS.

	Resistant population(%) / DHC (mM) / T*			
	1st	2nd	3rd	4th
mutant				
UV401	TNTC/0.15/1	0.56/0.15/1	57.8/0.15/1	0.0/0.15/2
UV402	TNTC/0.15/1	0.61/0.15/1	85.4/0.15/2	0.01/0.15/2
UV403	TNTC/0.15/1	0.26/0.15/1	31.5/0.15/2	0.12/0.15/2
UV404	TNTC/0.15/1	0.19/0.15/1	8.0/0.15/2	0.16/0.15/2
UV405	TNTC/0.15/1	0.0/0.15/1	1.3/0.15/2	0.250/.15/2

* T is bioassay time; 1: 8 h; 2: between 8 to 24 h; TNTC:Too numerous to count.

TABLE VI

DHC RESISTANCE OF *X. C.* PV. *MALVACEARUM* UNDER CONTINUOUS DHC SELECTION PRESSURE.

	Resistant population (%)							
	1st	2nd	3rd	4th	5th	6th	7th	8th*
Vials								
1	0.01	TNTC	TNTC	12.9	10.4	0.445	23.7	42.0
2	0.01	TNTC	TNTC	21.0	6.6	0.08	6.7	62.7
3	0.007	TNTC	TNTC	14.4	7.3	0.175	28.7	45.2

Assays 1 thru 4 were at 0.1 mM and 5 thru 8 were at 0.15 mM DHC.
TNTC: Too numerous to count.
* Two distinct morphologies was observed and named as O1, T1, O2, T2, O3 and T3 with the survivors where "O" means opaque, "T" means transparent.

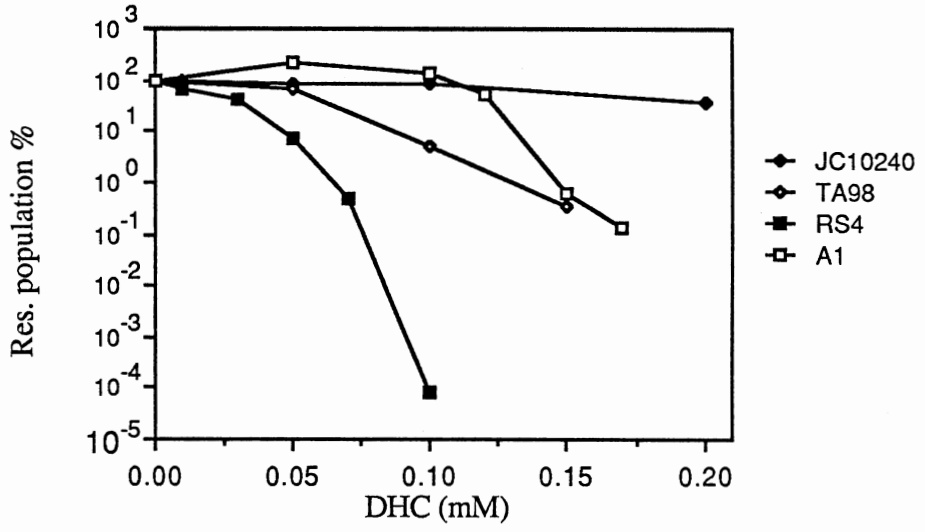


Figure 6. DHC sensitivity of A1, RS4, *E.coli* (JC10240) and *Salmonella typhimurium* (TA98).

but more resistant than RS4 to DHC (Figure 6). DHC was more toxic in the light than in the dark to RS4 (Figure 7).

Spontaneously occurring T-type bacteria from RS4 were more resistant to DHC than the O-type (Table VII). But the O-type survivors under the continuous selection pressure were more resistant to DHC than the T-type (Table VII); and the degree of resistance to DHC of the T-type was not enhanced after the 10 day incubation in the DHC-containing medium (Table VII). After 14 days incubation in Ac 44 leaves, O2 lost its DHC resistance and T2 maintained the same level of resistance (Table VII). However, after 14 days incubation in OK1.2 leaves, O2 and T2 (to a lesser degree than O2) both retained their DHC resistance. One revertant recovered from T2 in the OK1.2 was opaque and it had DHC resistance similar to an O2 (Table VII).

DHC^R mutants selection in EMB medium

The photodynamic action of DHC as described in Sections I, II suggested the second strategy for the selection of DHC^R mutants, which was to use commercially available photodynamic agents. The bacteria recovered from E, MB or EMBa plates were more resistant to DHC than those from NA plates (Table VIII). The DHC resistance was retained in the 2nd bioassay, regardless of the exposure to DHC during the 1st bioassay with bacteria recovered from dye-containing plates (Table VIII). All of them were consistently more resistant to DHC than the bacteria from NA medium that were never exposed to the dyes, indicating that the Xcm which survived from any of the dye-containing medium are indeed stable mutants. The results also indicate that the DHC resistance of these mutants is not simply due to physiological adaptation to dye in the first place. Single-colony isolates from the dye-containing media had a higher percentage resistant population than the parent strains (Table IX). Independent mutants recovered from the EMB plate were more resistant to DHC than the parent strain in both light and dark (Table X). Mutant EMB4 was more resistant to DHC at low concentration (0.2 mM)

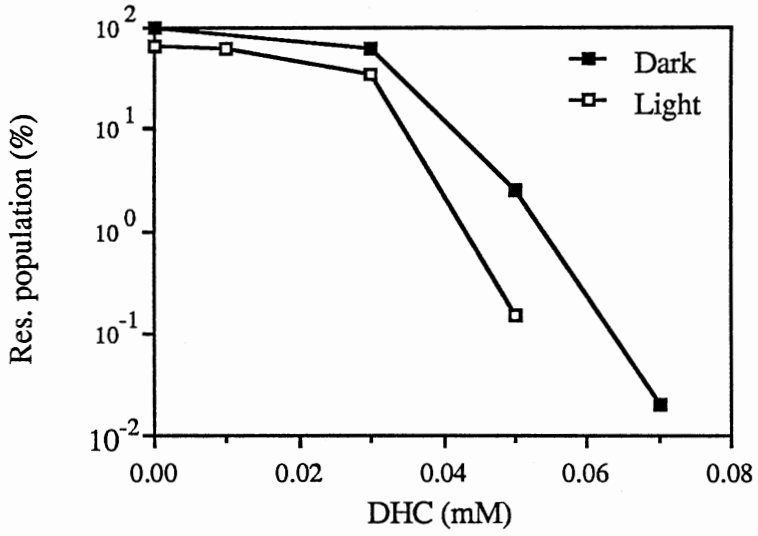


Figure 7. The effect of light (4 x 10^2 lux, white fluorescent light) plus DHC on RS4. Each point was an average of two replicate assays.

TABLE VII

DHC RESISTANCE OF RS4 WITH TWO DISTINCT COLONY MORPHOLOGIES WHICH WERE ISOLATED FROM NUTRIENT AGAR PLATE OR FROM COTTON LEAVES.

	Resistant population (%)		
O2	T2	RS4-O	RS4-T
27.4*	1.8*	0.054	1.15
< 0.08 (Ac44)	2.1 (Ac44)		
24.4 (OK1.2)	0.26 (OK1.2)		
	27.4 (OK1.2, O-type revertant)		

* average of two experiments.
RS4-O, RS4-T were single colonies out of NA plates without previous exposure to DHC.

TABLE VIII

TEST OF THE ROLE OF PHYSIOLOGICAL ADAPTATION IN DHC RESISTANCE OF *X. C.* PV. *MALVACEARUM* (RS4) RECOVERED FROM DYE-CONTAINING MEDIUM.

	Resistant population(%)		
Medium	1st bioassay	Exposure to DHC in the 1st assay	2nd bioassay
NA	15.9	no	5.5
		yes	3.4
E	48.6	no	60.8
		yes	79.2
MB	83.6	no	82.1
		yes	78.3
EMBa	45.2	no	71.5
		yes	32.6

NA: nutrient agar; E: eosin; MB: methylene blue; EMB: eosin plus methylene blue.

TABLE IX

DHC-RESISTANCE OF SINGLE-COLONY ISOLATES FROM E, MB, AND EMBA AGAR.

Resistant Populations(%)							
RS4 Parent				RS8 Parent			
Parent	EMBa	E	MB	Parent	EMBa	E	MB
0.05	42.8	31.0	25.8	0.03	24.1	82.1	19.2
	42.9	8.0	85.4			101.8	17.2
		9.9	33.1			87.9	17.8
		29.1	30.4			41.0	27.5
		10.1	38.1			76.2	
		12.1	48.5			95.8	
		1.1	43.3			81.2	
						98.4	
						95.1	
Average	42.8	14.4	43.5		24.1	84.4	20.4

TABLE X

DHC RESISTANCE OF INDEPENDENT MUTANTS RECOVERED FROM EMB MEDIUM.

		Resistant population (%)	
DHC (mM)	Strain	Dark assay	Light assay
0.1/Expt. 1	EMB1	44.4	37.4
	EMB2	6.7	25.0
	EMB3	33.7	51.0
	EMB5	3.4	4.9
	EMB6	2.1	21.8
	Parent (RS4)	<.0004	0.024
0.2/Expt. 2	EMB4	21.5	11.2
	Parent (H)	12.6	1.1
0.2/Expt. 3	EMB4	87.8	78.8
	Parent (H)	44.7	66.0
0.5/Expt. 4	EMB4	0.02	0.002
	Parent (H)	0.12	0.17

than the parent strain (H) but the opposite result was observed at high concentration (0.5 mM). Mutiplication of mutant EMB7*in planta* was not dramatically different from that of the parent strain RS4 (Figure 8).

The possible involvement of active oxygen species in the toxicity of DHC to Xcm and the possible induction of resistance factor(s) as suggested by the growth curves of those highly resistant strains, e.g. H, N etc. (Figure 3) suggest the possible protective role of enhancing superoxide dismutase (SOD). A condition which was known to enhance the Mn-SOD in *E. coli* was used to test if that could increase the DHC resistance of Xcm. Three strains of bacteria showed an increase in the resistance to DHC when Mn(II) was present in the growth and assay media (Table XI).

DHC^R mutant selection in an iron-chelated medium.

The Fe(III)-enhanced nicking of DNA by DHC in the dark suggested the third strategy for selection of DHC^R mutants. If Fe(III) is required for the full activity of DHC, then Xcm strains which could make Fe(III) unavailable would thereby increase their DHC resistance. Bacterial growth was not different from the control when 0.01, 0.03, or 0.05 mM of $Fe_2(SO_4)_3$ or 0.02, 0.06, or 0.1 mM Sequestrene 138 Fe (commercial iron chelator) was included in the NB. However, none of the concentrations (1.5, 2.0, 2.5, 3.0, 3.5 mM) tested showed that Sequestrene 138 Fe can effectively chelate Fe(III) to create an Fe-deficient medium. Of the five concentrations of 2,2'-bipyridyl tested (0.1, 0.15, 0.2, 0.25, 0.3 mM) 0.1 and 0.15 mM stimulated growth of the parent strain. The three higher concentrations were inhibitory, but only 0.2 mM 2,2'-bipyridyl permitted growth of mutants. This Fe(III)-unavailable medium was used to select mutants which are presumably siderophore overproducers. Six mutants were picked and further purified in the same medium. The frequency of obtaining these mutants is presented in Table XII. Mutants B1, B2, B3 and B4 were more resistant to DHC than their parent RS4 (Table XIII). Mutants B5 and B6 were not significantly different from their parents (Table XIII).

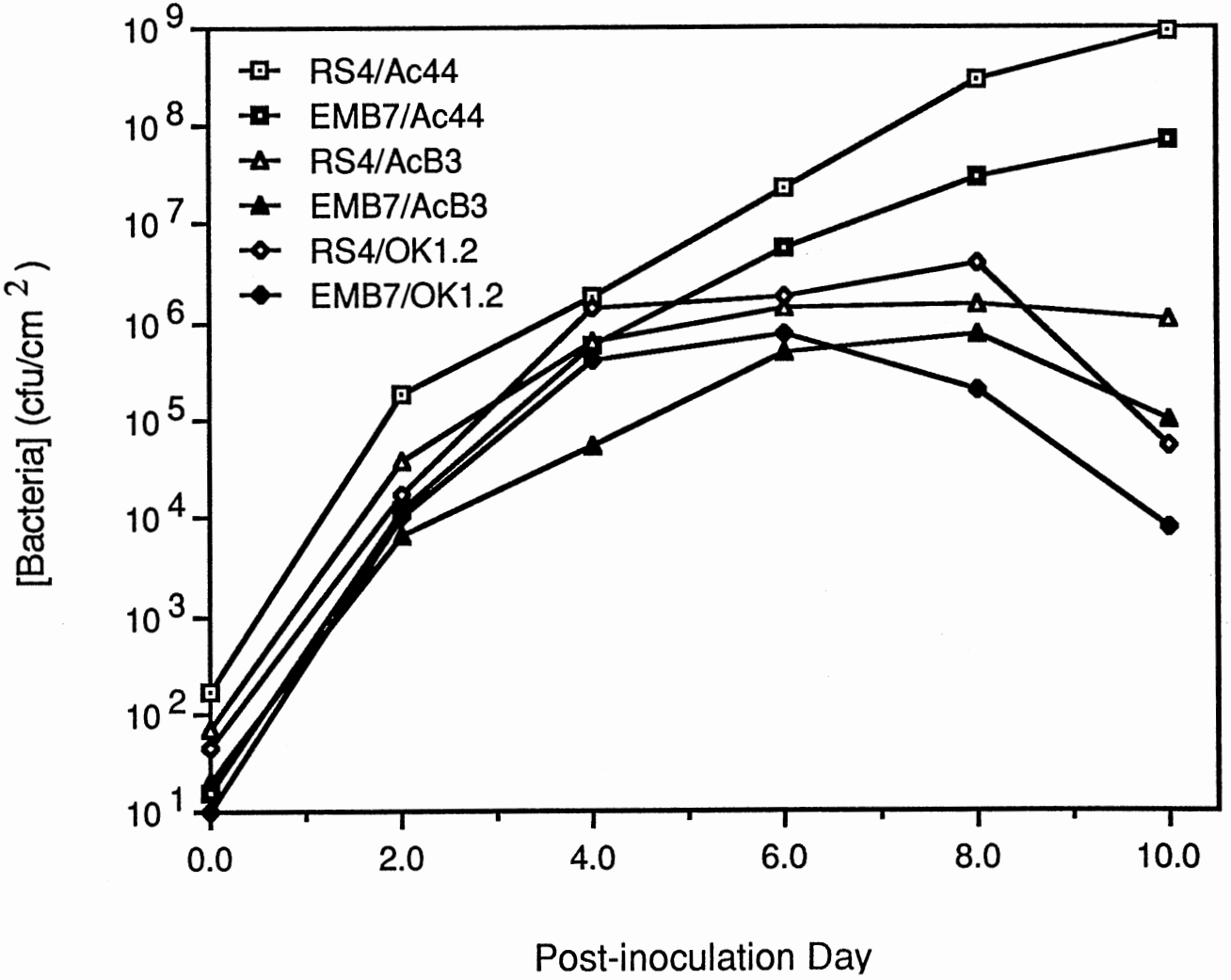


Figure 8. Multiplication of DHC^R mutant EMB7 and its parent (RS4) in Ac44, AcB_3 and OK1.2 cotton leaves. Each data point was obtained from one leaf of each lines. One plant of each line was used.

TABLE XI

DHC-RESISTANCE OF *X. C.* PV. *MALVACEARUM* FROM 0.1 MM $MnSO_4$-ENRICHED NUTRIENT BROTH.

	Resistant population(%)	
Strain	NB	Mn(II)-NB
RS4	0.3	55.5
RS8	8.1	57.5
EMB81/RS8	24.1	46.3

TABLE XII

THE ORIGIN AND/OR FREQUENCY OF THE POSSIBLE SIDEROPHORE OVERPRODUCING AND EMB^R MUTANTS.

Mutant	Parent strain	Mutation Frequency
B1	RS4	5.34×10^{-7}
B2	RS4	same as above
B3	RS4	1.45×10^{-7} to 2.08×10^{-8}
B4	RS4	$8.75 - 2.52 \times 10^{-8}$
B5	H	1.33×10^{-7}
B6	EMB4	9.76×10^{-8}
EMB1	MB41	
EMB2	MB1	
EMB3	MB2	
EMB4	H	
EMB5	RS4	
EMB6	RS4	

TABLE XIII

DHC RESISTANCE OF INDEPENDENT MUTANTS RECOVERED FROM IRON-CHELATED MEDIUM.

		Resistant population (%)	
DHC (mM)	Strain*	Dark assay	Light assay
0.1	B1(RS4)	51.8	93.8
	B2(RS4)	44.9	64.6
	B3(RS4)	65.6	65.9
	B4(RS4)	47.9	61.3
	RS4	0.07	0.06
0.2/Expt. 3**	B5(H)	80.3	92.4
	B6(EMB4)	88.4	86.5
	EMB4(H)	87.8	78.8
	H	44.7	66.0
0.5/Expt. 4**	B5(H)	0.04	0.03
	B6(EMB4)	0.02	0.004
	EMB4(H)	0.02	0.002
	H	0.12	0.17

* mutant assayed (parent strain)
** cf: TABLE X

Does DHC cause the conversion of O to T-type Xcm?

If DHC causes the conversion of O to T-type bacteria or selects for T-type bacteria, then dilution (or removal) of DHC at various times during the bioassay would affect the occurrence of T (%). The increase of DHC in the medium not only resulted in a decrease in the resistant population with Race 3, but also resulted in an increase in the percentage of T-type bacteria (Figure 9). At the fixed DHC concentration (0.1 mM), the decrease of the resistant population percentage coincided with the increase of the percentage of the T-type bacteria (Figure 10, 11). The control group, which had no DHC, only showed a minor increase in T-type during the 8 h. bioassay (Table XIV, Figure 10). Ten fold dilution of the DHC, which was present in the culture medium, delayed the increment of T-type bacteria but did not prevent it (Table XIV). Contact of the full strength DHC (0.1 mM) with the tested bacteria for 0.5 or 1 h was not long enough to bring up the percentage of T-type equivalent to the control (0.1 mM for 8 h, Table XIV). Dilution of the no-DHC bacterial culture at the early times (0.5 and 1.0h), stimulated the T percentage from 13.5% (never diluted control) to 24.0 and 17%, respectively (Figure 11b). However, dilution of the same group at later times (1.5 and 2.0 h) did not result in the stimulation effect (Figure 11-b). Overall, the presence of DHC coincided with an increase in the percentage of T-type bacteria (Table XIV) as compared to the no-DHC groups (at each specified time has a no-DHC control).

The original O-type Race 3 was very sensitive to DHC and grew slower than the survivors of DHC of either type (Table XV). The T-type Race 3 showed no difference in sensitivity and growth rate before or after exposure to DHC, although the original T-type Race 3 was much more resistant to DHC and grew faster than the original O-type Race 3 (Table XV). An overnight culture of Race 3 was heavily populated with T-type bacteria which was shown by the high initial percentage of T type.

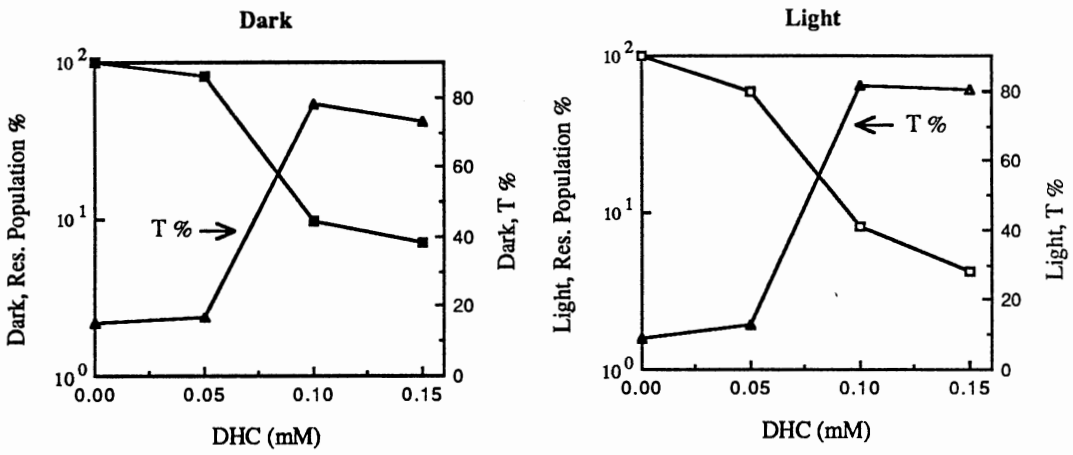


Figure 9. DHC dose response growth of Race 3 during 8 h and the occurrence of T-type in the dark or in the light.

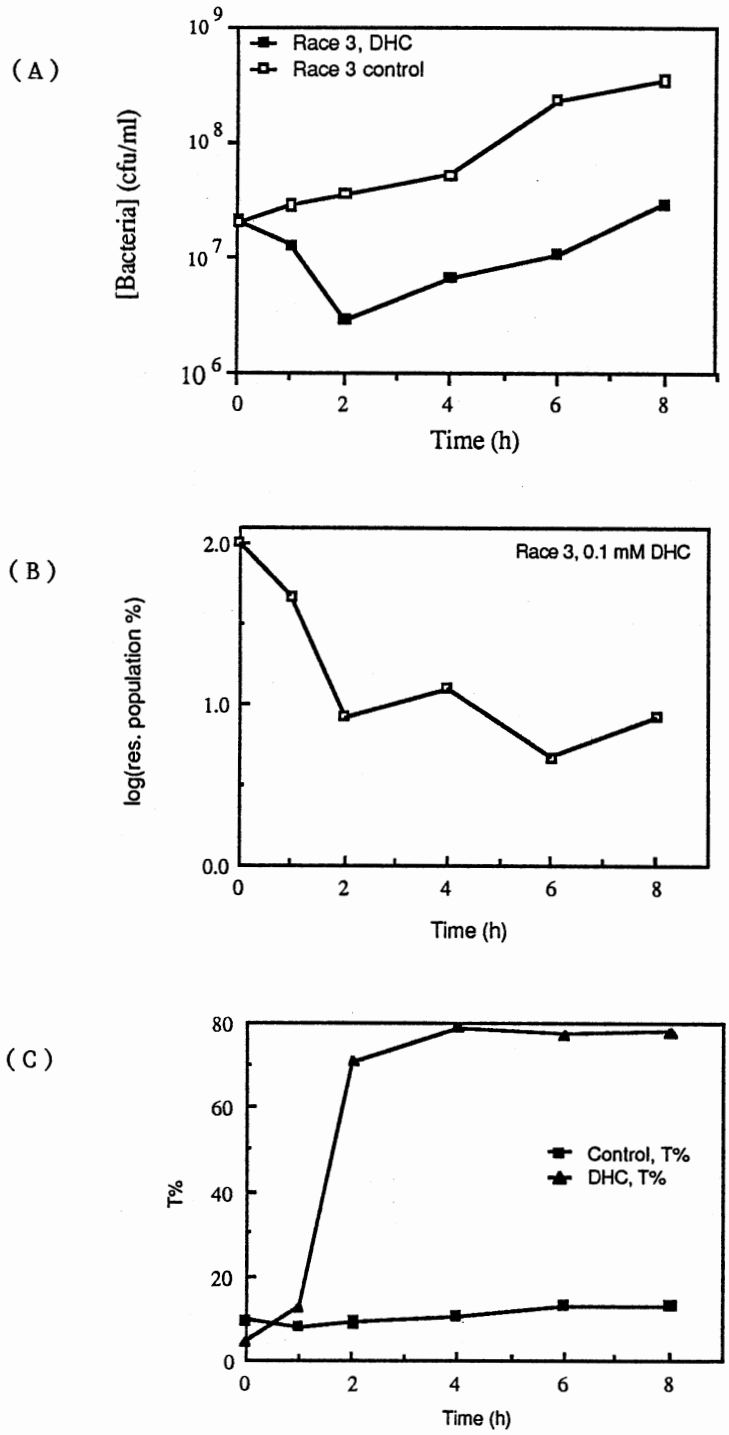


Figure 10. Time course of % DHC-resistant population and the occurrence of T-type bacteria in a culture of Race 3 during exposure to 0.1 mM DHC.

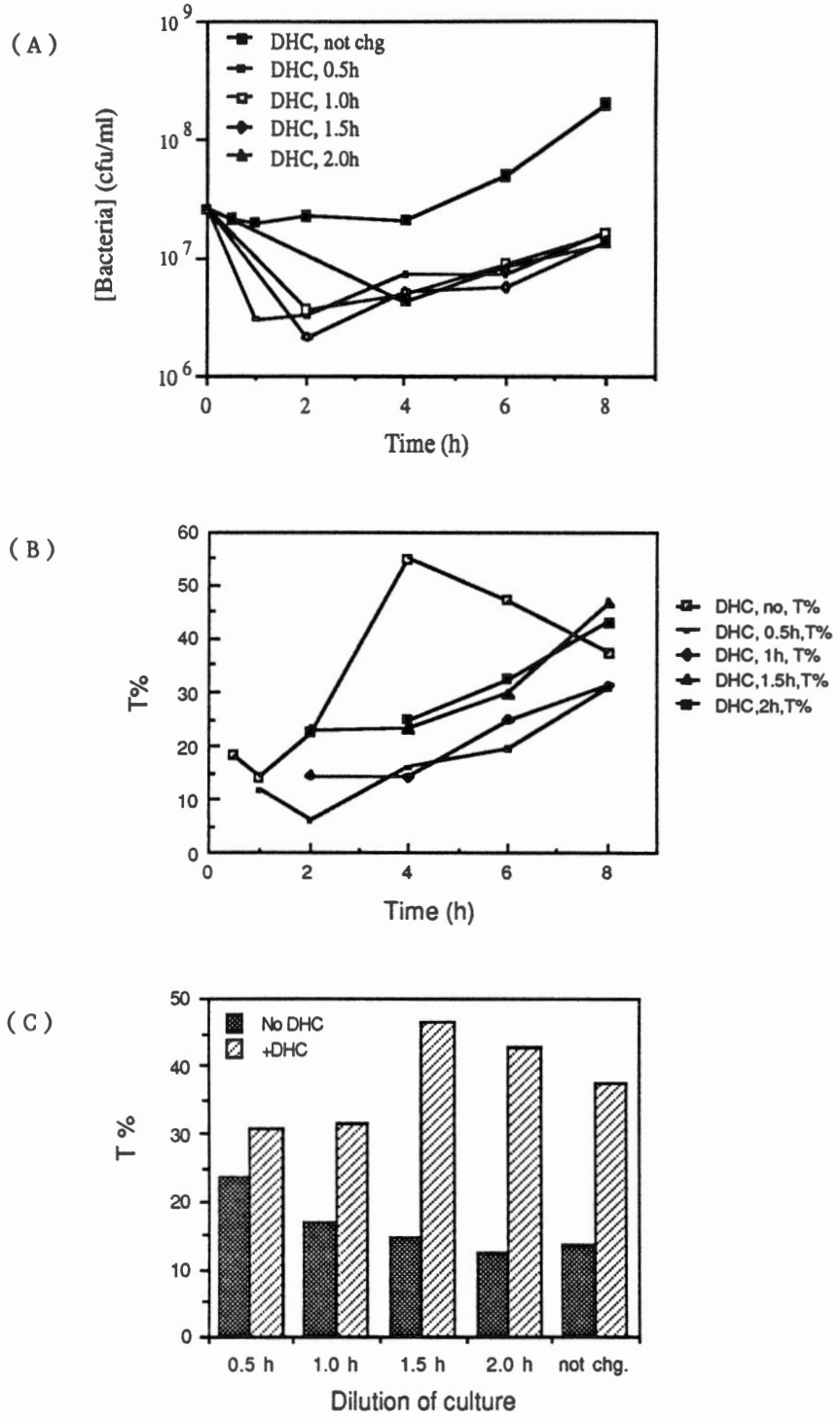


Figure 11. The effect of 10-fold diluting of the DHC (0.1 mM) at various times during the bioassay on the T-type occurence in Race 3. not chg: no dilution control.

TABLE XIV

VARIATION OF T(%) WITH DILUTION OF DHC (0.1 MM) AT VARIOUS TIME POINTS.

Dilution (h)	none		none		0.5		1.0		1.5		2.0	
DHC	-		+		+		+		+		+	
	(%)											
Time (h)	R*	T*	R	T	R	T	R	T	R	T	R	T
0	100	14.5										
0.5			69.6	18.5								
1.0			51.5	14.0	89.1	12.0						
2.0			40.6	22.5	73.6	6.0	75.7	14.5	43.0	23.0		
4.0			17.2	55.0	97.3	16.0	54.9	14.0	56.0	23.5	46.7	25.0
6.0			19.6	47.0	57.3	19.5	54.7	25.0	33.1	30.0	47.9	32.5
8.0	100	13.5	38.2	37.5	78.2	31.0	54.1	31.5	44.8	46.5	41.1	43.0
Dilution control					100	24.0	100	17.0	100	14.5	100	12.5

*R: resistant population ; T: T-type Xcm.

TABLE XV

RESISTANCE TO 0.1 MM DHC OF T AND O-TYPE RACE 3 BACTERIA BEFORE AND AFTER EXPOSURE TO DHC AND DOUBLING TIMES IN ABSENCE OF DHC.

Strains	Resist. Pop.(%)	Doubling time(min)
R3-O-b	0.04	153
R3-T-b	62.3	105
R3-O-s	72.3, 73.3	117
R3-T-s	68.6, 56.5	117
R3	8.5, 8.1, 9.8	

b: before exposure to DHC; s: survivor of DHC.
Independent assays are presented in some cases.

T-type characterization.

The frequency of occurrence of T-type bacteria from RS4 ranged from 1.2×10^{-2} to 4.0×10^{-4} (Table XVI), depending on the days of subculturing. In cotton leaves, single-colony lesions due to O2 (3.6×10^3 cfu/ml) or T2 (approximately 3×10^3 cfu/ml) in Ac44 and O2 in AcB_3 were visible to the naked eye; but lesions due to T2 in AcB_3 were visible only with the aid of a microscope (Table XVII). Lesions due to O2 and T2 in AcB_3 and T2 in Ac44 are shown in Figure 12. More concentrated inocula (aiming for 10^8 cfu/ml) were also used to check the T2/cotton interactions by syringe infiltration. Of six Ac44 plants tested, only one showed slight water soaking with T2, the rest of the plants all gave a hypersensitive response, some with redness in the epidermis. The responses to T2 in AcB_3, OK1.2 and Im216 were all weak hypersensitive reactions which looked like chlorotic lesions. The responses to the O2 group were normal as was the parent strain (RS4). T2 (aiming for 10^8 cfu/ml) cultured in a sucrose-containing medium and/or dispersed in sucrose-containing water just before inoculation, did not affect the responses *in planta* .

A 0.125% solution of xanthan gum (without bacteria) produced water soaking at day 6 in Ac44 (4 plants) yet at 1% produced no visible spots; at 0.25% one plant had water soaking; the other three had weakly water soaked lesions; and at 0.5% two plants had water-soaking and two plants had no visible spots. Xanthan gum itself at 0.125 or 0.25% elicited a hypersensitive response and at 0.5 or 1% elicited chlorotic response in AcB_3. In 0.125, 0.25, 0.5 or 1% xanthan gum, T2 produced water-soaked lesions in Ac44 and a normal level of hypersensitive response in AcB_3. After experiencing the variation of the responses to T2 *in planta* , the most suitable inoculum concentration to use for such purpose was determined to be 10^7 cfu/ml. Ten to 100 fold higher than 10^7 cfu/ml was too concentrated, and 10^2 to 10^6 cfu/ml was too dilute to get a clear response. With the presence of both xanthan gum (0.125%) and sucrose (1%) in the inocula T2 gave five

TABLE XVI

FREQUENCY OF OCCURRENCE OF T-TYPE BACTERIA FROM RS4 AFTER OVERNIGHT CULTURING OR MULTIPLE 1-DAY SUBCULTURING.

Source	T / (T+O)	Frequency of T
Cryovial	141/22631	6.2×10^{-3}
Single colony	8/19935	4.0×10^{-4}
Single colony	3/4367	6.9×10^{-4}
Cryovial, day 1	11/2735	4.0×10^{-3}
day 2	15/2412	6.2×10^{-3}
day 3	21/1982	1.1×10^{-2}
day 4	9/738	1.2×10^{-2}

TABLE XVII

APPEARANCE OF LESIONS DUE TO SINGLE-CELL COLONIES OF O2 AND T2 IN SUSCEPTIBLE (AC44) AND SINGLE RESISTANT GENE (ACB_3) COTTON LINES.

	O2 / Ac44	T2 / Ac44	O2 / AcB_3	T2 / AcB_3
Day 6	Macro, WS	N. V.	N. V.	N. V.
Day 9		N. V.	Macro, H*	N. V.
Day 15		Macro, H		N. V.
Day 16				N. V., Micro, H

WS: water-soaked lesions; N. V.: no lesion visible to the naked eye; H: hypersensitive response; Macro: macroscopic lesion; Micro: microscopic lesions.
* Total lesions were counted and one virulent mutant was recovered at 1.12×10^{-4}. Later four single colonies of this mutant confirmed its phenotype in AcB_3. Inocula concentration for O2 and T2 were approximately 3×10^3.

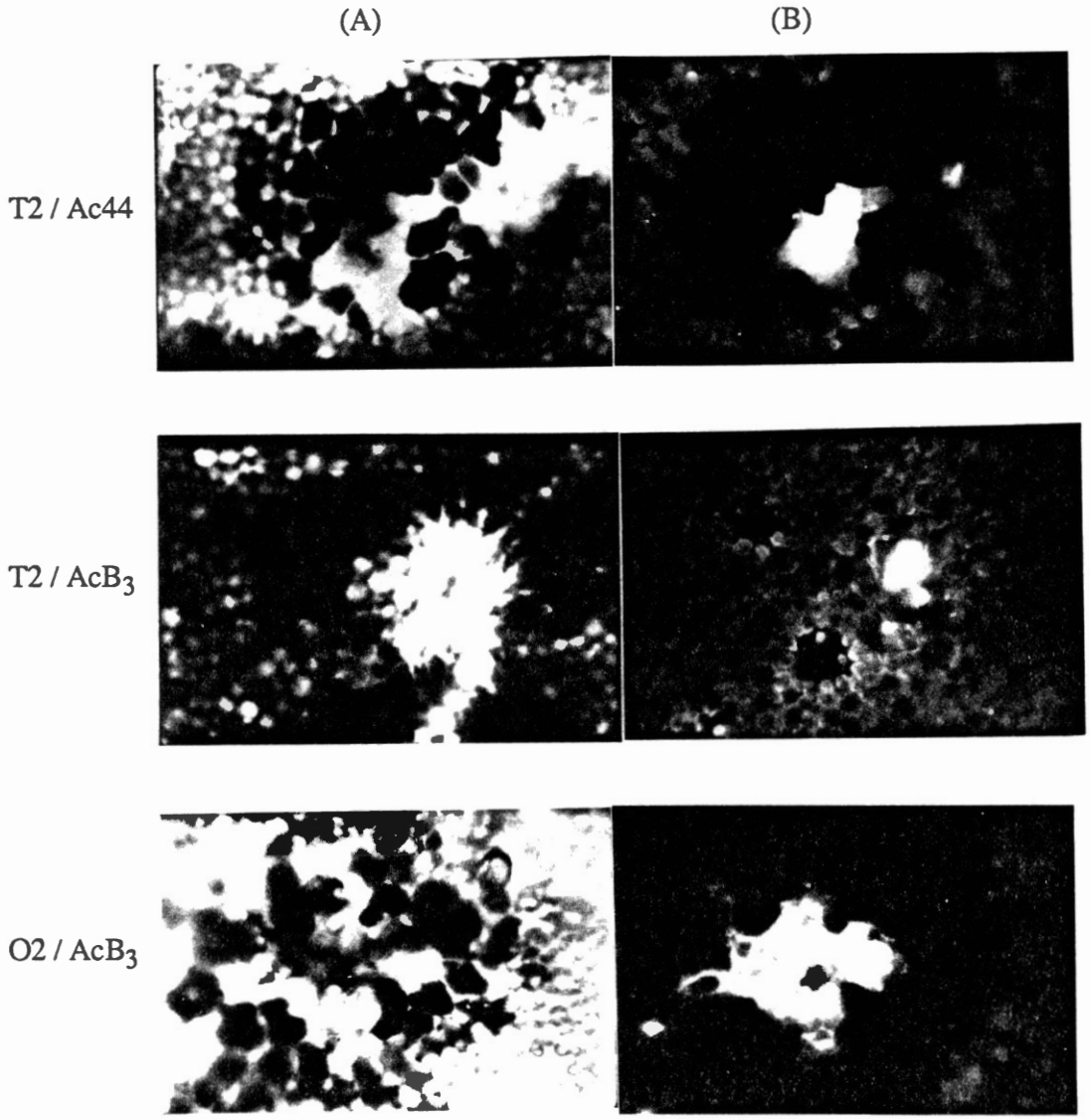


Figure 12. Lesions caused by single-colonies of strain T2 in cotton lines Ac44 and AcB_3 and by strain O2 in AcB_3. A. Transmitted white light, x110. B. Same fields as A; fluorescence.

times more bacteria per cm^2 leaf (Ac44) at day 11 than T2 dispersed in saturated $CaCO_3$ water and more clear water-soaked lesions (3-5-86).

A higher bacterial population was observed in inoculated Ac44 than in AcB_3, regardless of strains or solutions used in the inocula (Figure 13). T2 dispersed in a xanthan gum plus sucrose solution (XS) grew to a higher bacterial population in each cotton line when compared to T2 dispersed only in saturated $CaCO_3$ water (Figure 13). However, XS solution did not revert the bacterial multiplication of T2 to a density comparable to that of O2 within the same line (Figure 13). In the same experiment O-type revertants were recovered from plants inoculated with T2. From day 4 to day 8 a decreased % of O-type bacteria was observed with interaction of T2/Ac44 and T2-XS/AcB_3, but an increased % with interaction of T2/AcB_3 and T2-XS/Ac44 (Figure 14, Table XVIII).

No O-type revertants were recovered in a 1% sucrose-containing medium *in vitro*. However, the bacterial colony morphology observed in this medium was different from either T or O-types. It looked as if it had a T-type center surrounded with an O-type ring.

A significant fraction of T2 was protease-negative when tested. Interestingly, an extracellular protease-deficient isolate (2.6×10^6 cfu/ml) of T2 gave barely visible symptoms when syringe-inoculated into Ac44, AcB_2, AcB_N. This is a typical null reaction of a nonpathogen. Protease-positive T2 at a lower density (8.9×10^5 cfu/ml) produced chlorotic lesions in Ac44 and AcB_N, and hypersensitive lesions in AcB_2. Protease-positive O2 (2.6×10^6 cfu/ml) had water-soaked lesions in Ac44 and hypersensitive lesions in both AcB_2 and AcB_N. Total protein profiles of O2, T2, protease$^-$ T2 were similar, except protease$^-$ T2 had a significantly increased amount of p26 (Figure 15).

Recovery of spontaneous virulent mutants and their phenotypes.

Because of the possible mutagenic effect of DHC and the potential drawback of using Ames test as discussed in chapter II (pg. 62), I decided to test whether exposure to

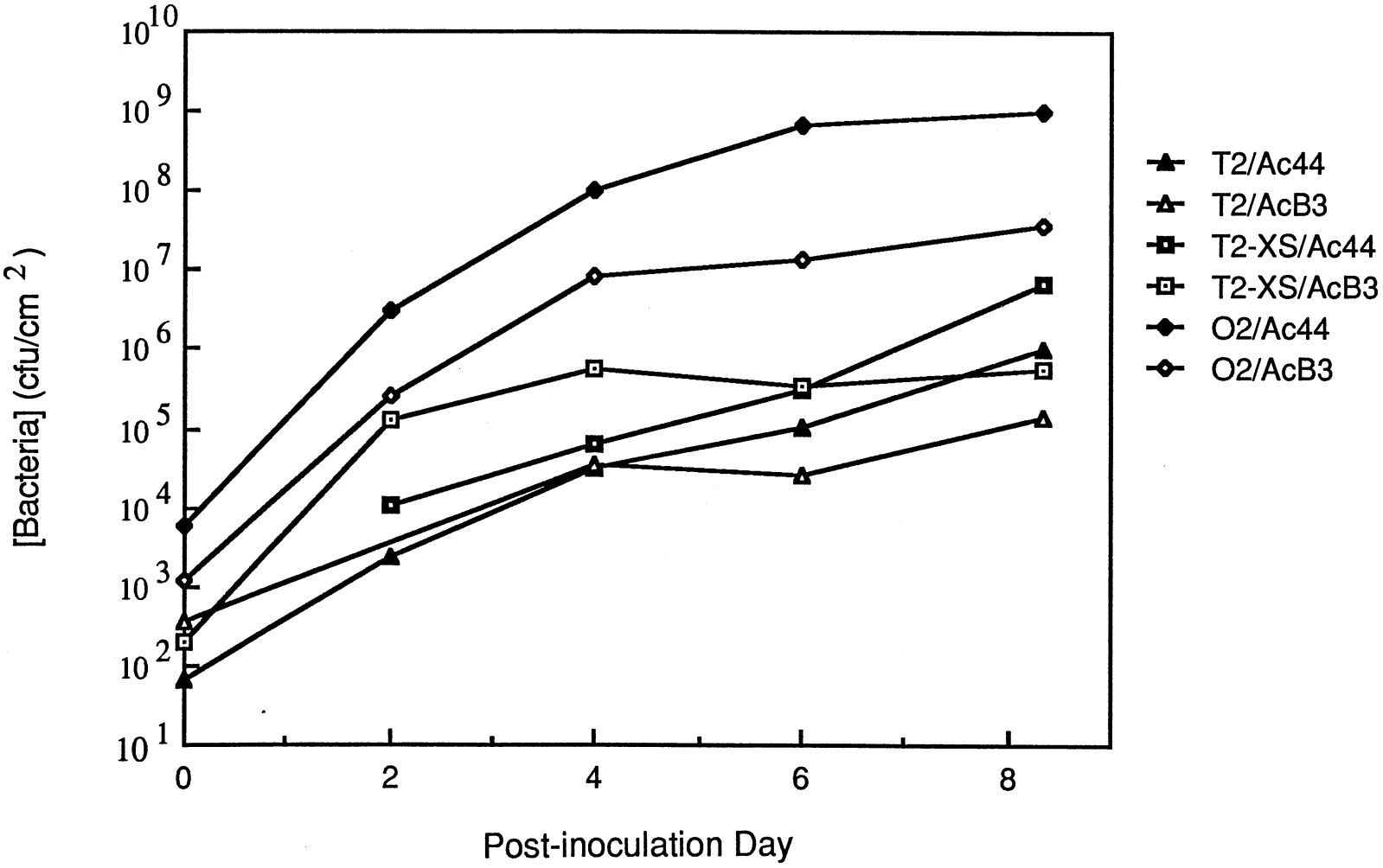


Figure 13. T2, O2 growth in Ac44, AcB_3 cotton leaves and the possible effects of the presence of xanthan gum (0.125%) and sucrose (1%) in the T2 inocula. T2-XS: T2 dispersed in xanthan gum (0.125%) and sucrose (1%) solution as initial inocula. T2, O2: T2 or O2 dispersed in saturated $CaCO_3$ solution as initial inocula.

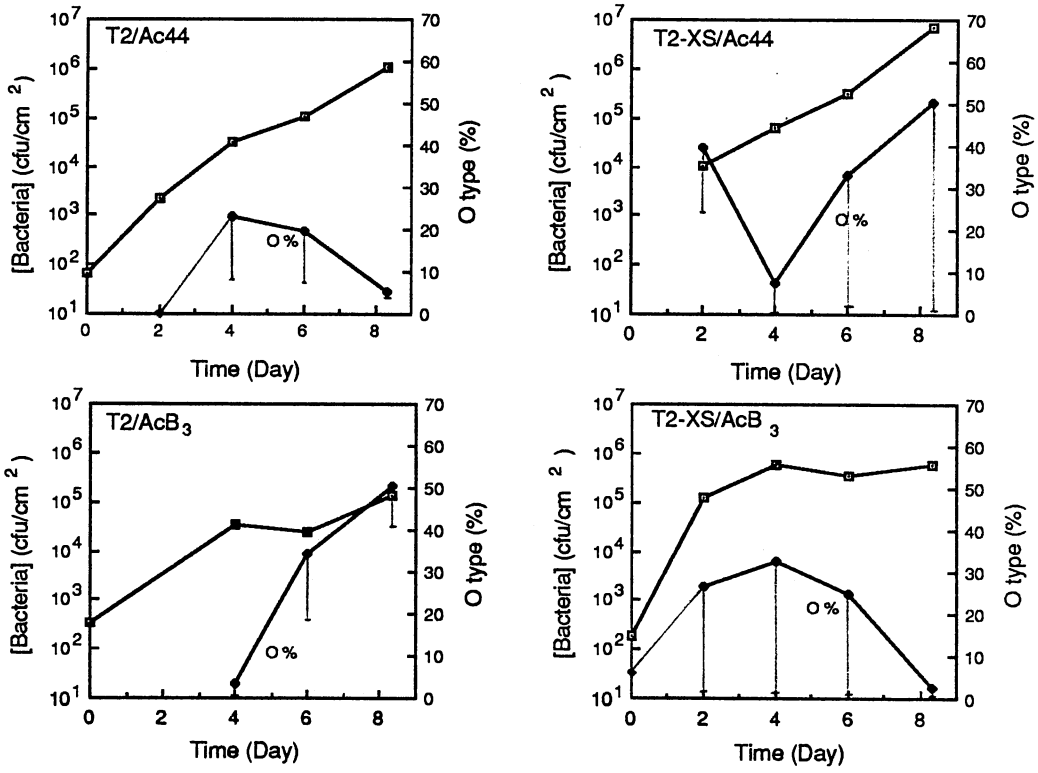


Figure 14. The occurrence of O-type revertants from T2 after incubation *in planta*. The error bars indicate the lower half of the range of each determination of O%.

TABLE XVIII

APPEARANCE OF O-TYPE BACTERIA (%) FROM T2 INCUBATED *IN PLANTA* (AC44, ACB_3) AND THE EFFECT OF XANTHAN GUM-SUCROSE PRESENCE IN THE INITIAL INOCULA.

	Day 2	Day 4	Day 6	Day 8.3
T2/Ac44, 1*	< 6.25	9.5	30.0	4.1
2*	-----	36.7	9.4	6.8
T2/AcB_3, 1	-----	< 0.6	49.5	60.0
2	-----	< 6.2	19.5	41.0
T2-XS/Ac44, 1	53.8	< 0.9	64.0	99.8
2	25.0	14.0	2.3	1.0
T2-XS/AcB_3, 1	51.2	63.9	47.8	0.6
2	< 1.7	< 1.5	< 1.7	4.6

*plant 1, plant 2; XS: xanthan gum (0.125%) and sucrose (1%) was present in the inocula.

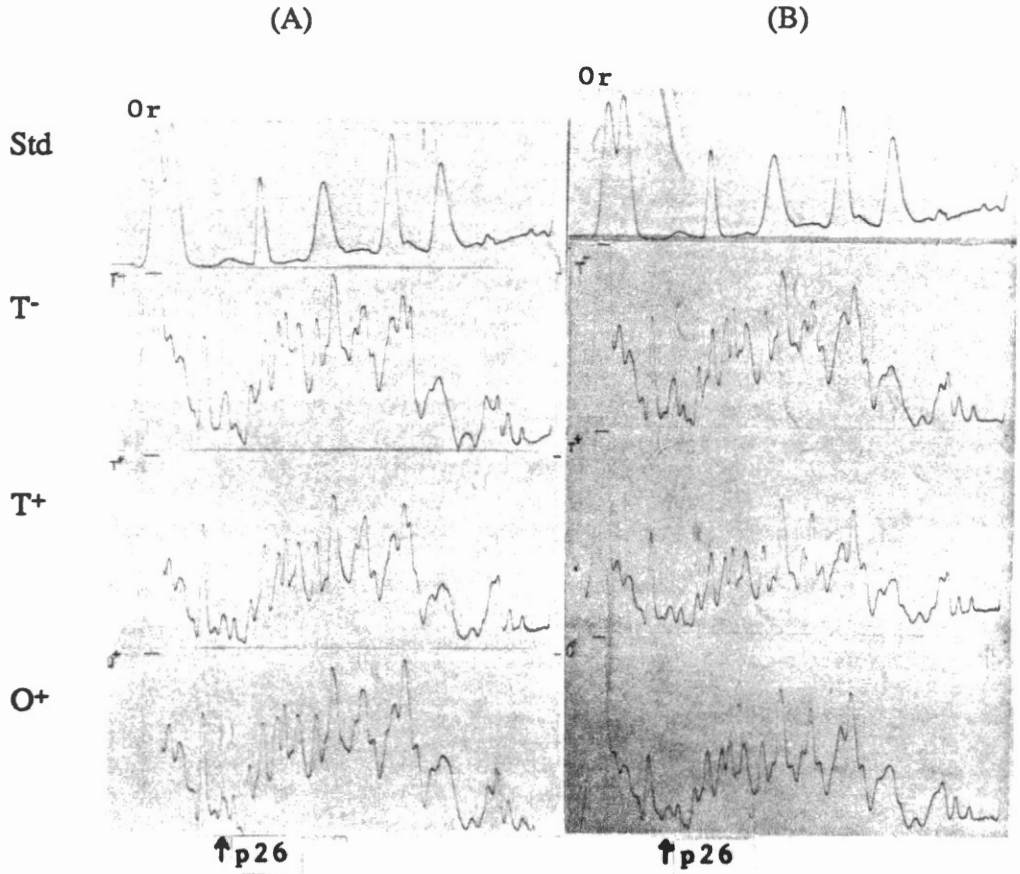


Figure 15. Protein profiles of T2-protease$^-$, T2-protease$^+$, and O2-protease$^+$ bacteria. Approximately 75µg and 100µg of protein were used in panel A and B respectively. Std: Molecular weight standard included lysozyme (14 000), soybean trypsin inhibitor (21 500), carbonic anhydrase (31 000), ovalbumin (45 000), bovine serum albumin (66 200), phosphorylase B (92 500). Or: Origin of the gel.

DHC increased the conversion of Xcm from avirulence to virulence. It was first necessary to establish the background occurrence of virulent mutants. The spontaneous virulent mutants with respect to differential cotton lines were obtained at frequencies of 2.5×10^{-3} to 3.3×10^{-4} (Table XIX). Since these frequencies were high, the initial objective of studying mutagenic activity of DHC was not pursued. However, the virulence phenotypes of the spontaneous mutants were checked in the differential cotton lines.

Of all the virulent mutants recovered, the mutants from AcB_N gained virulence in AcB_N only; the rest of the mutants all gained virulence in more than one cotton line, and one (B_4 (03)) was compatible in nine different cotton lines (Table XX). Virulent mutants obtained from AcB_1 were virulent not only in AcB_1, but also in AcB_3 and Acb_7. Independent virulent mutants recovered from AcB_3 or Acb_7 were also virulent in the other two lines. This kind of group is called "cluster" in this work. Of all these simultaneous conversions to virulence, three clusters were found which are virulent against AcB_1-AcB_3- Acb_7, AcB_4- AcB_5, or AcB_6- (Stoneville-2B-S9) - Gregg. All virulent mutants obtained from each of the following cotton lines, AcB_1, AcB_3, Acb_7 and AcB_N, had the same phenotype when checked in differential cotton lines. Only one virulent mutant was isolated from each of the lines AcB_5 and Gregg. Two kinds of virulent mutants were isolated from AcB_6 and three kinds from AcB_4. One mutant which gave a clear incompatible reaction in Ac44 (the standard susceptible line) was also recovered from AcB_N.

Plasmid variation & virulence

The instability of Xcm expressed during selection of DHC^R mutants, as well as the high frequency of occurrence of T-type and virulent mutants suggested that an unstable genetic factor may exist in Xcm. Plasmids can be unstable factors due to uneven distribution during cell division. The major reason for checking the plasmid pattern of these virulent mutants was my extreme curiosity about them. The size of the plasmid from

TABLE XIX

THE FREQUENCY OF RECOVERY OF SPONTANEOUS VIRULENT MUTANTS FROM RS4 IN INCOMPATIBLE COTTON LINES.

	compatible lesions / total numbers of lesions			
Cotton line	Expt. 1	Expt. 2	Expt. 3	Avg. frequency
Ac B_1	6/8968(2)*	3/3792(3)	6/6270(3)	8.1×10^{-4}
Ac B_3	6/7482(2)	37/10213(2)	-----	2.2×10^{-3}
Ac B_4	7/3804(2)	7/5154(3)	-----	1.6×10^{-3}
Ac B_5	-----	-----	-----	-----
Ac B_6	1/2035(2)	6/9545(3)	-----	5.6×10^{-4}
Ac b7	4/6958(2)	-----	-----	5.8×10^{-4}
Ac B_N	39/10077(2)	11/10708(2)	-----	2.5×10^{-3}
Gregg	5/7719(3)	-----	-----	6.5×10^{-4}
S-2B-S9**	1/3010(3)	-----	-----	3.3×10^{-4}

* (2): Growth chamber 2 (Conviron); (3): Overflow growth chamber 3 (Percival).
**Stoneville-2B-S9.

TABLE XX

PHENOTYPES OF SPONTANEOUS VIRULENT MUTANTS DERIVED FROM RS4 AND RECOVERED FROM INCOMPATIBLE COTTON LINES.

			Acala												
			44	B_1	B_2	B_3	B_4	B_5	B_6	b_7	B_N	1-10B	S-20	S-2B-S9	Gregg	Phenotypical cluster*
Parent			+	-	-	-	-	-	-	-	-	-	-	-	-	
Mutants	No. of independent mutants	No. of individual isolates														
B_1	3	7	+	+	-	+	-	-	-	+	-	-	+	-	-	1
B_3	3	24	+	+	-	+	-	-	-	+	-	-	+	-	-	1
b_7	1	1	+	+	-	+	-	-	-	+	-	-	+	-	-	1
B_4-1	2	3	+	-	-	-	+	+	-	-	-	-	-	-	-	2
B_4-2	2	2	+	-	-	-	+	+	+	-	-	-	-	+	+	2, 3
B_4-3	1	1	+	+	-	+	+	+	+	+	-	-	+	+	+	1, 2, 3
B_5	1	1	+	-	-	-	+	+	+	-	-	-	-	+	+	2, 3
B_6-1	2	2	+	-	-	-	-	-	+	-	-	-	-	+	+	3
B_6-2	2	2	+	-	-	-	+	+	+	-	-	-	-	+	+	2, 3
B_N	3	18	+	-	-	-	-	-	-	-	+	-	-	-	-	
Gregg	1	1	+	-	-	-	-	-	+	-	-	-	-	+	+	3

+: Compatible interaction; -: Incompatible interaction.
*1: B_1-B_3-b_7 cluster; 2: B_4-B_5 cluster; 3: B_6-(Stoneville-2B-S9)-Gregg cluster.
S-20: Stoneville-20; S-2B-S9: Stoneville-2B-S9.

the virulent mutants isolated from AcB_1, AcB_3 and Acb_7 was the same as pTJS from the parent RS4 (Figure 16-a,b). Three of the four AcB_4 mutants (Figure 16-a), one of the two AcB_6 mutants (Figure V1a), and 10 of the 18 AcB_N mutants tested had apparently changed their plasmid size (Figures 16-b, c, d). Race 4 and Race 10 had plasmids smaller than pTJS of RS4 (Figure 17-a). Strain N had two plasmids, both of which were smaller than pTJS (Figure 17-a). Race 7 had a plasmid larger than pTJS (Figure 17-a). One mutant recovered from AcB_4 (03) had an extremely large deletion in its plasmid (pTJS403). The plasmid migrated to the same position as the smaller plasmid of strain RN1 (Figure 17-b). The pTJS was approximately 80.4 kb as determined by adding up the size of SstI fragments (data not shown). The pTJSn13 plasmid migrated to the same position as pTJS (Figure 16-c) and gave an identical restriction pattern (Figure 21). The pTJSn12 plasmid apparently lost a 7.5 kb Sst I fragment (Figure 21). The pTJSn06 and pTJSn19 gave similar restriction patterns and the size changes from pTJS as estimated by EcoRI, SstI, and Pst I digestion were 20.5, 19.4, 14.5 kb respectively. The pTJSn17 plasmid gave restriction patterns different from those of the pTJSn19. Estimates of the deletion relative to pTJS obtained from EcoRI, SstI, PstI digestion fragments sizes were 16.9, 15.7 and 12.3 kb, respectively. The deletion in pTJSn03 was estimated by PstI digestion as 18.6 kb. All this data are shown in Figure 21.

DNA rearrangement?

The relatively high frequency of obtaining virulent mutants and the major variations of plasmid size in some of these mutants suggested the possible involvement of an insertion sequence element (IS) or transposon (Tn) in creating the observed phenomena. Both elements could also be candidates for the unstable factors in Xcm mentioned in the previous paragraph. From the work of Gabriel et al (1986), the dominant effect of avirulence genes of Xcm was known. Therefore the virulent mutants obtained in this work are assumed to have crippled avirulence genes. The possibility of inactivation of avirulence genes by the

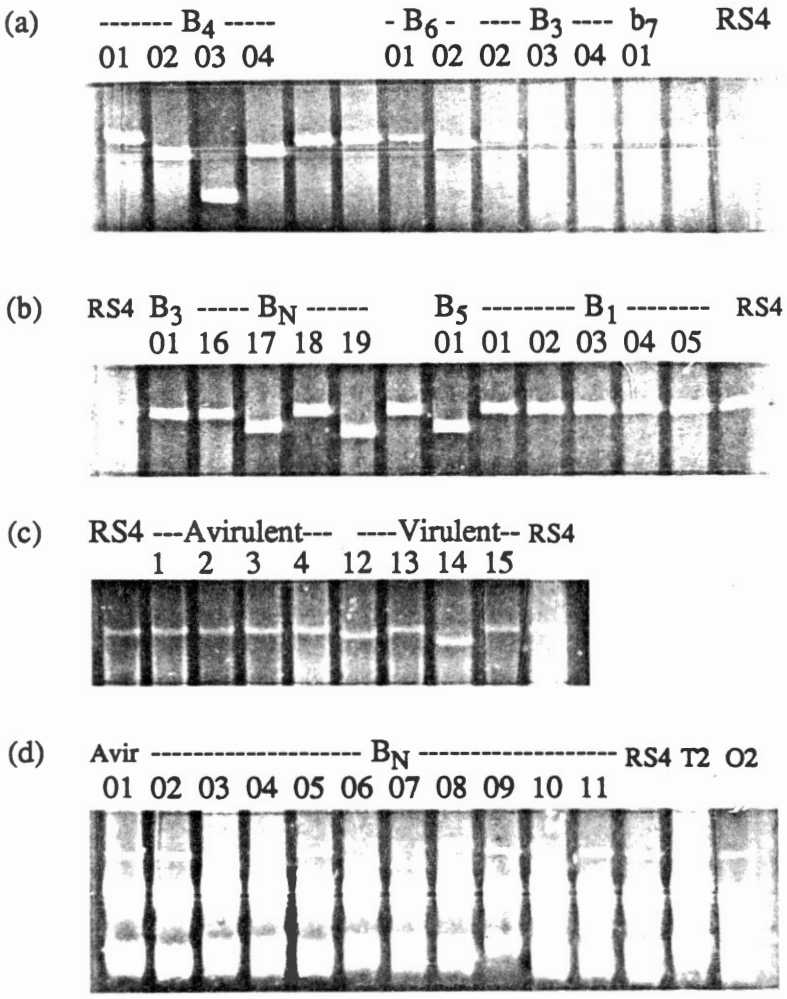


Figure 16. Plasmid profiles of the virulent mutants from (a) AcB_3, AcB_4, AcB_6 and Acb_7; (b) AcB_1, AcB_3, AcB_5 and AcB_N; (c) AcB_N; (d) T2, O2, AcB_N and one mutant incompatible in Ac44.

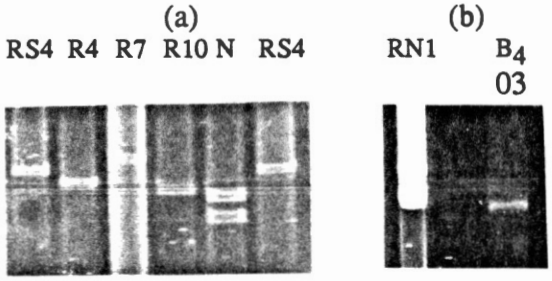


Figure 17. Plasmid profiles of some known races and strains that are compatible in AcB_N.

putative indigenous IS or Tn in these spontaneous virulent mutants was tested. Chromosomal DNA preparations from eight virulent mutants against AcB_3 were restricted and probed with the cosmid which contained the $AvirB_3$ gene. The same experiment was also conducted with 17 virulent mutants of AcB_N (02 to 19 except 09) and one mutant (Ac B_N (01)) which was avirulent in all tested cotton lines (Ac44 as well) and probed with the cosmid which harbors the $AvirB_N$ gene. There was no difference in the pattern of the hybridized bands between mutants that are compatible in AcB_3 and their parent (RS4) (Figure 18) or AcB_N mutants and their parent (RS4) (Figure 19). And there was no difference of hybridized bands (Figure 18) between the parent (RS4) and the strain H, the donor of the cloned avirulence genes. When the restricted total DNA (Figure 20a) of the virulent mutants of AcB_N was Southern blotted and probed with the indigenous plasmid (pTJS) of the parent, new bands appeared in some mutants. Of nine mutants which had deletion in their plasmids (n03, n04, n06, n07, n10, n12, n14, n17, n19) six of them had extra chromosomal bands that hybridized with pTJS (n03, n04, n06, n07, n10, n19) (Figure 20-b). The other three (n12, n14, n17) were missing one band but another band of higher molecular weight hybridized (Figure 20-b). One mutant (n08), which had an insertion in the plasmid, had a missing band and also had extra bands being lighted up. Only one mutant (n13), which did not have an apparently changed plasmid, lost band IV in exchange for a larger band. The rest of the mutants which had the same size plasmid as the parent had the same pattern as the parent. When the same blot (of Figure 20-b) was rehybridized with the $AvirB_N$ clone, it was apparent that the bands identified by the two sets of probes were not the same (Figure 20-c).

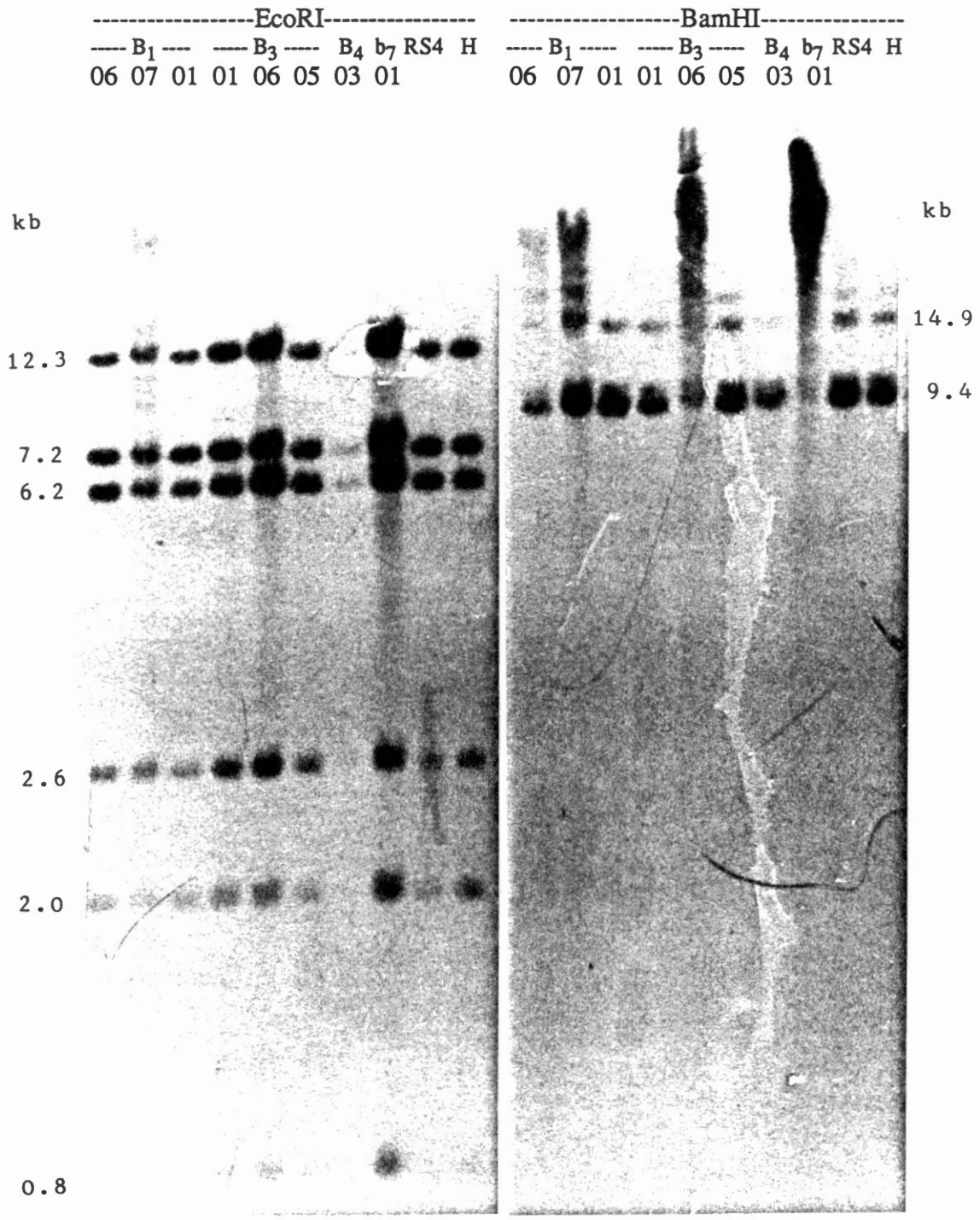


Figure 18. Chromosomal DNA of mutants virulent against AcB_1 - AcB_3 - Acb_7 restricted with EcoRI or BamH1 and probed with clone pUFA717, which contains the Avir B_3 gene.

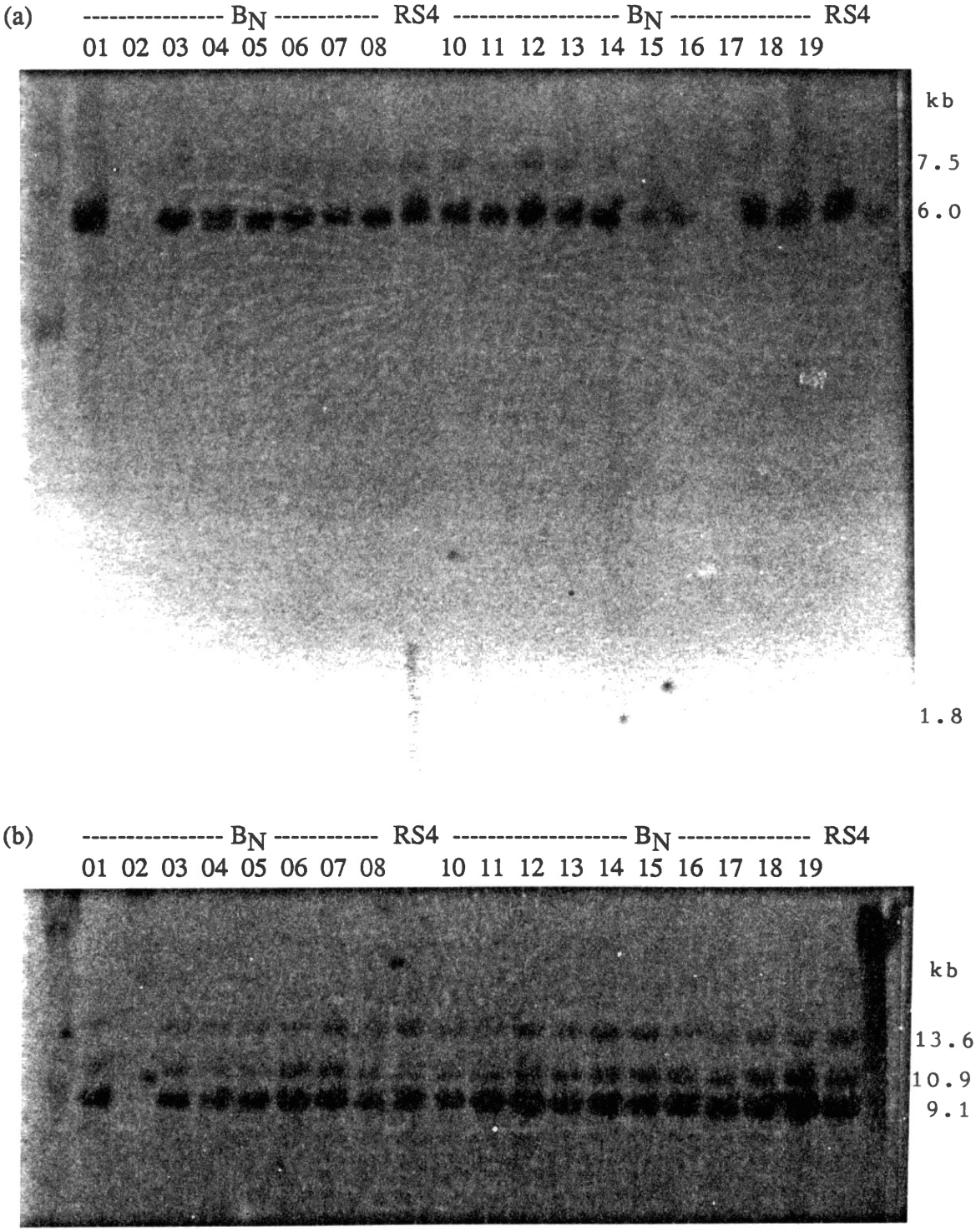


Figure 19. Chromosomal DNA of mutants virulent against AcB_N restricted with (a) EcoRI or (b) SalI and probed with clone pUFA-H1 which contains the Avir B_N gene.

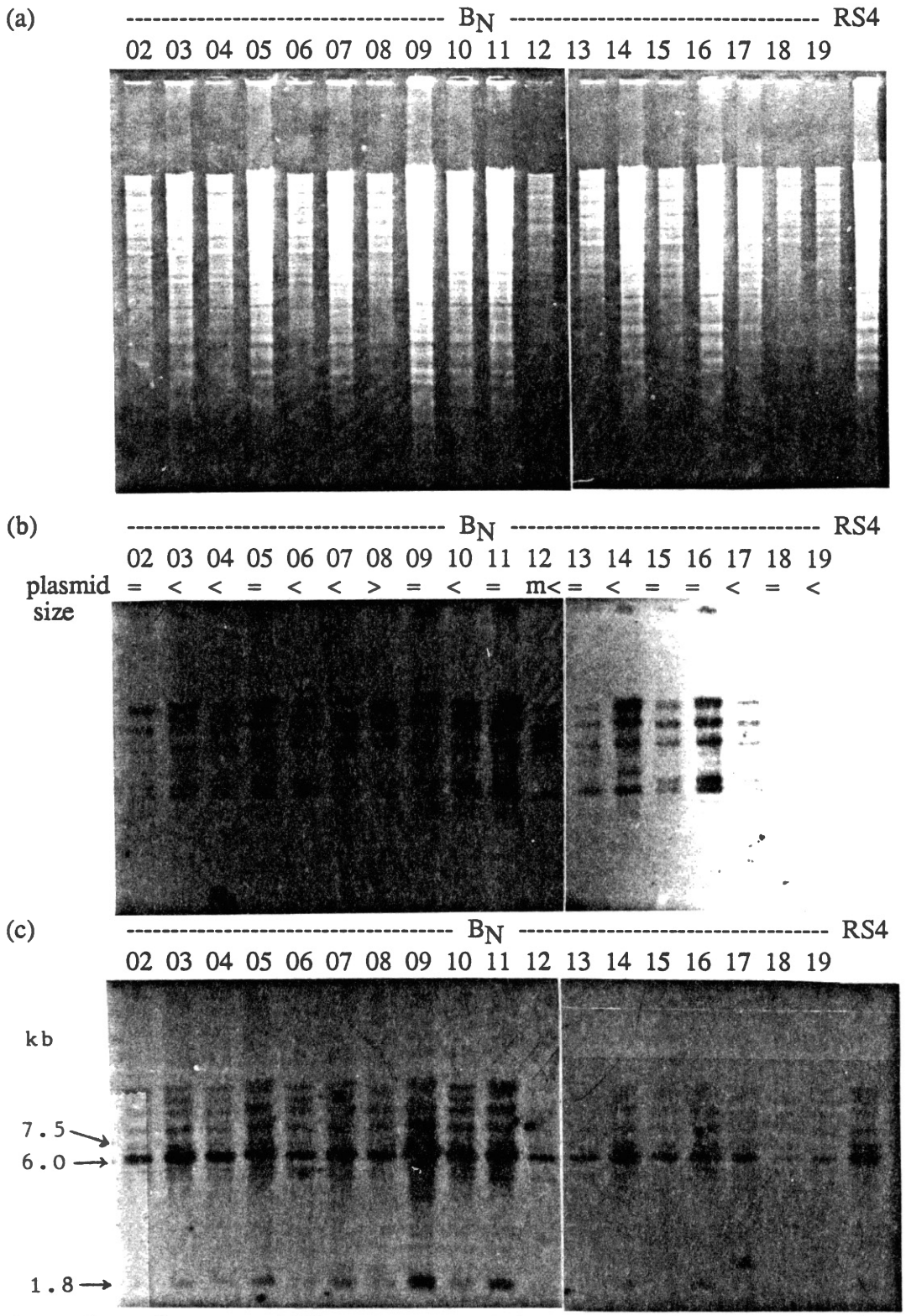


Figure 20. Chromosomal DNA of mutants virulent against AcB_N were (a) restricted with EcoRI, after Southern blotting the blot was (b) probed with indigenous plasmid pTJS (RS4) and then (c) probed with pUFA-H1 which contains the Avir B_N gene.

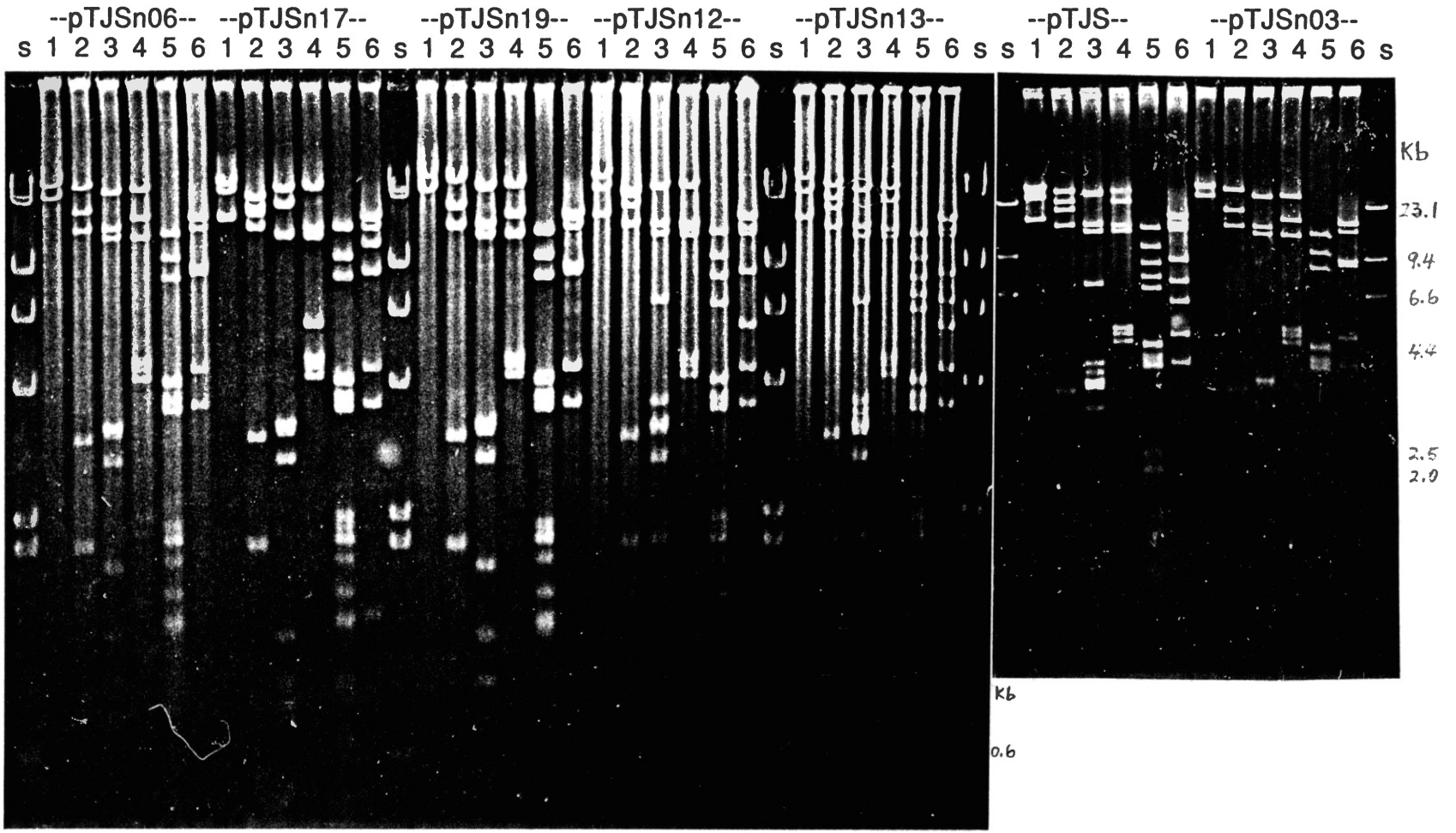


Figure 21. Plasmid restriction patterns of some mutants virulent against AcB_N and their parent (RS4).
S: molecular weight standard (lambda phage restricted with HindIII); 1: HindIII; 2: SalI; 3: BamH1; 4: EcoRI; 5: PstI; 6: SstI.

CHAPTER V

DISCUSSION

Inactivation of MDH by DHC

Since DHC (0.7 mM) in the dark was inactive toward plasmid DNA and DNase I, it is surprising that about 30-40 % of MDH was inactivated in the presence of 0.3 to 1.0 mM DHC in the dark within 1 min (Figure 1). This suggests that the MDH assay system is more sensitive than the DNase I and the plasmid DNA assay system. It also suggests that two different events are responsible for the light-dependent inactivations and for the inactivation of MDH in the dark. Photodynamic compounds have been reported to inactivate enzymes (Spikes et al, 1969; Spikes, 1977). Thus the finding of inactivation of MDH and DNase I by DHC in the light is in agreement with the hypothesis that DHC is a photodynamically active compound.

Unlike most photodynamic agents, the light absorbance maximum of DHC overlaps the absorbance bands of DNA and proteins. Therefore it is possible that the tested materials also contributed to the photodynamic activities observed in this study. However, without the presence of DHC a similar effect was not observed, which minimizes the likelihood of the tested materials being responsible for the effect.

Effects of iron on DNA nicking by DHC

DHC is activated both by light and by Fe(III) in the presence of oxygen. That the single strand DNA band appeared in the presence of Fe(III) and DHC in the dark (Figure 2) is an effect similar to using alkali treatment to separate the double stranded DNA (Fig. 3 of section I). During the possible process of producing superoxide and hydroxyl radicals by

DHC and Fe(III) regardless of whether by the Fenton or the Haber-Weiss reaction, OH^- is simultaneously being produced. This means an increase in pH which could cause "melting" of double stranded DNA (ds. DNA). The melting of ds. DNA in the presence of Fe(III) and DHC in the dark is another piece of indirect evidence suggesting the involvement of the active oxygen species. The extensive degradation of DNA by Fe(III) in the light plus argon is consistent with others' observations (Cernohorsky et al., 1971, Singer et al., 1965).

The photoactivation is not likely to be unique for DHC and LC alone. Other compounds that are important factors in plant disease resistance such as flavonol dihydroxy phenols (catechin, gallocatechin and their condensed proanthocyanidin derivatives)(Bell et al, 1978), and gossypol and the related compounds, may also be light-activated to give them their full biological activity. This is supported by the observation of the nicking of plasmid DNA by catechin plus light (data not shown).

When EDTA at 0.1 mM forms a complex with iron, the EDTA-iron appears capable of catalysing the production of the hydroxyl radical from superoxide plus hydrogen peroxide (Brawn et al, 1981). The addition of Fe(III) reveals the dark action of DHC toward DNA (Figure 2) and the possible presence of iron as a trace contaminant in the TE buffer, which would be chelated by EDTA (1 mM), may have contributed to the light plus DHC nicking of DNA. These considerations suggested that iron is probably the limiting factor in my experimental condition (plasmid DNA assaying system) and the action of DHC in the dark or light may still share a commom mechanism.

Chain reaction initiators used in the polymer industry can be classified into three categories : a) light activated; b) thermoactivated; and c) redox-activated (D'Alelio, 1952). In the process of studying conditions that activate DHC, some other observations besides the role of Fe(III)(redox-activation of DHC) were also interesting. When ammonium persulfate (APS, an initiator used frequently in making polyacrylamide gels) was tested in the plasmid DNA assaying system, it nicked DNA itself in the dark and DHC quenched

this nicking (data not shown). Therefore DHC effectively competed with DNA for the radicals generated by APS. Further experiments could find conditions to minimize the APS degradation of DNA and then test the activation of DHC by APS. After separation of the phytoalexins on a thin layer chromatography (TLC) plate, the conversion of DHC to LC was sped up by heating it in the air (Essenberg et al, 1982). This may be due to the thermolabile character of DHC. By this hypothesis the DHC free radicals generated upon the thermolysis were quenched by oxygen and ended up as LC (an oxidized-DHC). Light activation of DHC which acts as an initiator was demonstrated continuously in this study (Sections I, II). Oxygen is a very special molecule which is extremely vulnerable to free radicals and therefore acts as a powerful quencher in polymerizations involving radicals. It is undesirable both in the polymer industry and in the laboratory (removal of oxygen is necessary in making a polyacrylamide gel).

The involvement of peroxidase, the most abundant enzyme in plant cell walls, in conjunction with catechin and H_2O_2 in producing an inhibitory effect on Xcm has been reported (Venere, 1980). This suggested the possibility of the dark activation of DHC by peroxidase, although DHC does not have *ortho*-phenolic groups, which are characteristic of the best substrates for peroxidase. The conversion of hemigossypol by the action of peroxidase to gossypol (Veech et al., 1976), indicated that the presence of two phenolic groups on the same molecule may not be required for peroxidase action. But due to the nicking of DNA observed with the addition of peroxidase (Sigma)(data not shown) itself, the work was not followed up. Whether this observation is real or due to contamination of DNase in the peroxidase is not known. The beauty of the hypothesis of peroxidase activation of DHC is that in the process of releasing a toxic compound, the plant cell would potentiate it to the maximum toxic state right at the place where the pathogen is located (the cell wall).

DHC^R mutant selection in DHC medium

The instability observed with both spontaneous and UV mutagenized Xcm delayed the isolation of stable DHC^R mutants to test the importance of DHC in the role of cotton's resistance to Xcm. The presence of spontaneously occurring T-type Xcm which had fairly stable DHC resistance, but lower pathogenicity, also complicated the selection of the DHC^R mutant. At least with mutant A1 the instability was not due to the loss of indigenous plasmid pTJS (data not shown). The more gradual and stable increase in the resistant population (%) during the 10 day continuous-selection pressure *in vitro* (Table VI), and the retention of DHC resistance (Table VIII) by O2 in an incompatible reaction *in planta* , which is accompanied by high PA production (Essenberg et al, 1986) suggested that the presence of DHC is required for the maintainance of DHC resistance. The discontinuity of DHC in the medium during the selection of spontaneous mutants A1, A2, A3, A4 and UV mutants 401, 402, 403, 404, 405 may partially account for their reversion. The complete loss of resistance to DHC by O2 in the compatible interaction (very little PA production) is consistent with the theory discussed above. It also suggests the possible disadvantage of retaining the DHC resistance by the pathogen.

In the study of the antibiotic resistance of Xcm, two types of resistance were common (Brinkerhoff, 1970). One type originated apparently as multiple-step resistance and was unstable when passed through the host, or grown on media without the antibiotic. The other type was a single-step resistance and was stable when passed through the host or grown in media without the antibiotic, e.g. streptomycin (Brinkerhoff, 1970), rifampicin (this study). The toxicity of DHC toward bacteria other than Xcm, e.g. *E. coli*, *Salmonella typhimurium* (Figure 6), qualified DHC as an antibiotic, not merely a phytoalexin. The instability observed during the selection of the DHC^R mutants and the necessity of the presence of selection pressure to maintain this character, suggests the DHC resistance is also a multiple-step resistance. The EMB^R Xcm probably possessed the single-step

resistance, therefore it is stable in the absence of selection pressure. If DHC^R requires multiple-step mutations, then single step mutations in Xcm would be expected to give only a partial resistance to DHC. The partial resistance of EMB^R mutants supports this hypothesis.

The fact that they failed to show complete resistance at the concentration used to select these mutants, but did show partial resistant to a lower concentration of DHC may suggest several inferences. First, the partial resistance qualified them as DHC^R mutants. Secondly, complete DHC resistance may be lethal to Xcm. Finally, since the concentrations used in these experiments were far lower than the estimated local concentration of DHC *in planta* (Essenberg et al, 1986), it predicts that differences of the *in planta* bacterial growth between mutants and the parent strain would occur only briefly during accumulation of phytoalexins.

After incubation in the DHC-containing medium for 10.1 days (approximately 72.3 generations) the resistant population (%) of these bacteria ranging from 42 to 62 % (Table VI), was similar to the resistance of mutants isolated by other means (E, MB, EMB, 2,2'-bipyridyl)(Table IX, X, XIII). All of them failed to show complete DHC (0.1 mM) resistance.

The observed frequencies of the DHC^R mutants were higher than the commonly accepted spontaneous mutation frequency (10^{-7} to 10^{-9}). The survivors of the DHC selection were only partially DHC-resistant when subjected to the same level of DHC. My observation of the instability of the DHC-resistant character of Xcm is consistent with the high genetic variability of Xcm that has long been recognized (Brinkerhoff 1963). Other possible explanations for one or both of these observations include: a) DHC itself is a mutagen; therefore at the right concentration it will kill a majority of the Xcm and induce mutations in the rest of the Xcm; b) Xcm has very active transposons which can be activated by DHC and the result of the translocation of a transposon either inactivates the gene(s) that is responsible for the sensitivity to DHC or activates the expression of the

DHC-resistance gene(s) in Xcm. Both instability and high-reversion frequency are characteristic of transposon involvement; c) DHC-resistance gene(s) are encoded in a plasmid which is unstable in the absence of DHC. Plasmids are autonomous self-replicating extra-chromosomal circular DNAs which often carry genes that are not essential for host cell growth. These include genes for antibiotic resistance and for biodegradation of hydrocarbons. Since one can view DHC as an antibiotic, it is possible that the DHC-resistance gene(s) are also carried in a plasmid. Plasmid involvement can easily explain the instability of the DHC survivors and the high reversion. This is due to the uneven distribution of plasmids during bacterial division; d) DHC-resistance is a physiological response of Xcm that is induced only in the presence of DHC or other antibiotics with similar modes of action.

DHC^R mutant selection in EMB medium

Growth of *E. coli* in simple media enriched with Mn(II) resulted in the elevated activity of the Mn-containing SOD (Pugh et al 1984). The enhanced resistance to DHC of RS4, RS8 and EMB81/RS8 with the presence of Mn(II) in the culture NB (Table XI) could be partially due to the elevated activity of inducible SOD (Mn-SOD). It is probably not due to Mn(II) as a limiting nutrient factor in NB, because the doubling time of each tested strain showed no difference with or without the presence of Mn(II). Because exposure of the EMB^R to Mn(II) led to additional enhanced resistance (Table XI), it could be suggested that two mechanisms were responsible for the increased DHC resistance by Mn(II) and selected by EMB.

The possibility that a physiological adaptation of bacteria recovered from a dye-containing medium was responsible for the DHC resistance was ruled out (Table VIII), since bacteria exposed to dyes during a first bioassay were not more resistant to DHC than those that had not exposed to the dyes in a second bioassay. It is thus clear that preadaptation to dye(s) is not responsible for the DHC resistance of the survivors recovered

from E, MB, or EMB plates. It is also clear that isolates recovered from these dye-containing media were mutants whose DHC resistance was stable throughout the three culture periods of the experiment. With RS4 as the parent strain, the DHC resistance of mutants recovered from the MB was similar to that of the EMBa plate but higher than that of the E plate (Table IX). With RS8 as the parent strain, the DHC resistance of mutants from the E plate was higher than that of the MB and the EMBa (Table IX). Again mutants from MB and the EMBa were similar in degree of DHC resistance (Table IX). These findings suggest that different mechanisms are probably involved in Xcm gaining resistance through the selection with different dyes. From the different responses to DHC by mutants derived from two parent strains, it can be suggested that RS4 & RS8 are independent mutants. This is consistant with the time course experiment which revealed the different resistance levels of RS4 and RS8. The highest achieved DHC resistance, assayed at 0.1 mM with mutants derived from RS8, was an isolate out of the E plate. Eosin and methylene blue are well known photodynamic agents. Several EMB^R mutants revealed their DHC resistance both in the dark and in the light (Table X). Since mutant(s) selected for resistance to other photodynamic agents in the light also possessed resistance to DHC in the light, this supports the hypothesis that DHC is a photodynamic agent.

Local concentrations of phytoalexins in the Xcm-cotton system were very low in compatible interactions (e.g., 0.002-0.012 mM DHC) and were higher in incompatible interactions (e.g., 0.23-1.47mM DHC) (Essenberg et al, 1986). In two of the five incompatible interactions tested, the local concentrations appeared to be too low in comparison with the ED_{50} and ED_{90}, values which were determined in the dark, to account for the inhibition of Xcm that occurred *in planta*. However, since light potentiates the toxic effect of DHC to Race 1, EMB7 (Fig. 8, Section I) and RS4 (Figure 7), it is likely that the local phytoalexin concentrations of all the tested incompatible interactions were high enough to be responsible for the inhibition of the bacterial growth that occurred *in planta* in a well-lighted growth chamber. Nature is clever in designing a molecule

(phytoalexin) and using the normal growth condition (light) of plants to activate it and to defend the plants against the invaders (Xcm).

When the *in planta* (Figure 8) and the *in vitro* (Fig. 8, Section I) bacterial growth curves of EMB7 are superimposed, the killing effect of light plus DHC (0.1 mM) *in vitro* seems parallel with that *in planta.* The killing effect observed in the line with at least two resistance genes (OK1.2) occurred earlier than in the line with a single resistance gene (AcB_3). This delay may reflect the phytoalexin dose difference between these two lines. The similar bacterial killing pattern also suggests that the *in vitro* bioassay under the growth chamber light condition, mimics the later period of the *in planta* bacterial growth.

The EMB^R mutant derived from H, a relatively DHC-resistant strain (Figure 3), showed DHC resistance at 0.2 mM, but was completely sensitive when tested at 0.5 mM (Table X). The enhancement of resistance observed at 0.2 mM DHC relative to the parent strain (H) was much less than that of the mutants derived from the most sensitive strains (RS4, RS8). These observations suggest that there is an upper limit to possible resistance reflected either in the resistant population of the bacteria or in the tolerable dose of DHC. These limitations also predict that the limited DHC-resistance gained by these mutants may not be reflected *in planta* by a higher bacterial yield than that of their parents. Several other factors also support this prediction. The DHC resistance of these mutants was observed at a concentration lower than that which develops*in planta.* At least three phytoalexins are present *in planta* in the incompatible interaction, therefore gaining partial resistance to only one of them probably is not enough to combat the phytoalexin flood. The bacterial growth of EMB7 in AcB_3 and OK1.2 as compared to the parent (Figure 8) is consistent with this prediction.

DHC^R mutant selection in iron-chelated medium

In the presence of Fe(III) the absorption spectrum of 2,2'-bipyridyl is altered, which indicates the interaction of these two molecules (data not shown). The mutants that

survived in a medium which had its iron tightly bound by 2,2'-bipyridyl must produce compounds which have higher affinity for iron than 2,2'-bipyridyl and/or produce more of this compound in order to scavenge the iron for surviving. If Fe(III) plays an important role in potentiating the toxicity of DHC to Xcm, then a mutant that could chelate the Fe(III) from the bioassay medium and make it unavailable for DHC, would be expected to be more resistant to DHC. Indeed all the mutants selected in iron-chelated medium were more resistant to DHC (Table XIII). Yet none of the mutants were 100% resistant to DHC at the concentrations tested. This possibly means that the toxicity of DHC is not completely due to the production of superoxide and the hydroxyl radical as deduced from the reaction sequence involving the Fenton reaction (pg. 60). Alternatively, the production of active oxygen species by DHC and Fe(III) may be faster than the production of siderophores. Should the second possibility be the case, then none of the six tested mutants are constitutive siderophore-producing mutants. The direct proof that these mutants are overproducing siderophores will be necessary in the future. The third possibility is that other metal ions present, e.g., Cu(II) may also react with DHC to produce active oxygen species via a similar sequence of reactions and the siderophore produced by the mutants cannot chelate those ions. The iron that was scavenged by the siderophores might enhance DHC-resistance by incorporation into more SOD, catalase to defend against the possible increase of active oxygen species.

Although there is no report of Fe-2,2' bipyridine as a photosensitizer, Ru-2, 2' bipyridine (Ru is in the same group in the periodic table as Fe) is a photosensitizer. Should Fe-2,2' bipyridine also act as a photosensitizer, then the underlying mechanism of resistance to DHC of both the B series and the EMB series of mutants may be similar. This hypothesis is supported by: a) the selection of B series mutants was conducted in a 24 h continuously well illuminated room, and b) a similar degree of DHC resistance of the mutants that survived in the photodynamic agent-containing medium or in the 2,2'-bipyridine-containing medium. However, when mutants B1, B2, B3 and B4 were

inoculated on EMB or EMB plus 2,2'-bipyridyl plates at 10^8 to 10^9 cfu/plate none of them gave a lawn of bacteria which was expected should these two types of mutants share the same mechanism to the same degree. Further experiment could determining whether EMB mutants have resistance to 2,2'-bipyridyl.

The toxic effect of DHC in the dark toward Xcm may still be due to a reaction similar to the photodynamic action of DHC but accomplished by light-independent*in vivo* activation of DHC via processes mentioned by Cilento (1980). This may be why the MB^R, E^R, EMB^R and 2,2'-bipyridyl^R mutants all give a similar degree of resistance to DHC regardless of whether the assay is in the dark or in the light.

Variation of resistance of different strains

The existence of the relatively DHC resistant strains (Figure 3) suggested the possibility of gaining DHC resistance by the sensitive strains. The lag period expressed by those strains that are most resistant to DHC also suggests the induction of a possible defense mechanism in bacteria, either by detoxifying the DHC or by other means. An alternative explanation is the spontaneous oxidation (decomposition) of DHC which occurs during the lag period when the stress (DHC) is eliminated and bacteria start to multiply normally. Since the half life of DHC at 30°C, measured by its fluorescence, is approximately 3 h and because of the presence of strains RS4 and RS8, which never recovered during the 8 h bioassay, it is unlikely that the second hypothesis is the case.

All the strains that are most resistant to DHC probably have a history of exposure to high concentrations of phytoalexins (incompatible interaction) *in planta*. JH and MP were derived from Race3 by selection for growth in Acb7, which is incompatible with Race 3, and they were only partially resistant to DHC. This is consistent with the observation of increasing DHC resistance of R3-O after the exposure to DHC *in vitro* (Table XV).

In summary, when known photodynamic reagents eosin (E) and methylene blue (MB) were used to select E^R, MB^R or EMB^R Xcm from RS4 and RS8, a 280 to 2800 fold

increase in the percentage of the population resistant toward DHC resulted. These compounds are known for producing singlet oxygen, superoxide and H_2O_2. When Xcm was grown in Mn(II)-enriched nutrient broth and then bioassayed in the same medium, presumably with enhanced Mn-SOD, a similar increase in resistance to DHC was also observed. When 2,2'-bipyridyl was used as iron chelator in a selective medium the surviving putative siderophore-overproducers also possessed DHC resistance. Race 1, Race 2, strain N, Race 4 and strain H, which were most resistant to DHC, exhibited a 2-3 h lag before the onset of logarithmic growth, which might be the period of induction of defense mechanisms like SOD, catalase or siderophores. All these results suggest the involvement of detoxification of active oxygen species in the bacterial resistance to DHC.

Does DHC cause the conversion of O to T-type Xcm?

The coincidence of the presence of DHC and the enhancement of T-type bacteria (Table XIV, Figures 9, 10) could be due to any of the following situations : a) T-type bacteria are more resistant to DHC than the O-type; b) T-type bacteria grow faster than the O-type bacteria; c) DHC caused the conversion of the O to T. After determining the growth rate and the DHC sensitivity of both types of bacteria existing in Race 3, it is clear that the partial DHC resistance of Race 3 (Figure 3b) was due to the presence of two subpopulations; the O-type was very sensitive to DHC and grew slowly, whereas the T-type was more resistant to DHC and grew faster (Table XV). Since the O-type bacteria that survived the 8 h DHC selection gained resistance to DHC comparable to the T-type (Table XV), it seems possible that such a conversion from sensitive to resistant bacteria also contributed to the partial DHC resistance of Race3, although the dilution from the DHC at the early hour of the selection delayed the increment of the T-type bacteria (Table XIV). Unfortunately that is not enough evidence to support the third hypothesis mentioned above.

T-type characterization

The idea that T-type Xcm is deficient in xanthan gum and that addition of xanthan gum might restore its virulence to cotton plants was tested. The water-soaked lesion observed with 0.125% xanthan gum alone (pg. 108) was not reproducible, although it did give visible chlorosis in AcB_3. The plant response to sucrose (0.5%) per se was also unreproducible. The positive responses observed previously (as mentioned in Chapter IV) were not due to wounding (the responses were not in ring shape as one would expect from a blunt-ended syringe wounding) nor to possible microbial contaiminants present in the xanthan gum (the gum solution was sterilized). It is most likely that the inoculated spots were too close to each other, therefore the solution itself acted chemotactically to attract the bacteria of the neighboring inoculated spots.

Macroscopic hypersensitive lesions were typical in incompatible interactions of avirulent Xcm and cotton with a single resistance gene. Microscopic hypersensitive lesions were previously observed in incompatible interactions of avirulent Xcm and Im216, which is a line with three resistance genes. The microscopic lesions observed with T2 in the line with single resistance gene B_3, and the macroscopic hypersensitive lesion with T2 in susceptible line Ac44, which does not have any known resistance gene, raised an interesting question. Line Ac44 is used as a standard line to test whether to accept or reject an isolate as a pathogen for cotton, and only those giving watersoaked lesions were counted as cotton pathogens. By this criterion T2 is a non-pathogen to cotton, alternatively one says T2 lost its pathogenicity. However, the hypersensitive lesion in Ac44 to T2 was surprising, since it resembled the reaction of Ac44 to heterologous pathogens (e.g., *X. campestris* pv. *campestris*), rather than to nonpathogens (no visible lesion). Is it possible that Ac44 has an unknown resistance gene which is only revealed by T2? The up-graded resistance responses elicited by T2 in lines possesing a single resistance gene could then be easily explained by the resistance gene dose. If one set aside the issue of whether T is a

pathogen or not and focussed just on the avirulence of T *in planta*, is it possible that T-type bacteria are "super-avirulent" and expressed *in planta* by higher avirulence gene dose ?

The O-type revertant was observed only when T-type bacteria were incubated inside the cotton plant. The presence of sucrose or ground cotton leaf debris did not show a similar reverting effect (data not shown). More work needs to be done to determine the factors which are responsible for the observed *in planta* reversion of T to O-type bacteria.

The frequency of obtaining T-type bacteria from RS4 was in a similar range to that of the spontaneous virulent mutants derived from RS4, although only the macroscopic lesions were counted when spontaneous virulent mutant frequencies were obtained. The possible presence of microscopic lesions due to the T-type bacteria, which were not included in the determination of the virulent mutants frequency, should be negligible, because addition of a small number (microscopic lesions due to T-type Xcm which were not counted) to a large number (macroscopic lesions of O-type Xcm which were counted) which is one to ten thousand fold larger and which was used as a denominator will not result in much change in the ratio. Since continuous subculturing during a 4 day period greatly enhanced the occurrence of T-type bacteria (Table XVI), it not only suggests that the *in vitro* condition increased the loss of pathogenicity but also agrees with the observation that prolonged incubation enhanced the loss of pathogenicity (parasitism) (Brinkerhoff, 1963, Gabriel, 1986). Because of the different physiological responses elicited by T-type and O-type Xcm using prolonged subcultured Xcm to do an experiment could complicate the interpretation of one's data such as gaining avirulence.

In an incompatible reaction, the presence of a resistance gene from the host and the avirulence gene from the pathogen are necessary. The avirulence genes of Xcm are believed to be dominant (Gabriel et al, 1986). The loss of the avirulence gene X will produce a compatible interaction *in planta* (X-line) and gaining the avirulence gene Y will produce an incompatible interaction *in planta* (Y-line). The frequencies of obtaining T-type bacteria and virulent mutants from RS4 were similar and are higher than the expected

spontaneous mutation frequency. The phenotypical behavior of these bacteria in relation to congenic cotton lines and the possible involvement of different avirulence gene doses leads to a speculation as to the relationship between these two types of mutants. Since well-isolated water-soaked lesions of the virulent mutants appeared at the same time, regardless of the cotton line used, it is less likely that the mutations are induced by the plant or by phytoalexins. It more likely was an event occurring during the *in vitro* culture of the inoculum. Interestingly, out of the same parent emerges one subpopulation which gained avirulence (T-type) and another subpopulation (virulent mutants) which lost avirulence at a comparable rate. The possible involvement of a transposable element can easily explain the observed comparable high frequency, as well as the lost and gained phenotype of these two subpopulations. It is tempting to speculate that a very unstable factor (insertion element, transposon) was involved in the process. If this hypothesis is true, theoretically one should be able to find a colony with a sector of T-type and a sector of a race-changed mutant.

The T-type bacteria produce colonies that look yellower and thinner than the O-type bacteria and the color difference was more profound in a minimal medium. Perhaps T-type produces more xanthomonadins so that it looks yellower and produces less xanthan gum and/or membrane protein so that it looks thinner. The highly double-bonded xanthomonadin resembles the structure of the carotenoids chemically and is likely to mimic the singlet oxygen quencher ability of the carotenoids. The possibly higher level of xanthomonadin produced by T-type bacteria may partially contribute to its greater resistance to DHC than the O-type bacteria by acting as an endogenous active oxygen quencher.

The microscopic lesions of dilute inocula of T2 in AcB_3 could be due to several causes: 1) T is a "super" elicitor in AcB_3, either in terms of the amount of phytoalexins being produced, or in terms of the earliness of the production of phytoalexins. The consequence effectively restricts T to give a response mimicking the incompatible reaction in cotton with more resistance genes. 2) T is supersensitive to phytoalexins, therefore there

is much less bacterial multiplication and much smaller lesions. The bioassays of *in vitro* toxicity of DHC toward these two types of bacteria (Table VII) does not support this hypothesis.

Pathogenicity negative mutants of *Xanthomonas campestris* pv. *campestris* were reported to be deficient in extracellular protease and pectate lyase (Daniels et al, 1984). An extracellular protease-deficient mutant of Xcm has decreased pathogenicity (Gholson, Rogers and Essenberg, unpublished work). Protease deficient T2 elicited barely visible symptoms when syringe inoculated into several cotton lines. Yet protease proficient T2 at a comparable inoculum concentration did give visible chlorotic lesion in Ac44. Therefore, the involvement of protease in the *in planta* response of T2 is consistent with the role of protease in pathogenicity (Daniels et al, 1984). The polygalacturonate lyase activity of T2 remains to be determined.

Gabriel (1986) mentioned the commonly observed loss of parasitic ability of plant parasites upon repeated transference in agar media. The enhanced occurrence of T-type bacteria under continuous selection pressure to select for DHC^R mutants and the similar enhancement during multiple subculturing are consistant with that statement.

Some EPS deficient *Erwinia* produced only occasional small lesions that were not water- soaked (Panopoulos et al, 1985) resembling the *in planta* response of T-type bacteria. Avirulent mutants of *P. solanacearum* which had rough (incomplete) LPS were noticed. Pleiotropic phenotypes of many studied avirulent mutants of *P. solanacearum* are not due to the loss of plasmid (Panopoulos et al, 1985). Although T2 acted avirulent in Ac44 and the colony morphology resembled that of avirulent *P. solanacearum* , unfortunately the EPS or the LPS profiles of T2 remain to be determined. However, T2 did retain the plasmid which has the same size as the pTJS (Figure 16d).

Race-changed mutants

The relatively high spontaneous mutation frequency observed with respect to the recovery of virulent mutants from incompatible cotton lines is consistant with the observed frequency of T-type bacteria. Both of the frequencies were much higher than that of spontaneous drug-resistant mutants (e.g. 2.5 x 10^{-8} for rifampcin). The ease of obtaining virulent mutants presumably due to inactivation of the corresponding avirulence gene, suggests the possible involvement of an unstable element such as an insertion element. Unfortunately, attempts to use available*E. coli* cosmid clones which harbor cultivar-specific avirulence genes as probes to explore this possibility failed to support this hypothesis. Of the two sets of mutants which were virulent in AcB_3 or AcB_N, no apparent polymorphism was observed between mutants and the parent (Figures 18, 19).

Several possibilities exist; the simplest is that there were no transposons or insertion elements involved in these race-changed mutants. Secondly, there may be insertion element involvement, but the size of this element is small with no restriction sites for EcoRI, BamH1 or SalI. Therefore, the resulting insertion was beyond the limit of resolution of the gel. Thirdly, the involved element may not be small, but have restriction sites for EcoRI, BamH1 and SalI. The insertion was close to the restriction sites of the chromosome, so that upon digestion of the chromosomal DNA with these restriction enzymes, the resulting pattern of mutants was not different from that of the parent. Fourthly, the race-changed mutants may have inactivated control genes which are necessary for expression of the normal avirulence gene. Therefore using clones which harbor corresponding cultivar-specific avirulence genes as probes, one would fail to detect any insertional inactivation in the control genes. Under this theory one also assumes a) the recipient strain N in the reported work of cloning cultivar-specific avirulence genes (Gabriel et al, 1986) was defective in the avirulence gene itself but has normal a control gene; b) the donor strain H in the same work was normal in both the avirulent and control genes; c) the parent strain of these race-changed mutants was as normal as strain H.

The simultaneous acquisition of virulence in several congenic cotton lines of many of the race-changed mutants supports the hypothesis that control genes exist and are necessary in incompatible reactions. If a control gene could be in charge of several avirulence genes, then inactivation of one gene could result in the loss of avirulence in several lines simultaneously. The existence of the mutants which acquired virulence simutaneously in AcB_6, Stoneville-2B-S9 and Gregg, suggests the presence of an avirulence-controlling gene in Xcm or there is a common resistance gene in these three lines. This latter possibility could be tested by conducting a homology test crossing these lines with each other and checking the ratios of the F_2 progeny (Gabriel, 1986).

The consistent observation that approximately 50% of the race-changed mutants from AcB_N have suffered a major alteration in their plasmid size but that mutants from AcB_1, AcB_3 and Acb7 have not suggests that the avirulence-controlling gene of AcB_N probably is plasmid-borne. The possible correlation of plasmid with virulence in AcB_N was further strengthened by the observation of plasmid size differences among Race 4, Race 7, Race 10 and strain N, since all of these strains are known to be virulent in AcB_N. The avirulence-controlling genes of AcB_4-AcB_5 and AcB_6-(Stoneville-2B-S9)-Gregg may also be plasmid-borne due to the observed alteration in plasmid size.

The cross-hybridization of indigenous plasmid pTJS with the chromosomal DNA indicates the presence of homologous sequences. Since pTJS is fairly large (80 kbp), some of the qualities of a plasmid, which are the basis of methods for separating plasmids from the chromosomal DNA, may be diminished. Therefore, instead of chromosomal DNA one may easily obtain a total DNA. Because the restriction pattern of pTJS (Figure 21) was different from that of the pTJS-chromosomal DNA hybridization (Figure 20), it is legitimate to claim the homology shown in Figure 20 was between the plasmid and the chromosome. Since the fragments which were hybridized with pUFA-H1 did not overlap with the fragments that were hybridized to pTJS this is consistent with the finding that Avir-B_N was chromosome-borne (Gabriel et al, 1986). The presence of the same

repetitive sequences (RS) in both plasmid and chromosome, and evidence that some of these RS serve as primary sites of plasmid integration and excision is known for *P. syringae* pv. *phaseolicola* (Curiale et al, 1982, Quant et al, 1984, Szabo et al, 1984a,b). Such episomal properties have long been known for the F' plasmid of *E. coli* and were also documented in *Erwinia* spp. (Panopoulos et al, 1985). The polymorphism observed in the cross-hybridization of pTJS with the virulent AcB_N mutants' chromosome and its correlation of the major alteration in the plasmid size (Figure 20) suggests that the homologous sequences may serve as the sites of integration and excision. The sequence homology could enhance homologous recombination, resulting in integration of pTJS. By a similar mechanism, due to crossing over at a different site (imprecise excision), an alteration in the excised plasmid could result. Depending on the closeness of the integration and excision sites, both major and minor alterations are possible. The possible generation of new restriction sites and the alteration of size would result in polymorphism in both chromosome and plasmid. It is not known at this time if these homologous sequences in Xcm are repetitive sequences.

HindIII, SalI and BamH1 gave plasmid fragments larger than 23.12 kb. Due to the limitation of resolution of the gel, the size estimations of these fragment are not precise. Therefore, the size estimation of fragments from the other three restriction enzymes were used to determined the plasmid size. However, should a field inversion gel electrophoresis system (FIGE) was used the limitation mentioned previously will be overcomed by the better resolution. Although the plasmids are extremely large, the results of analysis of pTJSn12 in 0.4% agarose gel is sufficiently clear to reveal at least a difference of 7.5 kb in the intact plasmid. Since pTJSn12 had the least deletion among the plasmid-size-changed mutants, all the variations of the plasmids observed in this study involved major deletions or insertion (pTJSn08) greater than 7.5 kb. If one assumes 1 kb for each gene, then at least seven genes are "lost" or "gained" in all these mutants. But due to the existence of homologous sequences between pTJS and the chromosome it may be that these "genes" are

not lost but simply changed in location. Further, the loss of the specific SstI fragment of pTJSn12 as compared to the parent plasmid (Figure 21) may lead to the finding of the putative avirulence-controlling gene of AcB_N.

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