USEFUL MINIMAL MEDIUM FOR GROWTH OF *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM*, MOLECULAR BIOLOGICAL ANALYSIS OF AVIRULENCE IN *XCM*, AND IDENTIFICATION OF MULTIPLE RESISTANCE GENES IN THE SEGREGATING *GOSSYPIUM HIRSUTUM* LINE ACALA B5

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LIST OF ABREVIATIONS

AA	amino acid
Ac	Acala
avr	avirulent
CERL	Controlled Environment Research Laboratory
cfu	colony forming units
IS	insertion sequence
kbp	kilobasepair
KB(I)	King's medium B
LB	Luria-Bertani medium
MM	minimal medium
NB	Nutrient broth
OD	optical density
R	resistance
SR.CM	spontaneous race-change mutant
Tn	transposon
vir	virulent
Xcm	*Xanthomonas campestris* pv. *malvacearum*

CHAPTER I

INTRODUCTION

The host–pathogen interaction between cotton, *Gossypium hirsutum* L., and its bacterial pathogen, *Xanthomonas campestris* pv. *malvacearum* (E.F. Smith) Dye (*Xcm*), has as one of its primary characteristics race/cultivar specificity. That is, there are multiple races of *Xcm* that interact with *Gossypium* sp. carrying either none, one, or several resistance (R) genes such that the interaction is either compatible or incompatible. It was the presence of race specificity analogous to that of certain fungal/plant interactions in which the genetics of both the host and the pathogen could be studied by sexual crosses that led Brinkerhoff (1970) to propose that the *Xcm*/cotton system constitutes a gene–for–gene genetic system. That is, if *Xcm*/cotton posessess a true gene–for–gene interaction, *Xcm* would be found to contain avirulence (avr) genes conferring recognition by host lines that possess the corresponding, specific resistance (R) genes. Bacterial blight of cotton has been extensively reviewed by Verma (1986).

Verification of gene–for–gene genetics for the *Xcm*/cotton system constituted the primary goal of this work. To do this, a molecular biological approach was undertaken. My role in this project was to obtain mutants of *Xcm* by transposon mutagenesis having altered race/cultivar specificity and then to clone the transposon–labeled avr gene. Concurrently, a shot–gun approach was undertaken by Dr. Gabriel. Both approaches encountered difficulty due to the recombination proficiency of *Xcm* and required extensive searching for molecular biological protocols and tools suitable for *Xcm*. Chapter 3, "Useful minimal medium for growth of *Xanthomonas campestris* pv. *malvacearum*," deals partially with the search for useful tools.

Since the use of transposon mutagenesis directly into *Xcm* was not productive and putative avr genes were being identified by Dr. Gabriel (1986), the approach undertaken was modified to characterization of of a putative avr gene–containing clones. Chapter 4, "Molecular biological analysis of avirulence in *Xcm*," handles this. Cosmid clone, pUFA809, was one of four avr clones which gave activity when screened on the cotton line Ac B_5. Ac B_5 was noted to be segregating for an unknown number of resistance genes when single progeny rows were screened for resistance to race 1 in the field in the summer of 1984. As a necessary adjunct to the molecular characterization of pUFA809 and the other avr B_5 clones, a study of this segregating population was done to identify plants possessing single R genes and new races of *Xcm* differentiating among them, Chapter 5, "Identification of multiple resistance genes in the segregating *Gossypium hirsutum* line Ac B_5."

CHAPTER II

LITERATURE REVIEW

Introduction

The following is a brief description of aspects of the physiology and genetics of Xcm, cotton and its interaction. This review is not comprehensive and simply provides a framework for the experimental chapters. The molecular genetics of host/pathogen interactions was, at the start of these studies, a budding field. It was exciting to see the molecular genetics of host/pathogen interactions blossom rapidly during the somewhat brief period of my graduate training.

Xcm / Cotton

The race/cultivar specificity by *Xcm* to various cotton lines was described in detail by Brinkerhoff (1970). Since *Xcm* is a haploid organism with little–understood genetics, it was not possible to apply the techniques of classical genetics to prove whether the system obeyed gene–for–gene genetics. The presence of multiple races of *Xcm* differing in their reactions toward unique R genes constitutes a prerequisite for a gene–for–gene system, as does the need for the host genetics to be well defined. Hunter and Brinkerhoff (1963) developed many single R gene lines of cotton having well–defined genetics.

The physiology of the interaction is well characterized. *Xcm* is known to induce the accumulation of phytoalexins in cotton cells adjacent to infection sites in incompatible interactions at levels sufficient to be bacteriostatic in culture (Essenberg *et al.*, 1985 and Pierce and Essenberg, 1987). Physical events associated with the interaction are also well

defined (Cason *et.al* , 1977, 1978; Essenberg *et al.*, 1979a, 1979b; Al–Mousawi et al., 1982a, 1982b, 1983; Morgham *et al.*, 1988). It is not known if the accumulation of phytoalexins is the primary resistance response of recognition of *Xcm* by cotton.

Gene–for–gene Genetics

Flor (1947) analyzed the interaction between flax and its rust, *Melampsora lini*, in terms of both the pathogen and the host genetics. He proposed that the interaction could be understood by considering avirulence to be the dominant trait which in the presence of a corresponding resistance gene leads to the production of an incompatible interaction in a one–on–one, gene–for–gene manner. The other race and host combinations where complementary avr and R genes are not present lead to a compatible interaction.

The quadratic check shown in Figure 1 illustrates the reactions resulting when a dominant avr gene or its virulent allele interact with a specific, dominant R gene or its recessive allele. The interaction depicted is for a haploid pathogen. The only incompatible interaction is the one between the dominant R gene and the correponding dominant avr gene (upper left quadrant). All other interactions are compatible, except the lower left quadrant (the interaction between a vir allele of an avr gene and a dominant R gene) is weakly incompatible. This last sort of interaction might be the basis for combinations of R genes that lead to broad resistance, or immunity, against many races even when the proper gene–for–gene complements are lacking (Nelson, 1978.

Also shown in the figure is the result of a mutagenic action on the avr gene that converts the phenotype of the avirulence gene to that of virulence. This phenotypic difference would allow detection of the avr gene by hybridization, if the mutagen used is a transposon. This scheme was the theoretical basis for the Tn–tagging of avr genes attempted in *Xcm* in this work. A complication which might interfer with this is complementation of the Tn–tagged gene back to incompatibility must be possible when the

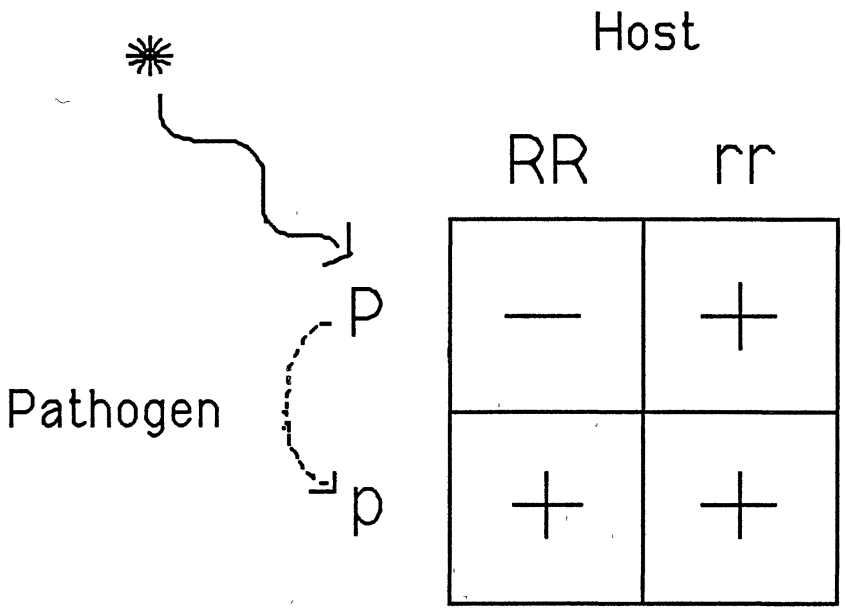


Figure 1. Quadratic Check Showing Result of Mutagenic Action on P. For the pathogen, P and p are symbols for an avirulence gene and its allele while, for the host, R and r are symbols for a resistance gene. The symbols + and -, respectively, indicate a compatible or incompatible interaction. The symbol, ✳, and the wavy dashed line indicate a mutagenic event acting on P while the curved line from P to p indicates the resulting phenotypic change of mutation from P to p.

avr gene and the Tn–tagged avr gene both are present as a merodiploid. Hence, the possible interaction may not be restored to as high a level of avirulence as the parent race prior to its mutation. If the plasmid undergoes marker exchange with the chromosome followed by loss of the vector, then the level of incompatibility might be indistinguishable from the level contributed by the parent strain of both the complementing plasmid insert and of the mutant being complemented..

Person (1959) described a mathematical treatment of gene–for–gene genetics of multiple races and cultivars. This tool allows statistical clustering methods to be used to refine the interpretation of many disease interactions, even poorly understood ones (Lebeda and Jendrulek, 1987). One disadvantage to this method is that fewer races are distinguishable in the absence of single–gene resistance plant lines than if all the resistance genes are represented independently in separate lines. For example if races 1 and 2 each carried 2 compatible alleles for avirulence toward lines A and B and had different alleles toward line C, then lines A and B would appear to contain the same R gene unless the R contributed by each gene was quantitatively different than for line C which is distinguished by races 1 and 2.

Cotton Genetics

The cotton used in this study is the species *Gossypium hirsutum* which is a new world upland cotton species. There are 31 wild species and three other cultivated species of *Gossypium* sp. worldwide (Verma, 1986). Cotton occurs both as diploid and as allotetraploid species with n = 13. Two of the cultivated species, *G. hirsutum* and *G. barbadense*, are allotetraploids possessing both an old world genome (the D–genome) and a new world genome (the A–genome). Some allotetraploids follow true diploid genetics, although multiallelism also may occur (Fehr, 1987).

Verma (1986) in his review of bacterial blight of cotton gives a table of the R genes known in cotton for bacterial blight. A synopsis of this table and one presented by Brinkerhoff (1970) is shown as Table 1, with the addition of the uncharacterized gene in the cultivar Gregg, the putative new genes found in the segregating cotton line Ac B_5, and the gene B_{12} found by Wallace and El–Zik (1989). The total number of R genes identified for bacterial blight is then at least 20. It is not known if all the R genes are unique, since homology tests for all pairwise combinations have not been done (Brinkerhoff, 1970). Certain of these genes are no longer available in cultivars (Brinkerhoff, 1970).

Brown (1961) was the first to note homologous pairings of A and D genomes during meiosis in *G. hirsutum*. Although no chiasmata were seen, it remains possible that such events do rarely occur. If they do, mixing of the genomes would occur. Perhaps such an event would allow breakage of a tight linkage group of R genes and distribute the genes among the A and D genomes. This conceivably could perturb *cis* regulation of expression and allow unseen genes to become observable. Barrow *et al.* (1973) describe their interpretation of virescent progenies with sectored, somatic twin spotting in cotton in this manner.

Brinkerhoff *et al.* (1984) obtained broad resistance, immunity, to all 17 then known races of *Xcm* by development of the line Im 216 containing the R genes B_2, B_3, and b_7 in the Acala background. Im 216 also may possess the polygenic complex from Stoneville 2B–S9, B_{Sm}. The broad resistance of Im 216 suggests that a portion of resistance may be due to recognition of other avr genes by non–complementary R genes in a way such that the sum of low specificity recognitions confers broader incompatibility. Van der Plank's (1963) concept of horizontal versus vertical resistance is of this category; the current understanding brought from the cloning avr genes from different sources indicates that all incompatible interactions may be explained in a gene–for–gene or gene–for–genes (where a single R gene conditions recognition of multiple avr genes) manner (Gabriel, personal communication).

TABLE 1

COMPILATION OF BLIGHT RESISTANCE GENES

Gene symbol	Source of resistance	Inheritance determined by
B_1	Uganda B31 (*G. hirsutum*)	Knight and Clouston (1939)
B_2	Uganda B31 (*G. hirsutum*)	Knight and Clouston (1939)
B_3	Schoeder 1306 (*G. hirsutum* var. *punctatum*)	Knight (1944)
B_4	Multani strain NT 12/30 (*G. arboreum*)	Knight (1948)
B_5	Grenadine white pollen (*G. barbadense*)	Knight (1950)
B_6	Multani strain NT 12/30 (*G. arboreum*)	Knight (1953a)
B_7	Stoneville 20 (*G. hirsutum*)	Knight (1953b)
B_8	*G. anomalum*	Knight (1954)
B_{9K}	Wagad 8 (*G. herbaceum*)	Innes (1966)
B_{10K}	Kufra Oasis (*G. hirsutum* var. *punctatum*)	Knight (1957)
B_{9L}	Allen 51-296 (*G. hirsutum*)	Lagiere (1959)
B_{10L}	Allen 51-296 (*G. hirsutum*)	Lagiere (1959)
B_{11}	Wagad 8 (*G. herbaceum*)	Innes (1966)
B_{12}	S295	Wallace and El-Zik (1989)
B_{In}	1-10B (*G. hirsutum*)	Green and Brinkerhoff (1956)
B_N	Northern Star 20-8 and 20-3 (*G. hirsutum*)	Green and Brinkerhoff (1956)
B_S	Stormproof No. 1 6-77 (*G. hirsutum*)	Green and Brinkerhoff (1956)
B_{Sm}	Stoneville 2B (*G. hirsutum*)	Bird and Hadley (1958)
B_{Dm}	Deltapine (*G. hirsutum*)	Bird and Hadley (1958)
$B_?$	Westburn 70 mutant (*G. hirsutum*)	Brinkerhoff *et al.* (1978)
B_{Gregg}	unidentified gene(s) (*G. hirsutum*)	Hunter *et al.* (1968)
B_5 44-21	Segregants from Ac B_5 (*G. hirsutum*)	This work.
B_5 65-7		"
B_5 55-18		"
B_5 65-31		"

Cotton was recently regenerated from explant callus in tissue culture (Umbek *et al.*, 1987). Eventually, application of the tools of genetic engineering to resistance in cotton may be possible. A full understanding of the interaction between an avr gene and a R gene awaits cloning and analysis of an R gene at the level of DNA. Perhaps, systematics may be applied to both the avr gene and R gene sequences and would describe the possible biochemical role each gene product plays.

Xcm, Physiology and Genetics

Xcm is a gram–negative, strictly aerobic, motile, yellow–pigmented bacterium. The species *Xanthomonas campestris* contains 125 pathovars, including *malvacearum*, distinguished by their pathogenicity on host species. This classification and nomenclature is unwieldy and probably in error since pathogenicity tests of every pathovar have not been done on every known host for a xanthomonad. Lazo and Gabriel's (1987) studies of RFLP linkage groups within *Xc* pvs. indicated that defined pathovar groups occur.

The yellow pigments or xanthomonadins of *Xanthomonas* spp. are a set of brominated aryl–polyene esters (Starr, 1983). Production of xanthomonadin is the primary factor distinguishing *Xanthomonas* spp. from the plant pathogenic *Pseudomonas* spp. Another feature of the physiology of *Xanthomonas* spp. is the production of copious amounts of exopolysaccharide slime, xanthan gum. This slime is most likely the factor which leads to the predominant water–soaking observed in compatible interactions. Under optimal conditions, lesions in the field have been noted to exude slime and are quite glossy in appearance (Brinkerhoff *et al.*, 1984).

Virulence factors within *Xanthomonas* spp. have begun to be characterized. Daniels *et al.* (1986) have identified genes for pectinolytic and proteolytic enzymes associated with the pathogenesis of *Xc.* pv. *campestris* on crucifers. Gholson *et al.* (1990) has identified several exo–proteolytic enzymes in *Xcm* that seem to be associated with

virulence. Prior work in this lab has shown that the phytoalexins 2,7–dihydroxycadalene and lacinilene C and lacinilene C 7–methyl ether are bacteriostatic to growth of *Xcm* in culture, and have greater toxicity to *Xcm* in the presence of light (Essenberg *et al.*, 1986; Sun, 1987; Sun *et al.*, 1988 a and b; Steidl, 1989; Essenberg *et al.*, 1990). At high enough concentrations the phytoalexins can kill *Xcm* grown in the dark. Although the production of phytoalexins is probably the biggest component of resistance, there is no direct evidence linking induction of phytoalexin production with avr gene expression. Correlative evidence given by Pierce and Essenberg (1987) found that the levels of phytoalexins in the dead plant cells adjacent to infection sites in an incompatible line contained sufficient levels of phytoalexins to be bacteriostatic while cells distant from the infection sites did not.

Brinkerhoff noted in the title of his 1970 review on *Xcm,* that it is a highly variable pathogen. The presence of possibly more than 20 R genes means that there are in excess of 20! or 2 X 10^{18} possible races. The actual number identified has been far lower. New races have appeared that overcome the resistance in current cultivars. In 1982, new isolates from Africa which overcame the immunity obtained by the B_2, B_3, b_7 gene combination appeared (Follin, 1981). A new cultivar was found by Girardot *et al.* (1986) which is highly resistant to both the African isolates and other known races. Wallace and El–Zik (1989) have shown it to contain a single new gene, B_{12}. However, this new gene may not prove to condition durable resistance. Table 2 gives the *Xcm* isolates and their sources used in these studies.

Brinkerhoff (1967) noted that sometimes colonies of *Xcm* form sectors on plates. I also saw this in one experiment (data not shown) where *Xcm* was grown in the presence of kanamycin for over one month. Schnathorst (1969) found race change mutants of *Xcm* occurring after successive passages of race 1 through leaves. Brinkerhoff (1963), Pierce (unpublished), Hall (unpublished), and Sun (1987) have all noted the high frequency with which spontaneous mutants having altered race phenotypes are obtained. Such spontaneous–race change mutants, SRCMs, have been seen in pvs. *vesicatoria* and *citri*

TABLE 2

SUMMARY OF *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM* STRAINS USED IN THIS STUDY

Strain	Purified from:	Source prior to purification	Reference
A	R2 Str^r b	M. Essenberg	Lazo *et al.* (1987)
B	R2 Str^r a	"	Lazo *et al.* (1987)
C	HV-1 a	L. Bird	Gabriel, unpublished
D	R2	"	Lazo *et al.* (1987)
E	R4-1	M. Essenberg	"
F	R1 Str^r a	"	"
G	R1 Str^r b	"	"
H	R4-2	"	Gabriel *et al.* (1986)
I	R3	"	Gabriel, unpublished
J	HV-3	L. Bird	"
K	HV-1 b	"	"
L	HV-7	"	"
M	R15	"	Lazo *et al.* (1987)
N	R2 a	"	Gabriel *et al.* (1986)
O	R2 b	"	Lazo *et al.* (1987)
Q	R1 a	M. Essenberg	Gabriel, unpublished
R	R1 b	"	"
S	R3-22	"	"
T	R18	"	"
U	R1	L. Bird	Lazo *et al.* (1987)
V	R3	"	"
W	R6	"	Gabriel, unpublished
X	R18	"	Lazo *et al.* (1987)
Y	R7a	"	"
Z	R7b	"	"
R3-22	R3-6 Str^r	M. Essenberg	Gabriel, unpublished
R1 Str^r		M. Essenberg	Hunter *et al.* (1968)
R2 Str^r		"	"
R3-6 Str^r		"	"
R7$_{1-4}$	R7	L. Bird	Essenberg, unpublished
R10(18)	R18	"	Hunter *et al.* (1968)
R18		M. Essenberg	Hussain and Brinkerhoff (1977)
RS4	R3 Str^r Rif^r	T.-L. Sun	Sun (1987)
TS5.1	RS4 via plant screening	T.-L. Sun	Sun (1987)
R18 Rif^r	selected on Rif KB plate from R18		This work
KM1, KM2	H via plant screenings		This work
KM3-KM46	H or KM1 via plant screenings		This work

(Stahl and Dahlbeck, 1981 and Tu *et al.*, 1989). For pathovars *vesicatoria* and *citri*, endogenous insertion sequence (IS) elements have been shown to be involved in mutational events (Kearney *et al.*, 1988; Tu *et al.*, 1989). The high rate of mutation indicates that *Xcm* also may possess endogenous IS elements. This last aspect is dealt with further in Chapters 4, 5, and 6.

Molecular Biology of *Xcm*

At the start of this project, no published accounts dealt with the application of molecular biology to any xanthomonad. Some success had been obtained with pseudomonads and this provided the framework for the molecular biological approaches taken. Since no prior work using a molecular biological approach to the study of *Xcm* had been published, considerable effort was expended to find a tools that would work in *Xcm*, for example, a conjugation system for plasmid transfer where the origin of replication would function in *Xcm* was needed. The major obstacle for all genetic transfer techniques is the presence of three type II restriction endonucleases, *Xma*I, *Xma*II, and *Xma*III, and one type I restriction endonuclease (Endow and Roberts, 1977; Kunkel *et al.*, 1979) which may degrade any DNA entering a cell prior to its methylation by the corresponding methylase function of the restriction/modification system in *Xcm*. Whether a piece of DNA enters the cell and remains stable is dependent on the balance between restriction and modification.

CHAPTER III

USEFUL MINIMAL MEDIUM FOR GROWTH OF *XANTHOMONAS CAMPESTRIS* PV. *MALVACEARUM*

Introduction

The goal of this work was to provide a medium for auxotrophic counter—selection of *Escherichia coli* strains in conjugation systems while maintaining growth of *Xcm*. Ornston and Stanier's medium (Ornston and Stanier, 1966), developed for growth of *Pseudomonas putida*, was compared to a modified Neidhardt's medium originally used for the growth of *E. coli* (Neidhardt, *et al.*, 1974). Other comparisons included determination of a suitable buffering system, carbon source, and amino acid additives. Some of the following data were presented at the American Phytopathological Society meeting of August, 1984 (McNally, *et al.*, 1984). Hereafter, the modified Neidhardt's medium is referred to as MOPS MM1.

Materials and Methods

Bacterial Strains and Growth Conditions

The following strains of *Xcm* were used in these studies: *Xcm* R3–22 (Essenberg, unpublished) and *Xcm* H and *Xcm* S for the bacterial growth curves and dry weight measurements, *Xcm* Y for the spectrum determination, and *Xcm* A through O and N through Z for the amino acid component tests (Gabriel *et al.*, 1986, Lazo *et al.*, 1987, Gabriel, unpublished). Descriptions of these strains appear in Table 2. *Xcm* strains were

grown in liquid culture at 30° C with rotary shaking at approximately 150 rpm while solid media on which *Xcm* were plated were also incubated at 30° C but without shaking.

Media

King's medium B (KB), Ornston and Stanier's medium, and Neidhardt's medium were used in this study. Table 3 gives the composition of each of the basal media. Stanier's medium was prepared according to Ornston and Stanier (1966) with 137 mM glycerol or 10 mM glucose as the C–source and sometimes 2 mM proline as an amino acid addititve. For Neidhardt's medium, usually both NaCl and H_3BO_3 were omitted as specified in Results and Discussion; otherwise, the medium was prepared as per Neidhardt (1972). Other media were based on the same inorganic ion components with the buffering system varying among Tris, HEPES, or P_i at pH 7.2 or no buffer (which had a small buffering capacity from the 4 mM Tricine and the amino acids added). For all the Neidhardt's media, the C–source was most often 137 mM glycerol with 68.5 or 10 mM glucose or 137 mM sorbitol (in the presence or absence of glycerol) being used less frequently. Table 3 gives the amino acid, vitamin, and nucleotide concentrations used when adding various combinations. When solid media were required, agar was added at 1.5% (w/v) prior to autoclaving.

Plate Tests for Combinations of Amino Acid
and Other Additives

Xcm R3–22 and A through O and Q through Z were streaked onto a KB plate using sterile toothpicks and allowed to grow for 2 days until growth along the streak was confluent. This KB plate then served as the plate for making replicas onto a series of eleven MOPS MM plates containing various combinations of nutritional supplements: casamino acids, yeast nitrogen base, amino acids, vitamins, and nucleotides. A twelfth

TABLE 3

MEDIA COMPOSITION AND ADDITIVE AMOUNTS

Medium	Amount	Component
King's Medium B (KB) (King, *et al.*, 1954)	2%(w/v)	Proteose Peptone No. 3
	137.0 mM	Glycerol
	8.61 mM	K_2HPO_4
	6.09 mM	$MgSO_4.7H_2O$
		pH 7.2 w/ HCl
Ornston and Stanier's (1966)	0.75 mM	Nitriloacetic acid
	2.26 mM	$MgSO_4$
	0.6 mM	$CaCl_2$
	1.62×10^{-4} mM	$(NH_4)_6Mo_7O_{24}.4H_2O$
	0.01 mM	$FeSO_4.7H_2O$
	7.6 mM	$(NH_4)_2SO_4$
	50 mM	KH_2PO_4
	50 mM	Na_2HPO_4
	10 mM	C-source
	0.1%(v/v)	Hutner's "Metals 44"
		pH 6.8
Hutner's "Metals 44" (Hutner, 1950)	8.6 mM	EDTA
	38 mM	$ZnSO_4.7H_2O$
	18 mM	$FeSO_4.7H_2O$
	9.2 mM	$MnSO_4.H_2O$
	1.56 mM	$CuSO_4.5H_2O$
	0.85 mM	$Co(NO_3)_2.6H_2O$
	0.46 mM	$Na_2B_4O_7.10H_2O$
		pH 2.0 w/ H_2SO_4
MOPS MM (Neidhardt, 1972)	40 mM	MOPS
	4 mM	Tricine
	1.32 mM	K_2HPO_4
	9.52 mM	NH_4Cl
	0.523 mM	$MgCl_2$

TABLE 3 (Continued)

Medium	Amount	Component
MOPS MM (continued)	0.276 mM	K_2SO_4
	0.010 mM	$FeSO_4.7H_2O$
	50 mM	NaCl
	5×10^{-4} mM	$CaCl_2$
	3×10^{-6} mM	$(NH_4)6Mo_7O_{24}.4H_2O$
	4×10^{-4} mM	H_3BO_3
	3×10^{-5} mM	$CoCl_2$
	1×10^{-5} mM	$CuSO_4$
	8×10^{-5} mM	$MnCl_2$
	1×10^{-5} mM	$ZnSO_4$
	1%(w/v)	C-source
		pH 7.2

Additives (Davis et al., 1980, Appendix 1, Part III):

Adenine	5.0 mM	Methionine	0.3 mM
Alanine	0.47 mM	Nicotinic acid	0.1 mM
Arginine	0.6 mM	Pantothenic acid	0.1 mM
Asparagine	0.32 mM	Phenylalanine	0.3 mM
Aspartic acid	0.3 mM	Proline	2.0 mM
Cysteine	0.3 mM	Pyridoxine	0.1 mM
Glutamic acid	5.0 mM	Serine	4.0 mM
Glutamine	5.0 mM	Threonine	0.3 mM
Glycine	0.13 mM	Thymine	0.32 mM
Guanine	0.3 mM	Tryptophan	0.1 mM
Histidine	0.1 mM	Tyrosine	0.1 mM
Isoleucine	0.3 mM	Uracil	0.1 mM
Leucine	0.3 mM	Valine	0.3 mM
Lysine	0.3 mM		

plate of KB served as a positive control for the replica plating. A second set of eleven combinations of additives was replica plated using plate the tenth media of the first test as the initial plate one week into the first test. A KB plate was used as an initial and final control for replica plating.

Growth along the streaks and within the replica areas for each *Xcm* was scored 0 to 4 as follows. A score of 0 was given if no growth occurred, 0.5 was given if small, pinpoint sized colonies were sparsely distributed, a score of 1 was given when the colonies were larger but did not touch, 2 was scored if the colonies began to touch or growth began to fill the replica area, 3 was given when growth was confluent but still defined by the replica boundary, and, finally, 4 was scored when growth filled the replica area and extended beyond the replica boundary. Daily assignments of scores were made relative to growth on each plate among the various strains for 10 days. Scores for these tests are given for the second day after every strain on the KB plates had a score of 4. An average composite test score was normalized to 1 by summing the scores for all strains for each medium and dividing by 4, the score for KB.

Visible Absorption Spectrum for *Xcm* Y Grown in KB(I)

Xcm strain Y was grown to an apparent A_{600} of 0.459. This culture was blanked against KB and scanned in a Hitachi spectrophotometer from 870 to 190 nm, as was a sample of the same KB used for the *Xcm* Y culture but blanked against dH_2O. The resulting spectra were digitized and edited.

Preparation of $Cu(En)_n.nH_2O$ Complex

The complex of cupric ion with ethylene diamine, $Cu(En)_n.nH_2O$, has an absorption spectra similar to that of xanthominidin, the yellow membrane pigment of

Xanthomonas sp. (Johnson and Bryant, 1934; Starr, 1981). Therefore, this complex was prepared to provide an absorbing species to measure the behavior of the Jr. Coleman spectrophotometer relative to that of the Hitachi spectrophotometer since both instruments were used for taking absorption readings. $CuSO_4$ 0.6250 g was added to 50 ml ethylenediamine and equilibrated for 4 hr at room temperature. The ethylenediamine was decanted away and enough dH_2O added to dissolve the crystals completely. The resulting solution was purple in color. This solution was empirically diluted to give a series for standard curve determinations on both instruments, and one of the dilutions was scanned from 800 to 500 nm on the Hitachi spectrophotometer using dH_2O as the blank with a spectrum of ethylenediamine versus dH_2O as the baseline control. Absorbance of the dilution series was measured at 600 nm on both the Jr. Coleman and the Hitachi.

Determination of Dry Weight vs. OD_{600} for *Xcm* H

Xcm H was grown in 250 ml of KB(I), MOPS MM1with 68.5 mM glucose, or MOPS MM1 with 137 mM glycerol and 2.39 mM total in 7 amino acids (alanine, arginine, asparagine, histidine, isoleucine, phenylalanine, threonine). When the OD600 measured on the Hitachi spectrophotometer of the cultures was at 0.481, 0.297, or 0.385, respectively, the cells were placed on ice for 15 minutes. They were then centrifuged at 16k x g for 30' in a Sorvall GSA rotor in 250 ml bottles at 4° C. The supernatant was discarded and the pellets were resuspended in 50 ml of sterile 0.9% NaCl. After this washing step, the cells were repelleted for 10' in the GSA rotor at 16k x g. The pellets were then resuspended in 5 ml 0.9% NaCl. The cell suspensions were then held on ice while the other steps were taken.

Aliquots of 750 ml were blotted onto predried and weighed nitrocellulose filters twice for a total of 1.5 ml / filter in duplicate for each medium. Sterile filter paper and paper towels provided a surface to absorb most of the liquid away from the cells. After the

surface of the cells appeared to be somewhat dry, the filters were dried to constant weight *in vacuo* at 60° C. The dry weight in g/ml of the concentrated cell suspension was calculated.

The cell suspension of the MOPS MM1 with glycerol and 7 AAs was used to make a dilution series in 0.9% NaCl whose OD_{600}s were then measured in the Hitachi spectrophotometer. The reference blank for these measurements was an aliquot of 0.9% NaCl. Dry weights for the dilutions were back–calculated from the value obtained for the undiluted sample.

Growth Curve Analyses

Liquid culture growth curves for *Xcm* R3–22, *Xcm* S, and *Xcm* H were determined with various media (Table 3) at 30° C for cultures inoculated with bacteria grown either in KB or in the media which was being tested. Measurements of OD were at 590 nm for sets 2, 3, and 4 and at 600 nm for sets 1 and 5 in a Jr. Coleman spectrophotometer and also at 600 nm in a Hitachi spectrophotometer for set 5 only. The ODs were measured in sidearm flasks with the Jr. Coleman blanked against a fresh aliquot of the corresponding medium (for each set). The ODs were corrected for the blank error by subtracting the measurement made in the flask used for the curve containing the corresponding medium prior to inoculation. For set 5, an aliquot of culture was removed at certain time points, diluted in 0.9% NaCl, if needed, and plated on KB(I) using the spiral plater (Spiral System Instruments, Inc., Bethesda, MD) for CFU/ml determinations. The plates were then incubated at 30° C for 2 to 3 days prior to counting.

Analysis of Growth Rates

For growth curves having an exponential phase, the OD values were replotted as log OD vs. time for the exponential phase, which was usually observed from 0.01 to 0.5

OD for the Jr. Coleman Linear regression lines were determined for the points lying within the general trend for the data with extremely aberrant points removed from the analysis. The doubling time, g, is given by the inverse slope of the regression line multiplied by the product of ln 2 and $\log_{10}$ of e (Ingraham, Maaloe, and Neidhardt, 1982, Ch.5). Regression line slopes, coefficients of correlation, and doubling times are reported. Sometimes for certain media, biphasic growth curves were observed; in these cases, values are given for the early phase and the late phase (corresponding most often to the exponential growth phase typically observed).

Results and Discussion

Xcm was found to grow in the modified Neidhardt's medium, MOPS MM1, both on plates and in liquid culture (data not shown). However, growth was too slow to be of practical use in experiments requiring bacteria to be in the exponential phase of growth. *Xcm* was found to grow in MOPS MM1 with both glycerol or glucose as the C–source without an appreciable lag time while little growth occurred in Stanier's medium (previously used by Essenberg *et al.*, 1982 and 1990). Figures 2 and 3 for growth curve set 1 demonstrate this.

Plate Tests for Combinations of Amino Acids

Since addition of amino acids to the medium would provide and additional source of carbon and a source of reduced nitrogen, a search for a subset of amino acids that stimulate the growth of *Xcm* while limiting the growth *E. coli* strains was undertaken. Combinations of amino acids and vitamins were tested for the ability to stimulate growth of 26 *Xcm* strains on plates relative to rich medium plates of KB(I). Data from the test appear in Tables 4 and 5. Ten amino acids (alanine, arginine, asparagine, cysteine, glutamic acid, histidine, isoleucine, phenylalanine, proline, threonine on plate 1.6) supported growth

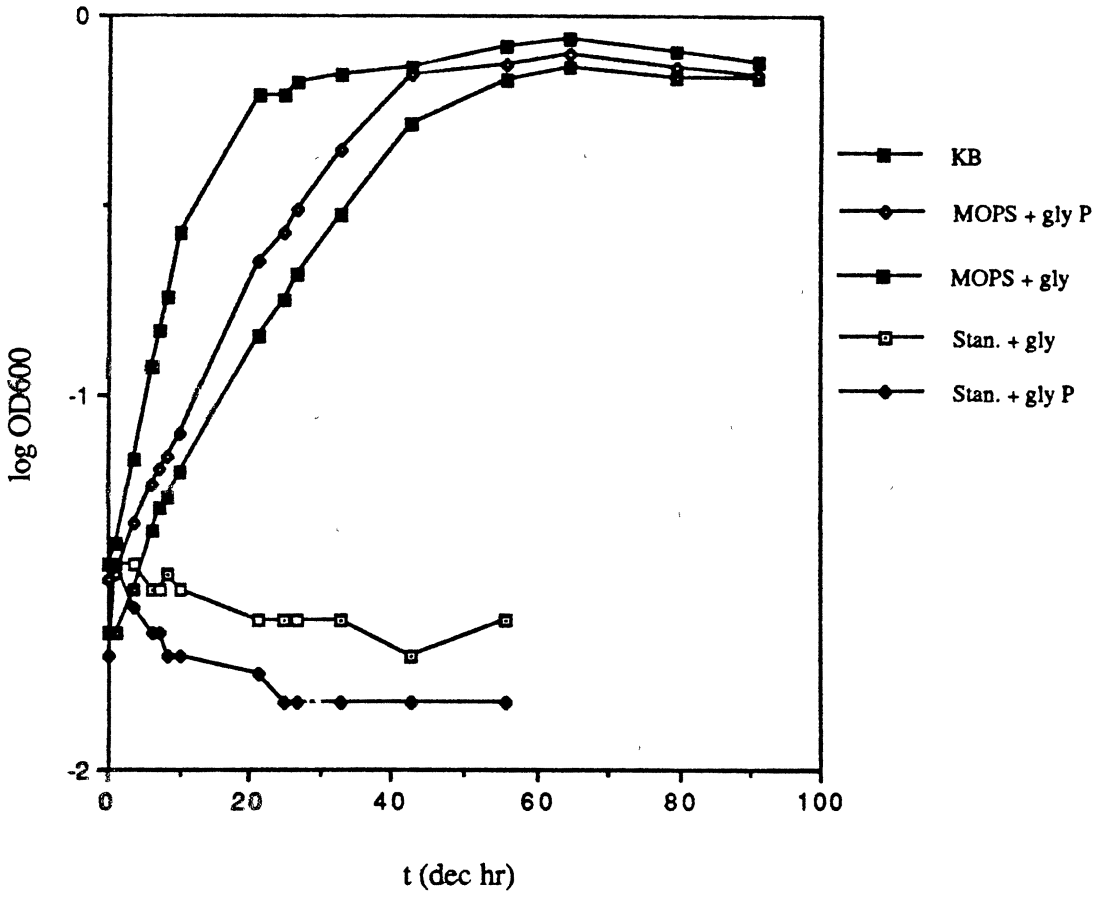


Figure 2. Growth Curve Set 1, Comparison of MOPS MM1 and Ornston and Stanier's with Glycerol and Proline. *Xcm* S was grown in KB(I) and in MOPS MM1 or Ornston and Stanier's medium, each supplemented with glycerol or with both glycerol and proline. Time (t) is reported in decimal hours.

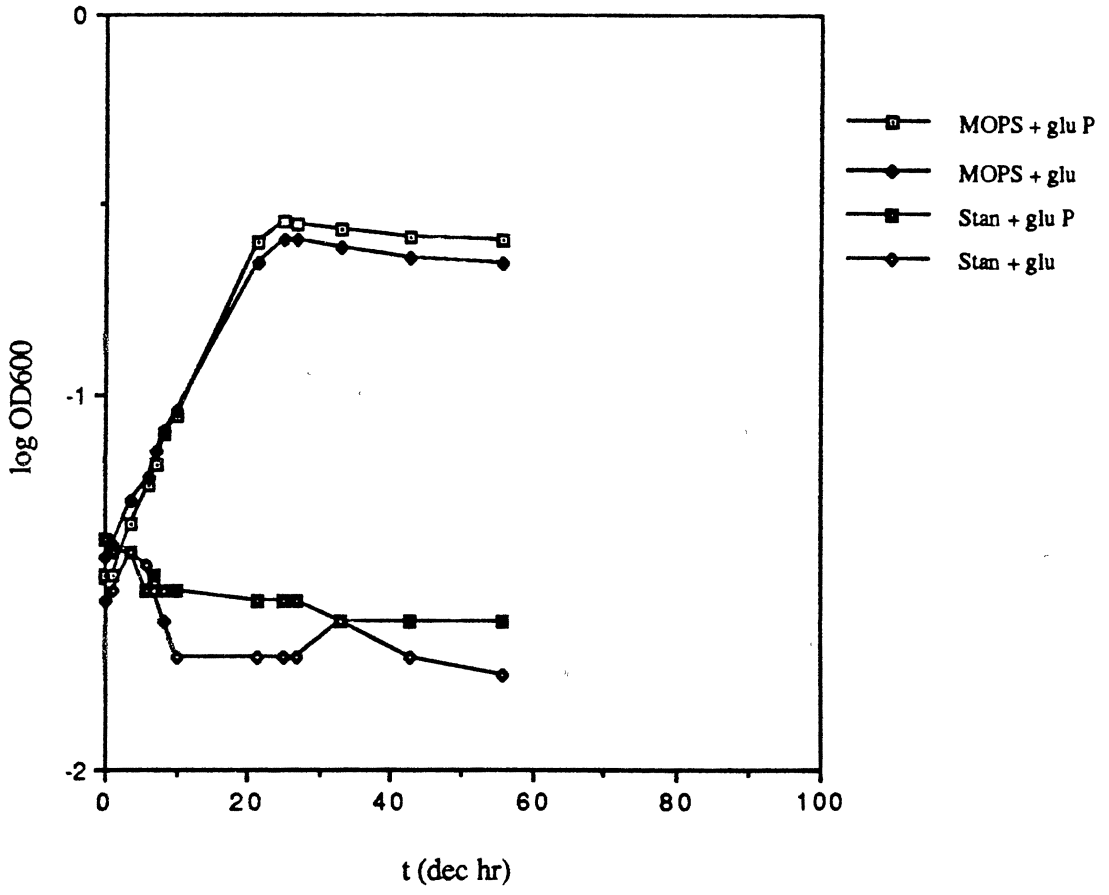


Figure 3. Growth Curve Set 1, Comparison of MOPS MM1 and Ornston and Stanier's with Glucose and Proline. *Xcm* S was grown in MOPS MM1 or Ornston and Stanier's medium, each supplemented with glucose or with both glucose and proline. Time (t) is reported in decimal hours.

TABLE 4

AMINO ACID ADDITIVE TESTS

	Plate												
Strain	1.0	1.1	1.2	1.3	1.4	1.5	1.6	1.7	1.8	1.9	1.10	1.11	1.12
A	4	3	4	1	4	4	4	0.5	3	2	4	3	4
B	4	3	3	1	4	4	4	0.5	3	2	4	3	4
C	4	3	3	1	4	4	4	0.5	3	3	4	2	4
D	4	1	2	0.5	4	3	4	0	3	1	4	2	4
E	4	1	3	0	4	3	3	0.5	2	1	3	1	4
F	4	2	2	1	4	4	4	0.5	2	1	4	1	4
G	4	3	4	1	4	4	4	0.5	2	2	4	3	4
H	4	3	4	2	4	4	4	0.5	2	2	3	3	4
I	4	2	2	0	4	3	3	0	2	1	4	2	4
J	4	3	4	1	4	3	3	0	2	1	3	1	4
K	4	3	2	0.5	4	4	4	0.5	3	2	4	3	4
L	4	3	3	2	4	4	4	0.5	3	3	4	2	4
M	4	0.5	3	0.5	4	3	3	0.5	2	0.5	2	2	4
N	4	3	2	0.5	4	4	4	0.5	3	2	4	2	4
O	4	3	4	2	4	3	3	0	2	1	3	1	4
Q	4	0.5	4	0.5	4	3	4	0	2	1	3	2	4
R	4	0.5	2	0	4	3	3	0	2	1	3	2	4
S	4	3	4	2	4	4	4	0.5	3	2	3	3	4
T	4	3	4	0.5	4	3	4	0.5	3	2	4	3	4
U	3	0.5	2	0	3	2	2	0	2	0	3	0.5	3
V	4	3	4	1	4	3	4	0	3	1	3	3	4
W	4	2	3	0	4	3	3	0	2	0.5	2	1	3
X	4	2	2	0.5	4	3	3	0.5	3	1	4	3	4
Y	4	2	3	0	4	3	2	0.5	2	1	2	2	4
Z	4	2	2	0	4	1	1	0.5	2	0	3	2	4
3-22	4	3	4	2	4	4	3	0	2	0.5	3	2	4
SUM	103	58	79	20.5	103	86	88	8	63	34.5	87	54.5	102
SUM/N	3.96	2.23	3.04	0.79	3.96	3.31	3.38	0.31	2.42	1.33	3.35	2.1	3.92
test	0.99	0.56	0.76	0.2	0.99	0.83	0.85	0.08	0.61	0.33	0.84	0.52	0.98

TABLE 4 (Continued)

	Plate												
Strain	2.0	2.1	2.2	2.3	2.4	2.5	2.6	2.7	2.8	2.9	2.10	2.11	2.12
A	4	4	4	3	3	3	3	3	3	3	3	3	4
B	4	4	4	4	3	3	3	3	3	3	3	3	4
C	4	4	4	3	3	3	2	3	3	3	4	3	4
D	4	2	2	2	2	3	3	3	2	3	3	3	4
E	4	2	0.5	2	1	2	1	2	3	2	3	2	4
F	3	4	3	0	2	2	2	2	3	2	2	3	4
G	4	4	4	1	1	1	2	3	4	3	4	3	4
H	3	3	3	3	3	3	3	3	2	3	3	3	4
I	4	2	2	3	2	3	3	3	2	3	3	3	4
J	4	2	2	2	2	2	2	2	2	2	2	2	4
K	4	3	3	3	3	3	3	3	3	3	3	3	4
L	4	4	4	0.5	1	2	2	2	3	3	3	3	4
M	3	1	2	1	2	1	2	0.5	0.5	1	0.5	0.5	4
N	4	3	4	4	3	3	3	3	2	2	3	3	4
O	4	2	2	2	2	3	2	2	2	2	2	2	4
Q	4	2	2	3	2	3	2	3	2	3	3	3	4
R	4	2	2	1	2	2	2	3	2	3	3	3	4
S	4	4	3	3	3	3	3	3	3	3	3	3	4
T	4	4	3	4	3	3	3	3	2	3	3	3	4
U	3	2	1	2	2	2	2	2	2	2	2	2	4
V	4	3	2	3	3	4	3	3	2	3	3	3	4
W	3	1	1	2	2	2	1	2	2	2	2	2	4
X	4	4	3	3	3	3	3	3	3	3	3	3	4
Y	3	1	1	2	2	2	1	2	2	2	2	2	4
Z	3	1	1	2	2	2	1	2	2	2	2	2	4
3-22	4	4	4	3	3	2	2	2	2	2	2	2	4
SUM	97	72	66.5	61.5	60	65	59	65.5	61.5	66	69.5	67.5	104
SUM/N	3.73	2.77	2.56	2.37	2.31	2.5	2.27	2.52	2.37	2.54	2.67	2.6	4
test	0.93	0.69	0.64	0.59	0.58	0.63	0.57	0.63	0.59	0.63	0.67	0.65	1

Table 4. Plates 1.0 and 1.12 are KB(I). Plate 1.1 is MOPS MM1 without supplementation. Plate 1.2 is MOPS MM1 with CAAs and yeast extract and NH_4SO_4 for 1.3. For plates 1.4 to 1.11, the AAs given in Table 5 were added. Plate 1.4 also contains adenine, guanine, nicotininc acid, pantothenic acid, pyridoxine, and thymine. Plates 2.0 and 2.12 are KB(I). For plates 2.1 to 2.11, the AAs given in Table 5 were added.

TABLE 5

COMBINATIONS OF AMINO ACIDS

plate	test value	**A**	**F**	**H**	**I**	**N**	**R**	**T**	C	E	P	D	G	K	L	M	Q	S	V	W	Y
1.4	0.99	+	+	+	+	+	+		+	+	+	+	+	+		+	+	+	+	+	+
1.6	0.85	+	+	+	+	+	+	+	+	+	+										
1.10	0.84					+			+		+				+						+
1.5	0.83	+	+	+	+	+	+		+	+	+	+	+	+		+	+	+	+	+	+
2.1	0.69					+			+		+										
2.10	0.67										+										
2.11	0.65					+			+		+				+						+
2.2	0.64					+			+						+						
2.5	0.63					+					+				+						+
2.7	0.63					+					+										
2.9	0.63										+				+						
1.8	0.61	+		+											+				+	+	
2.3	0.59					+					+										+
2.8	0.59								+		+										
2.4	0.58								+		+				+						
2.6	0.57					+			+												
1.1	0.56																				
1.11	0.52				+			+				+	+				+				
1.9	0.33		+				+			+						+		+			
1.7	0.08											+	+	+	+	+	+	+	+	+	+

Table 5. Test values from Table 4 were sorted in descending order for all amino acid containing plates. The combinations of amino acids are given. Those in bold lettering were the ones chosen for routine use at the end of this study.

almost as well as KB(I), plate 1.12, while the other 10 amino acids (plate 1.7) supported little growth. Subsets of amino acids overlapping these two groups did not support growth as well as on plate 1.6, although all supported better growth than on plate 1.7. The ten amino acids used for plate 1.6 were further analyzed by growth rate analysis in liquid culture.

Analysis of Dry Weight and Spectral Determinations of *Xcm*

In order to for OD to be a measure of growth, a determination of whether OD is a measure of cell mass as well as cell number was attempted. The visible spectrum of *Xcm* Y grown in KB(I) was scanned (Figure 4). This spectrum and that for KB(I) appear as Figure 4. *Xcm* Y was found to absorb strongly at 415 nm. This peak roughly corresponds to that for purified xanthomonidins (Starr, 1981). Both wavelengths 590 and 600 nm occur on the long shoulder of the peak for *Xcm* Y, with an OD at 590 and 600 nm half that of the peak maximum. This suggests that the contribution of the yellow pigments, xanthomonidins, to OD is significant. The OD's at 590 nm and at 600 nm are nearly the same so measurements made at the two wavelengths are directly comparable. Standard curves of A_{600} versus concentration of a $Cu(En)_n.nH_2O$ complex were made for both the Jr. Coleman and the Hitachi instruments. Beer's law behavior was observed for each machine (Figure 5) while absorbance values for the Hitachi are roughly 1.3 x those for the Jr. Coleman (slope of the regression line from Figure 6).

The best method for correlating cell number, cell mass, and OD is a dry weight analysis of growth. The dry weight of cells harvested at OD_{600}s on the Hitachi between 0.297 and 0.481 for KB(I), MOPS MM1 with glucose, and MOPS MM1 with glycerol and 7 amino acids was measured. These OD_{600}s are approximately 0.22 to 0.36 when converted to Jr. Coleman equivalents and are all within the mid to upper end of the exponential growth phase observed for *Xcm* regardless of medium composition as long as

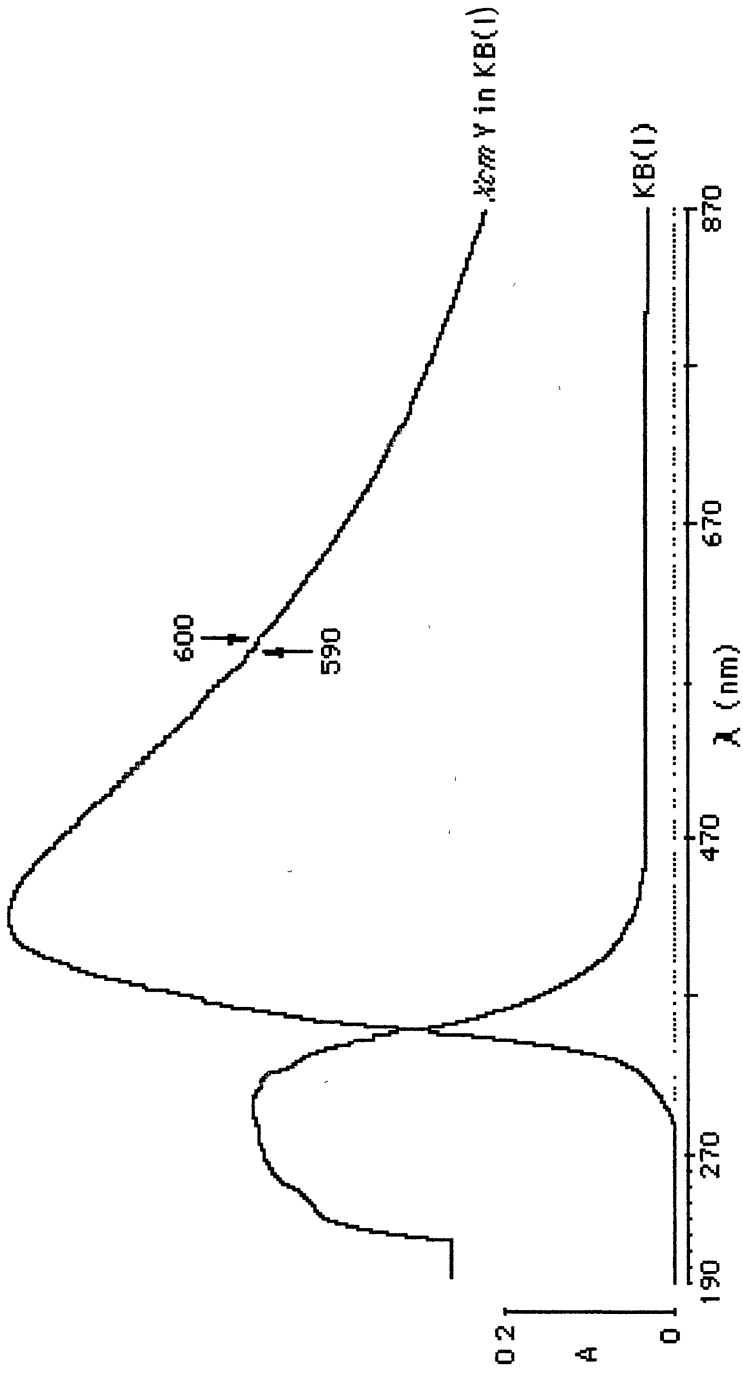


Figure 4 Visible Spectra of *Xcm* Y and KB(I).

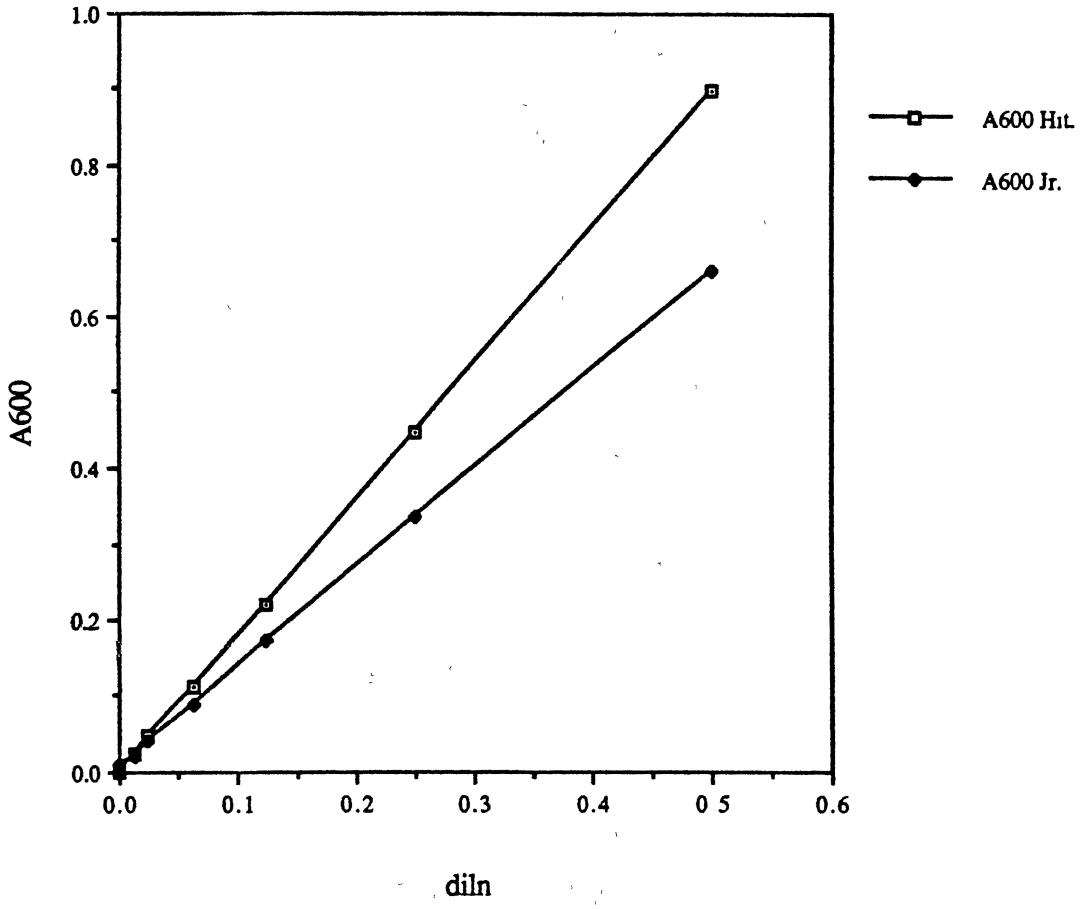


Figure 5. Beer's Law Behavior for Hitachi and Jr. Coleman Spectrophotometers. $Cu(En)_n.nH_2O$ complex was diluted with distilled H_2O and the absorbance of the dilutions was measured at A_{600} using both the Hitachi and Jr. Coleman spectrophotometers.

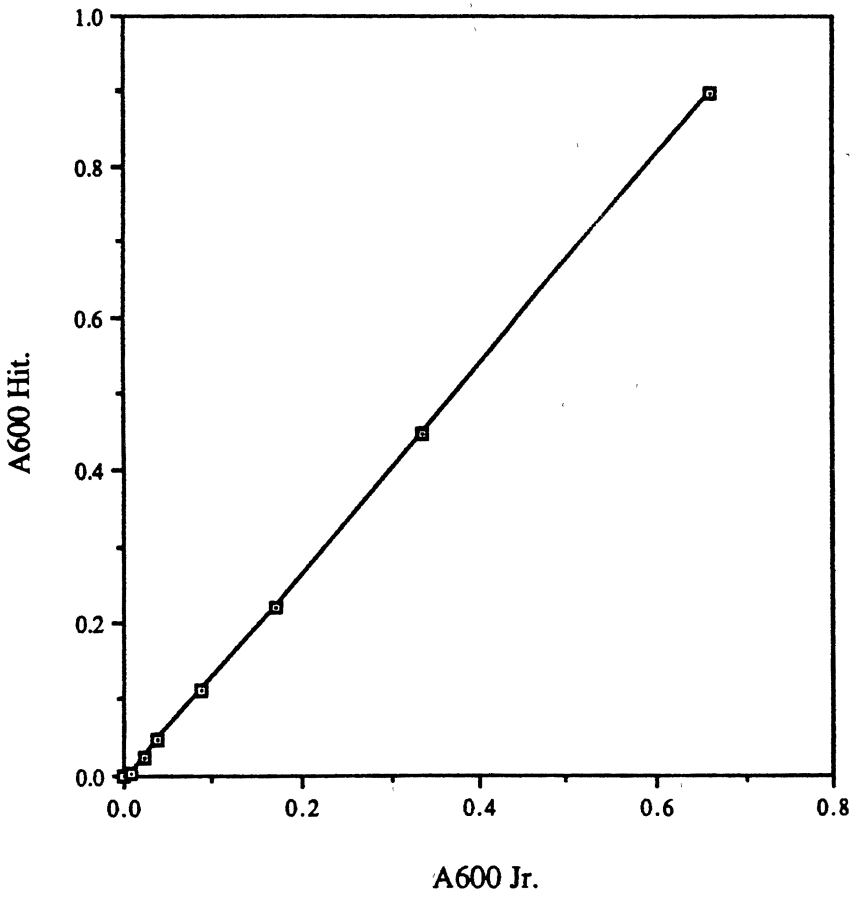


Figure 6. Relationship of A_{600} for Hitachi vs. Jr. Coleman Spectrophotometers. The absorbance values for each dilution measured in Figure 5 are replotted to show the relationship for absorbance at 600 nm between the HItachi and Jr. Coleman spectrophotometers. The line has a slope of about 1.3.

no component is limiting. For the MOPS MM1 with glycerol and 7 amino acids, a number of dilutions of the harvested cells were made, the OD_{600} was measured, and the dry weight was back–calculated for each of the dilutions. These data are plotted as Figure 7. At the time point of harvest, the suspensions of *Xcm* obeyed Beer's law.

Dry weights for growth in the other two media were calculated, and these values are plotted with the dry weight for the cell suspension as the independent variable in Figure 8. Linearity was not observed for these media. The divergence from linearity at the high end of exponential growth may indicate that cell size was changing as stationary phase was approached or that some medium component influenced the rate of growth in one medium and not the others. Perhaps the level of xanthomonidins per total cell components remained constant early in growth but late during growth decreased and hence lowered the OD.

For an accurate measure of the cell number in solution, OD is determined by light scattering from the bacterial cells and hence is influenced not only by the number but also their size. If the bacterial size changes during the exponential growth phase, then the cell number will not be directly comparable to the OD. The internal geometry of the measuring device also affects the component of OD due to scattering. In the Hitachi, the detector only sees light passing through a narrow slit parallel to the incident beam. A higher OD will be observed in a machine with this geometry compared to a machine such as the Jr. Coleman where a larger slit increases the likelihood that scattered light will reach the detector.

Lower ODs for the Hitachi than expected based on measurements taken at the same time point for the Jr. Coleman were observed as shown in Figure 9 for the medium KB(I). The Hitachi OD's also indicated a slower apparent growth rate. The lower growth rates could be due to a component of OD that is additive; thus when a log plot is made, the slope is decreased ($\log a + \log b = \log (ab)$). Hence, none of Hitachi growth curve data points are reported even though measurements were made for all of the time points of set 5 on both machines.

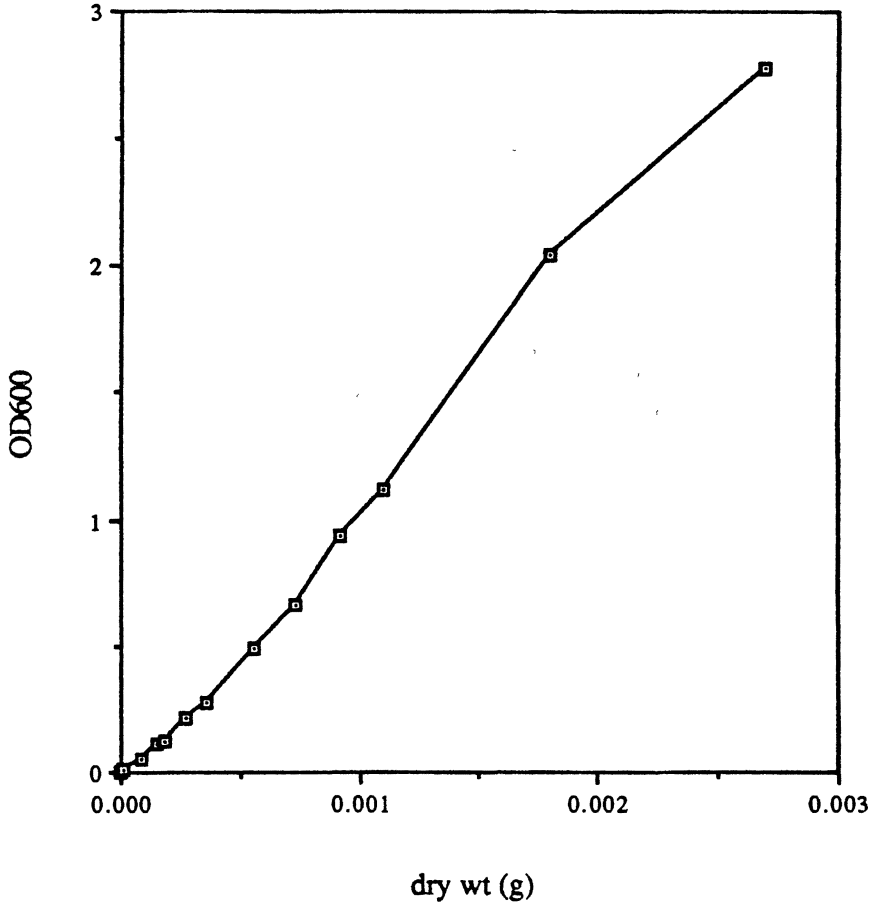


Figure 7. Dry Weight vs. OD_{600} for *Xcm* H. *Xcm* H grown in MOPS MM1 supplemented with glycerol and 7 amino acids was harvested at 0.385 OD_{600}. Dry weight for a suspension of cells was determined, and OD_{600} was measured for dilutions of the cell suspension. A dry weight per dilution was calculated.

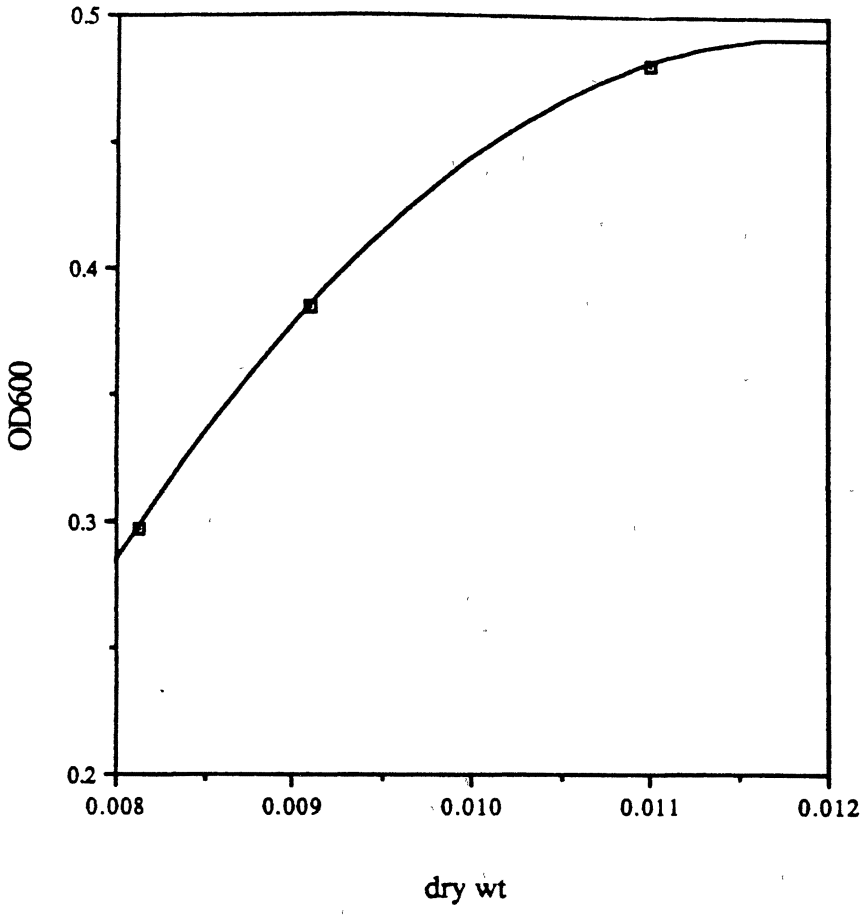


Figure 8. Dry Weight Measurements for Three Media. *Xcm* H was grown in MOPS MM1 with glucose (OD_{600} of 0.297), MOPS MM1 with glycerol and 7 amino acids (OD_{600} of 0.385), and KB(I) (OD_{600} of 0.481). OD_{600} is shown plotted against the dry weights for cells from each of the media.

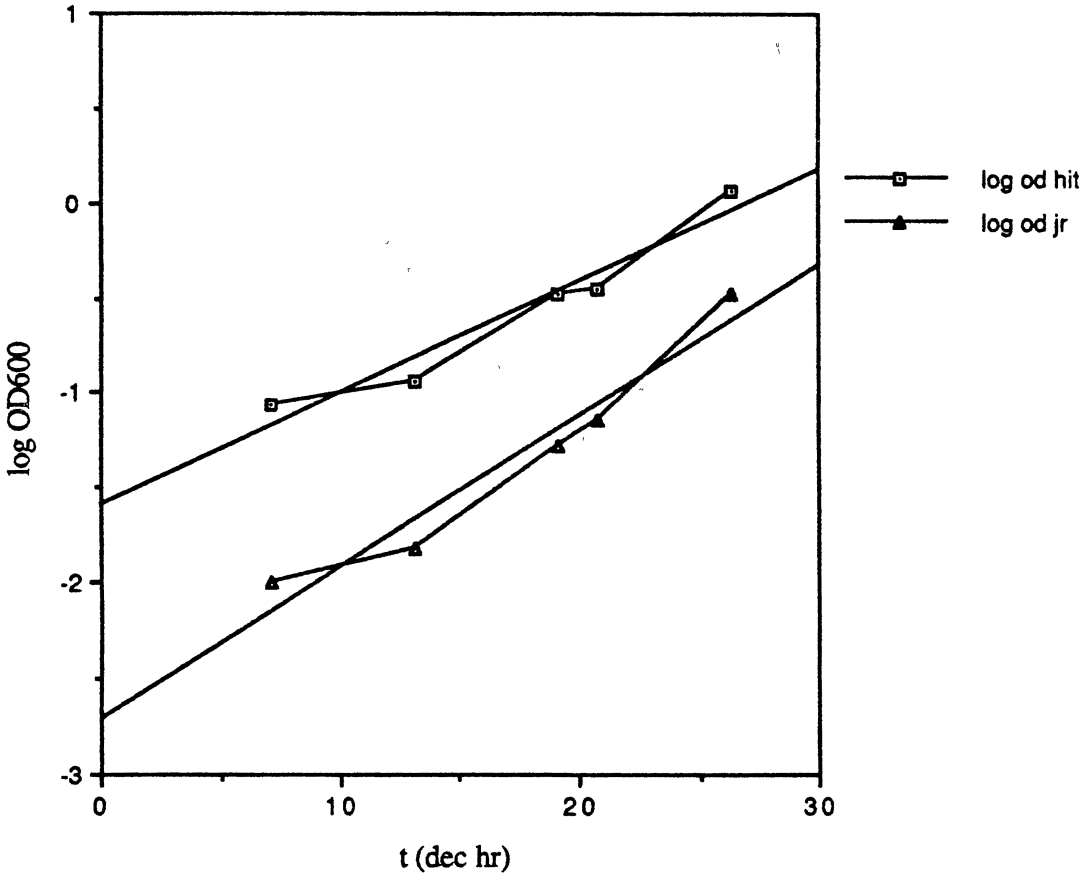


Figure 9. Lower Apparent Growth from Hitachi Measurements. *Xcm* H was grown in KB(I) with OD_{600} measured for the culture at various time points in both the Hitachi and Jr. Coleman spectrophotometers.

Growth Curve and Rate Analyses

Growth curve analyses were done five times with various combinations of buffers, C–sources, and amino acids. Although two wavelengths, 590 and 600 nm, were employed for measurement, growth rates between for these are comparable. Figures 2 and 3 and 10 to 22 show plots of log OD versus time for these growth curve sets while doubling times can be found in Table 6. In most cases, no lag phase prior to exponential growth was observed. Exceptions occurred when the medium or buffering system were inadequate as for Ornston and Stanier's medium (Figures 2 and 3). Initial inoculum concentrations for sets 1 to 4 were higher than those in set 5; while *Xcm* R3–22 was used in the former and *Xcm* strain H in the latter.

Growth curve analysis illustrated several features: Neidhardt's MM1 can be buffered by Tris, HEPES, P_i or MOPS as shown in figures 11, 15, 19, and 20. MOPS proved to be a more consistently reliable buffer than Tris, HEPES, or P_i. This seems to be due to its large buffering capacity at pH = 7.2, equivalent to its pK_a, since absence of any major buffering component was inhibitory (Figures 11 and 15). Growth curves in Figures 10 and 13 show that while sorbitol added to glycerol has a slight stimulatory effect, by itself it is inhibitory. Since *Xcm* colonies grown in the presence of glucose on plates produced copious amounts of exopolysaccharide (EPS), glycerol was chosen as the preferred C–source. EPS was known to interfere with DNA isolation, to decrease the efficiency of conjugation, and to inhibit infiltration of *Xcm* into cotton leaves.

Addition of proline to the medium was stimulatory when added at either 2 or 10 mM (Figures 12). Glutamic acid was also stimulatory, Figure 17. A mixture of alanine, histidine, isoleucine, and threonine stimulated growth slightly better than alanine plus histidine (Figure 14). When the set of ten amino acids from plate tests was tested, a larger stimulation was seen (Figure 17 and Table 6). Elimination of cysteine from the set of 10 resulted in decreased growth while further elimination of glutamic acid and proline led to an

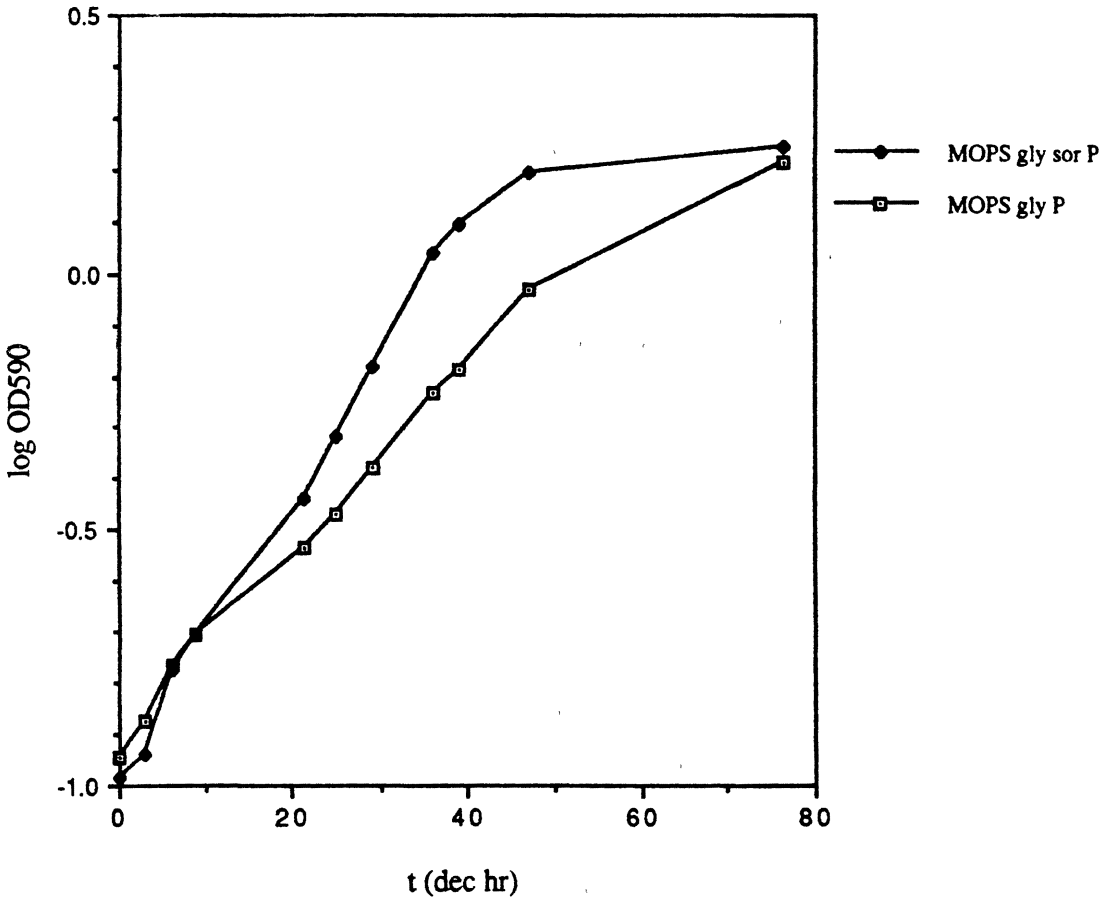


Figure 10. Growth Curve Set 2, Comparison of MOPS MM1 with Glycerol and Proline or Sorbitol. *Xcm* S was used for this comparison. Time (t) is reported in decimal hours.

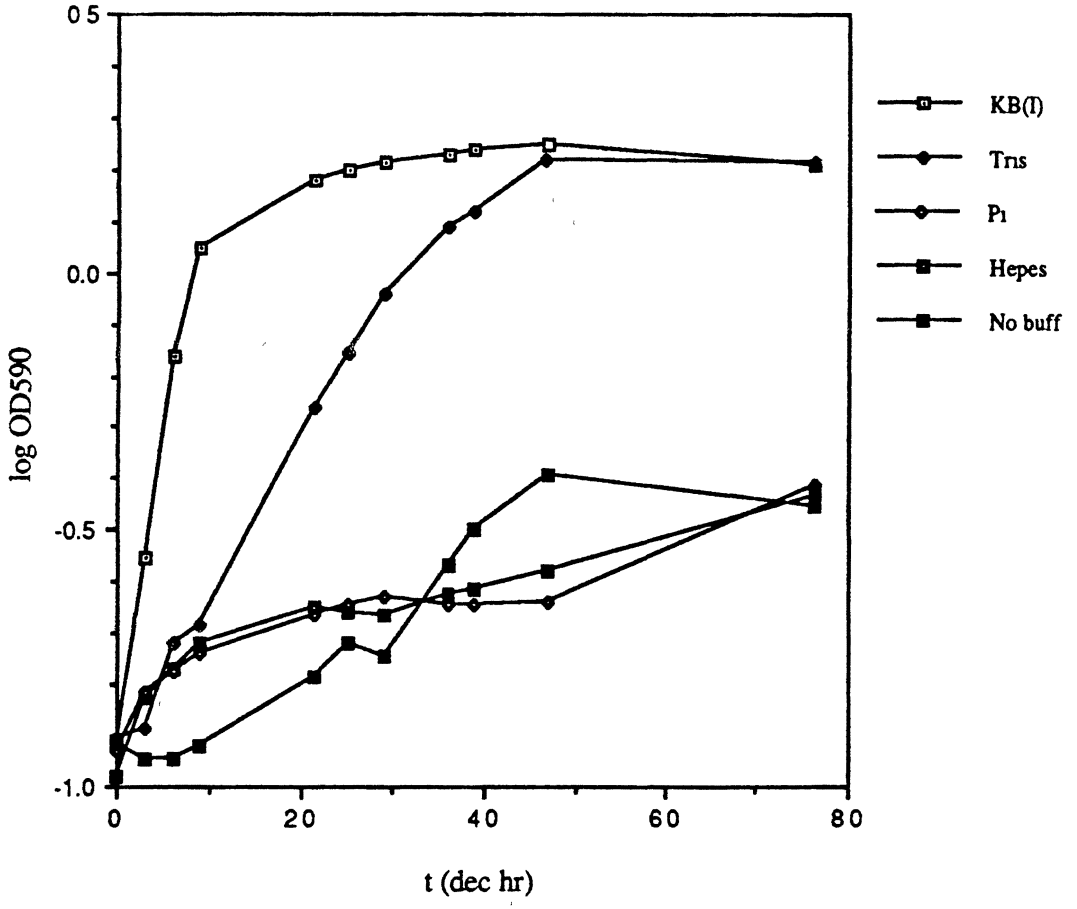


Figure 11. Growth Curve Set 2, Comparison of Tris, Hepes, or Phospate Buffers and No Buffer. *Xcm* S was grown in KB(I) or Neidhardt's medium buffered with Tris, Hepes, or phosphate (P_i) at pH 7.2 or in Neidhardt's medium without a major buffer. Time (t) is reported in decimal hours.

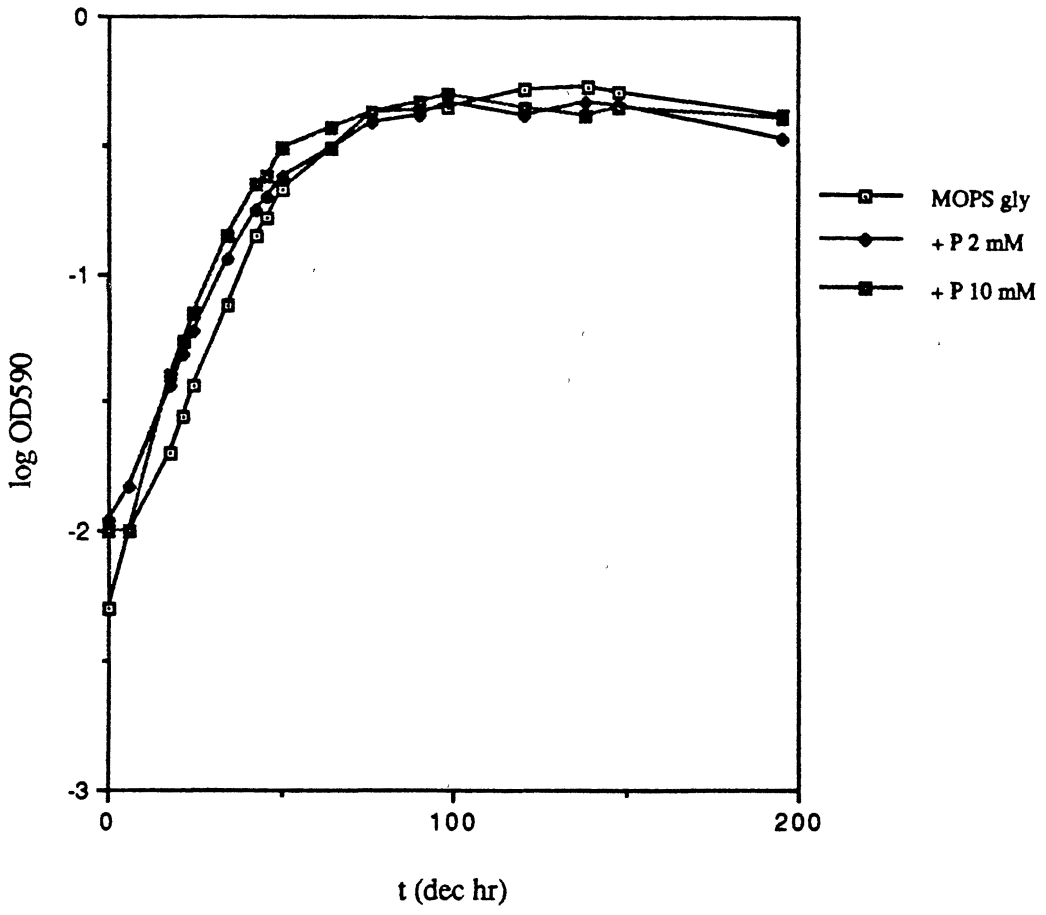


Figure 12. Growth Curve Set 3, Comparison of MOPS MM1 with Glycerol and/or Proline at Two Concentrations. *Xcm* S was used for these comparisons, proline was supplemented at either 2 or 10 mM to MOPS MM1 with glycerol. Time (t) is reported in decimal hours.

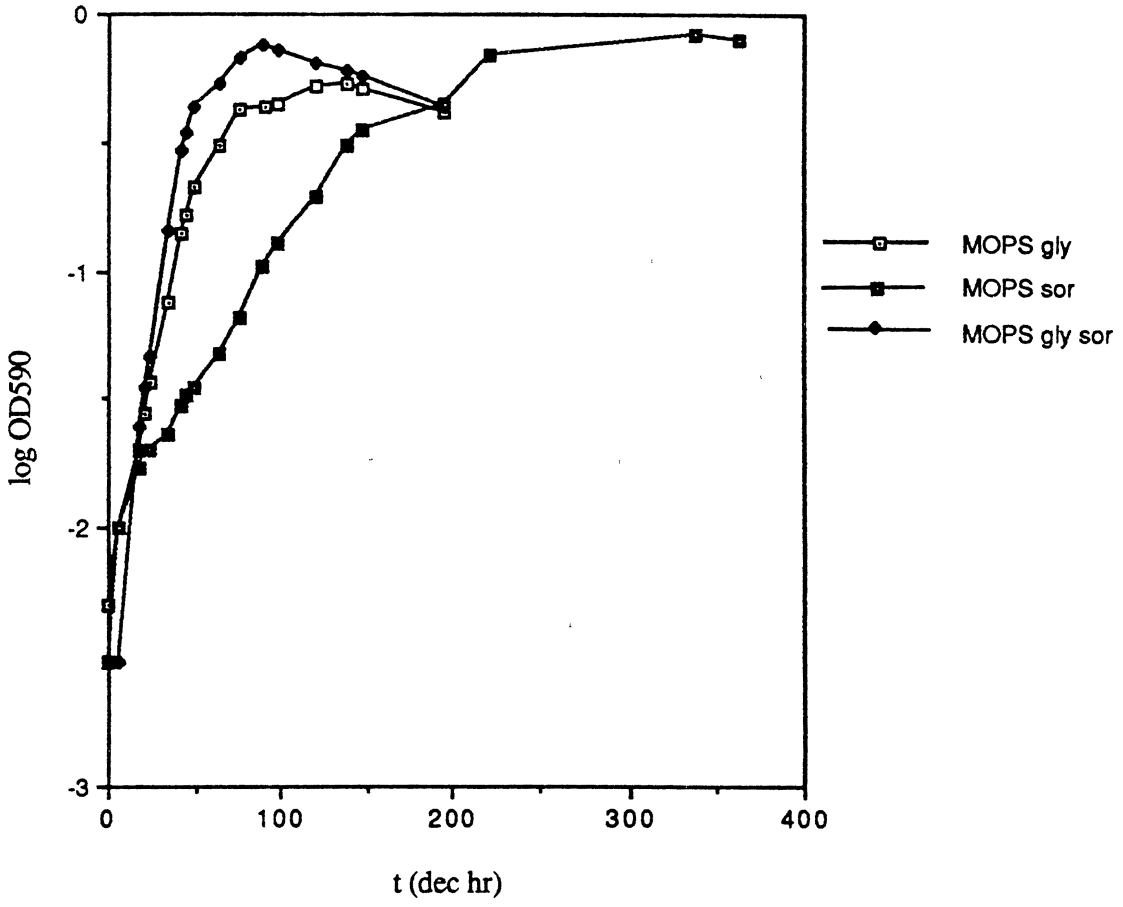


Figure 13. Growth Curve Set 3, Comparison of MOPS MM1 with Glycerol and/or Sorbitol. *Xcm* S was used for these comparisons, proline was supplemented at 2 mM to MOPS MM1 with glycerol and/or sorbitol. Time (t) is reported in decimal hours.

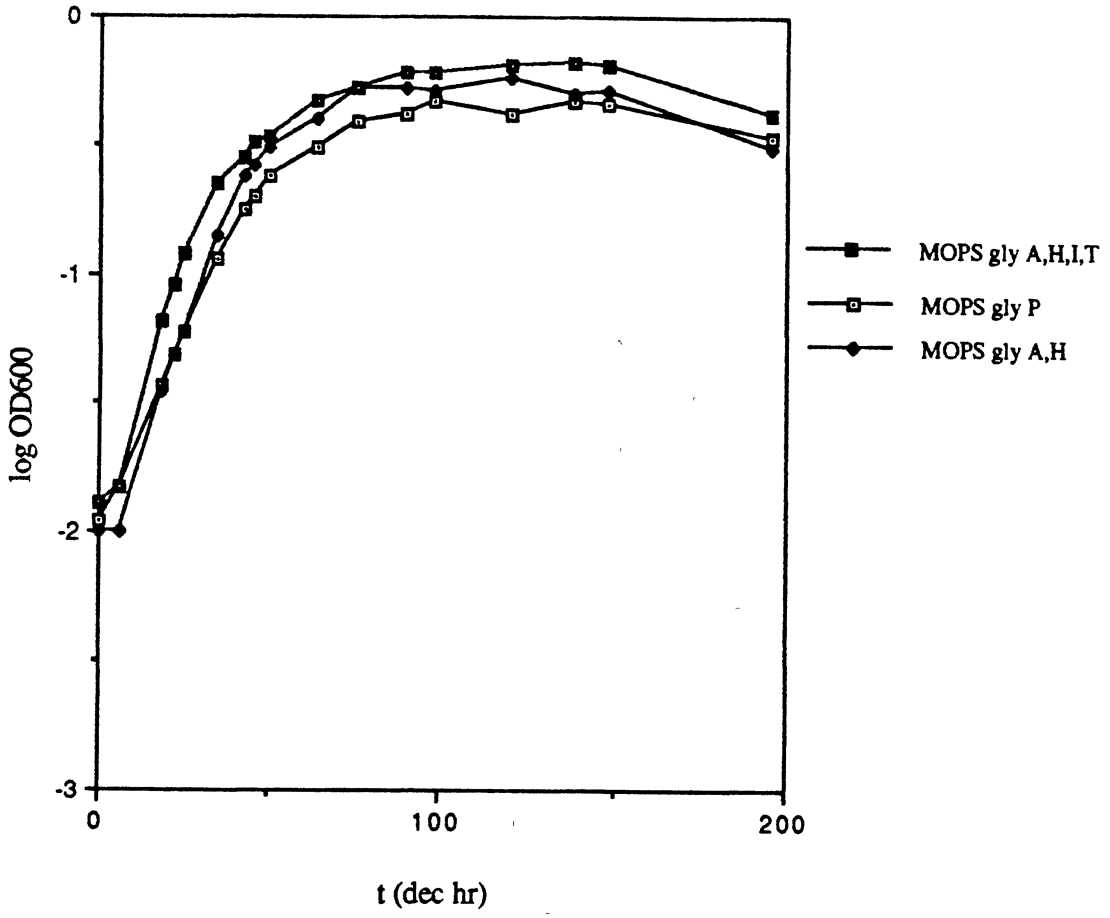
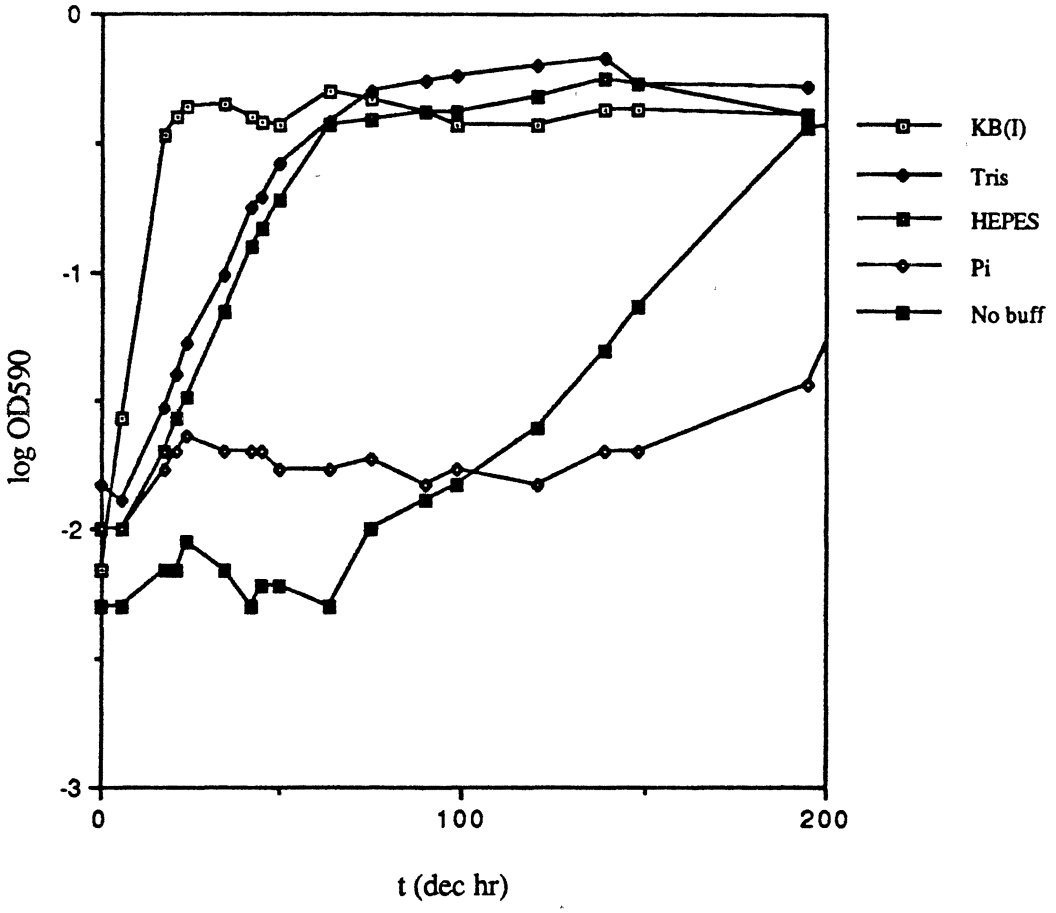


Figure 14. Growth Curve Set 3, Comparison of MOPS MM1 with Glycerol and Several Amino Acids. *Xcm* S was grown in MOPS MM1 with glycerol and proline or alanine and histidine or alanine, histidine, isoleucine, and threonine. Time (t) is reported in decimal hours.



Fugure 15. Growth Curve Set 3, Comparison of Tris, Hepes, or Phospate Buffers and No Buffer. *Xcm* S was grown in KB(I) or Neidhardt's medium buffered with Tris, Hepes, or phosphate (P_i) at pH 7.2 or in Neidhardt's medium without a major buffer. Time (t) is reported in decimal hours.

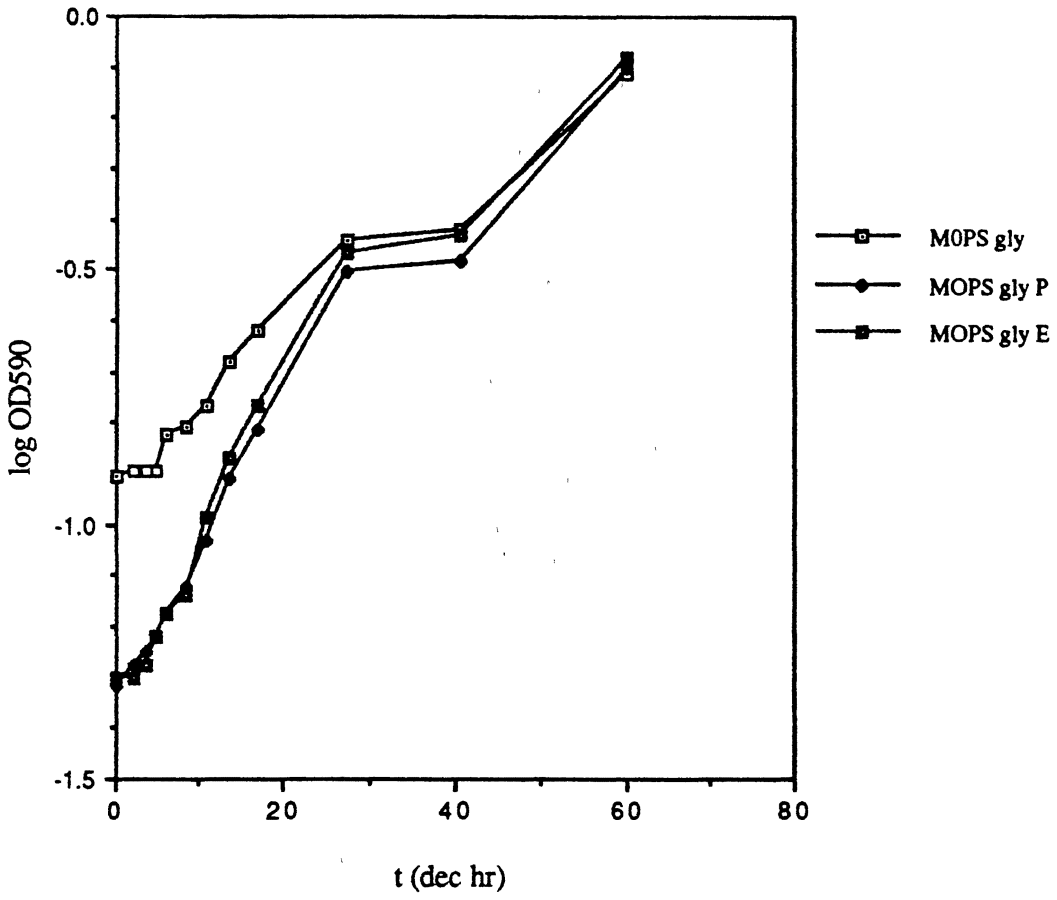


Figure 16. Growth Curve Set 4, Comparison of MOPS MM1 with Glycerol and/or Proline or Glutamic Acid. *Xcm* S was used for this comparison. Time (t) is reported in decimal hours.

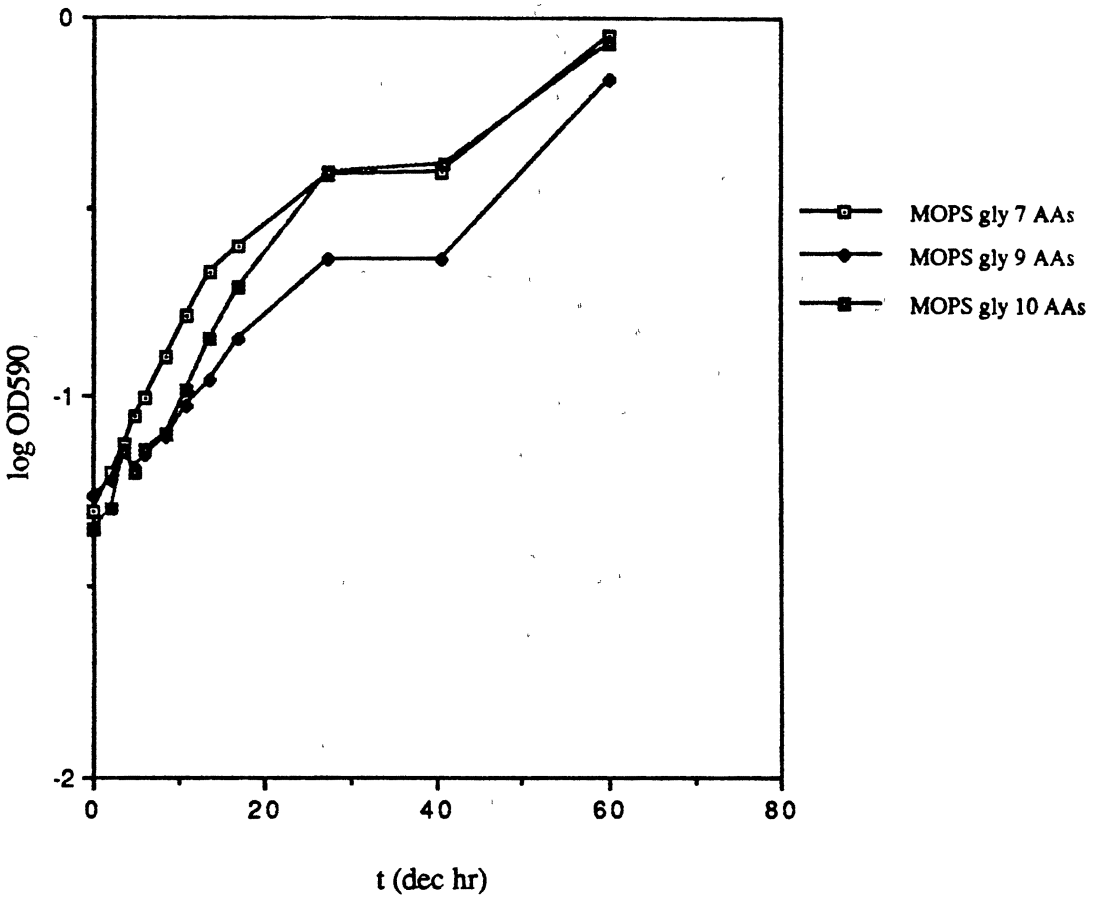


Figure 17. Growth Curve Set 4, Comparison of MOPS MM1 with Glycerol and Seven, Nine, or Ten Amino Acids. *Xcm* S was used for this comparison of the effect of supplementing MOPS MM1 with glycerol and seven amino acids (alanine, arginine, asparagine, histidine, isoleucine, threonine, and phenylalanine) or nine amino acids (the previous seven and glutamic acid and proline) or ten amino acids (the previous nine and cysteine). Time (t) is reported in decimal hours.

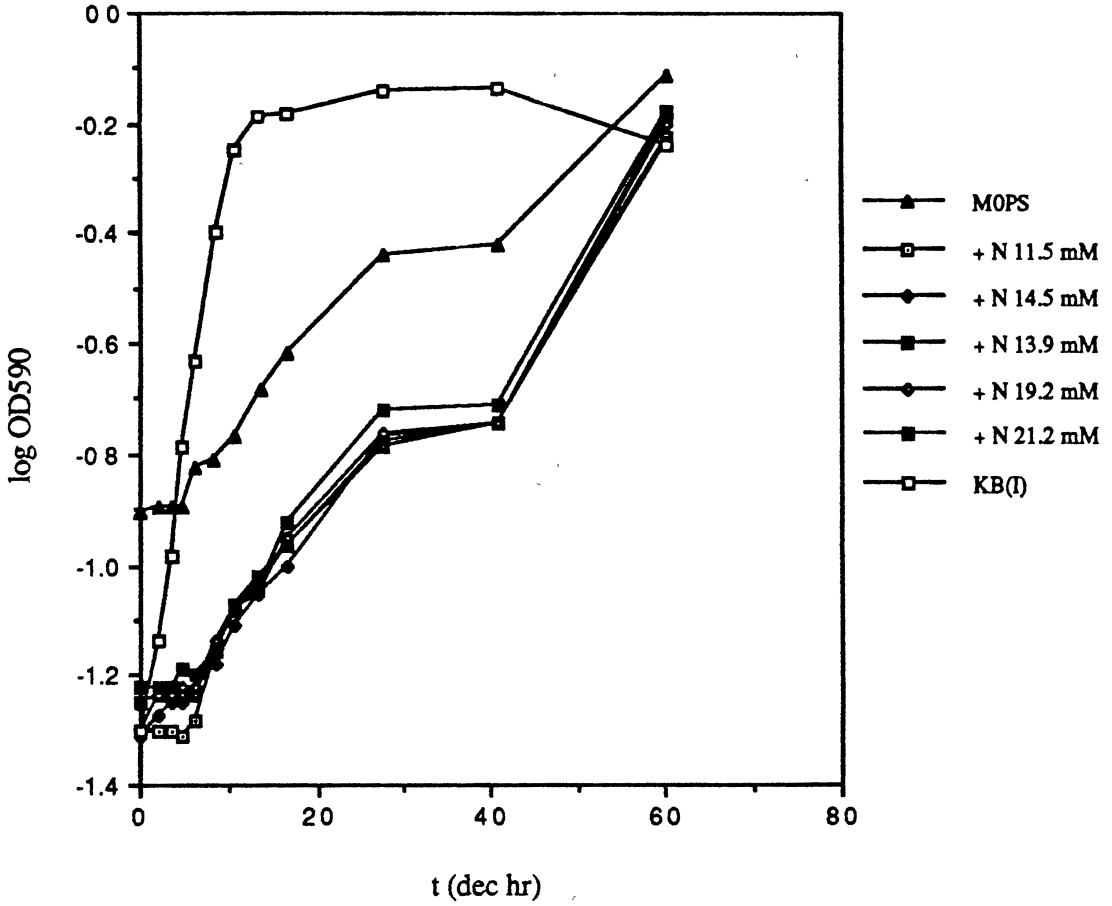


Figure 18. Growth Curve Set 4, Comparison of MOPS MM1 with Glycerol and Various Concentrations of NH_4Cl. *Xcm* S was grown in KB(I), MOPS MM1 with glycerol, and MOPS MM1 with glycerol and NH_4Cl at 11.5, 14.5, 13.9, 19.2 or 21.2 mM. Time (t) is reported in decimal hours.

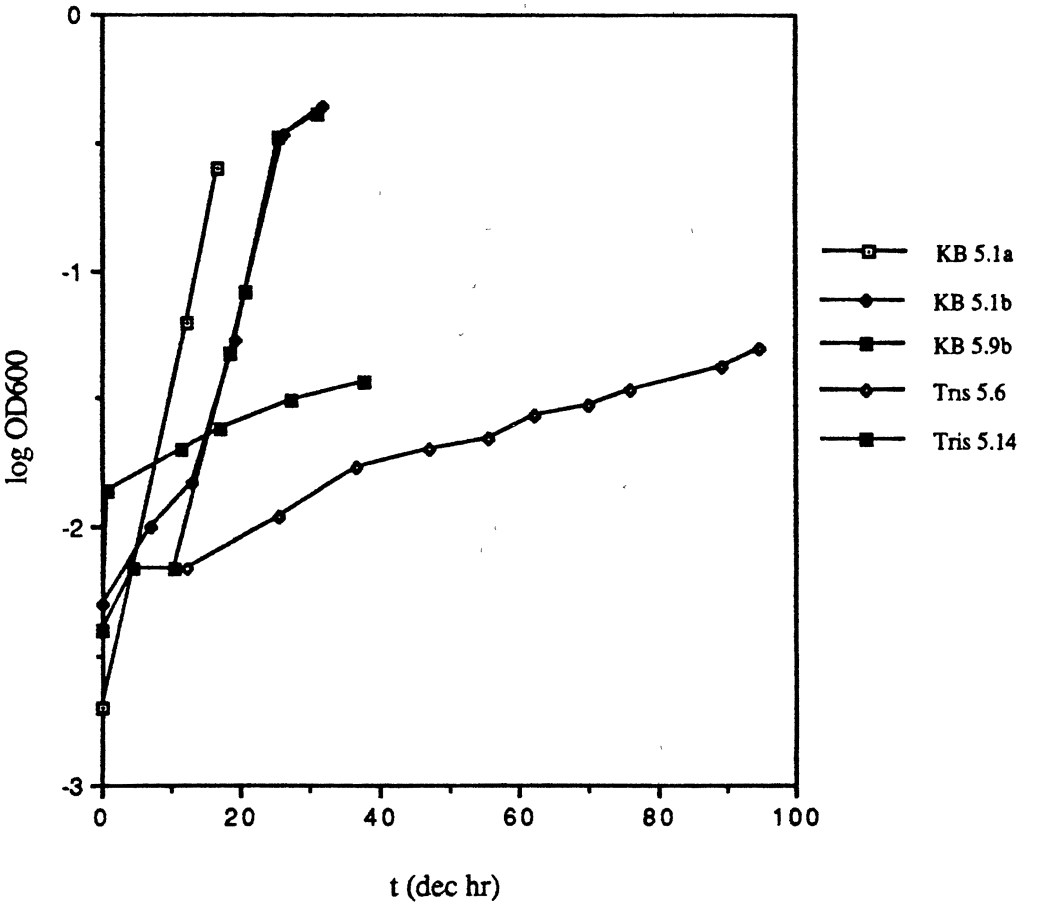


Figure 19. Growth Curve Set 5, Comparison of KB(I) and Neidhardt's Medium Buffered with Tris. *Xcm* H was grown in KB(I) or in Neidhardt's medium buffered with Tris pH 7.2 and supplemented with glycerol. Time (t) is reported in decimal hours.

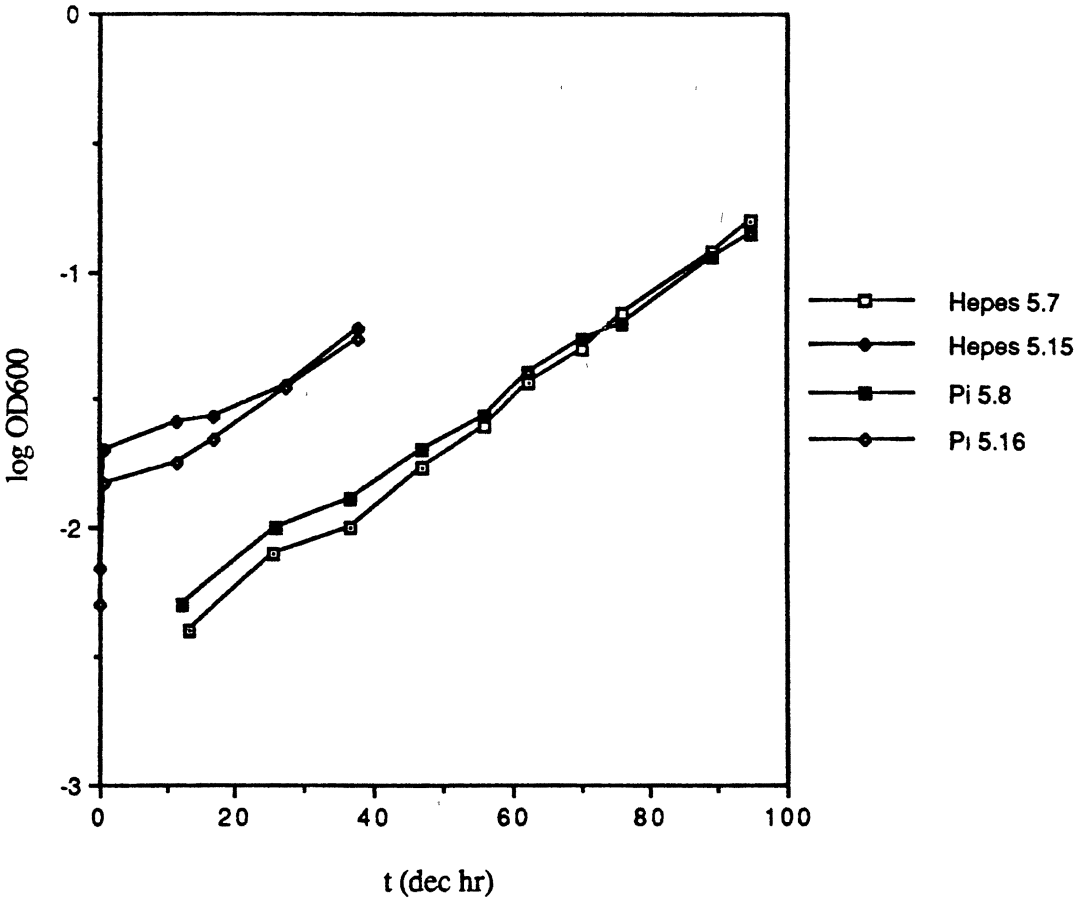


Figure 20. Growth Curve Set 5, Comparison of Neidhardt's Medium Buffered with Hepes or Phosphate. *Xcm* H was grown in Neidhardt's medium buffered with Hepes or phosphate (P_i) pH 7.2 and supplemented with glycerol. Time (t) is reported in decimal hours.

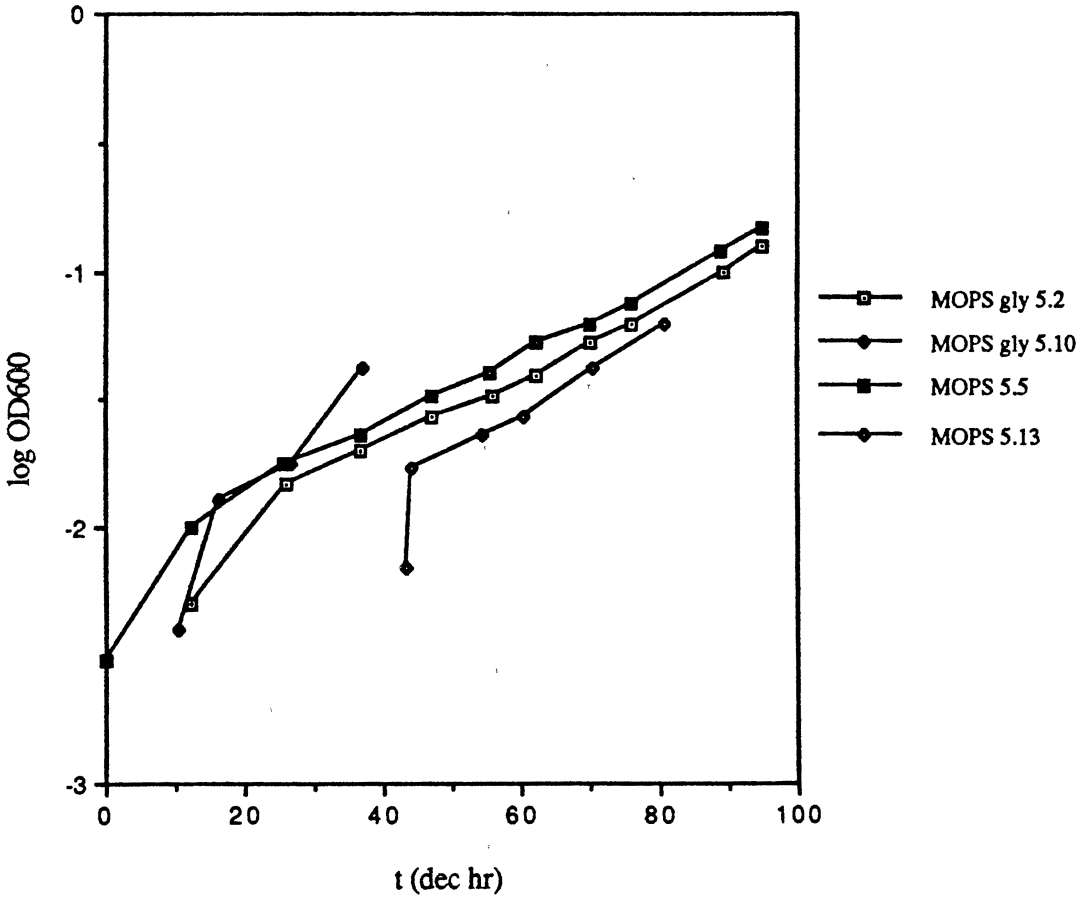


Figure 21. Growth Curve Set 5, Comparison of MOPS Medium with Glycerol and/or NaCl and H_3BO_3. *Xcm* H was grown in MOPS medium with glycerol and/or NaCl 50 mM and H_3BO_3 4 x 10^{-4} mM. Time (t) is reported in decimal hours.

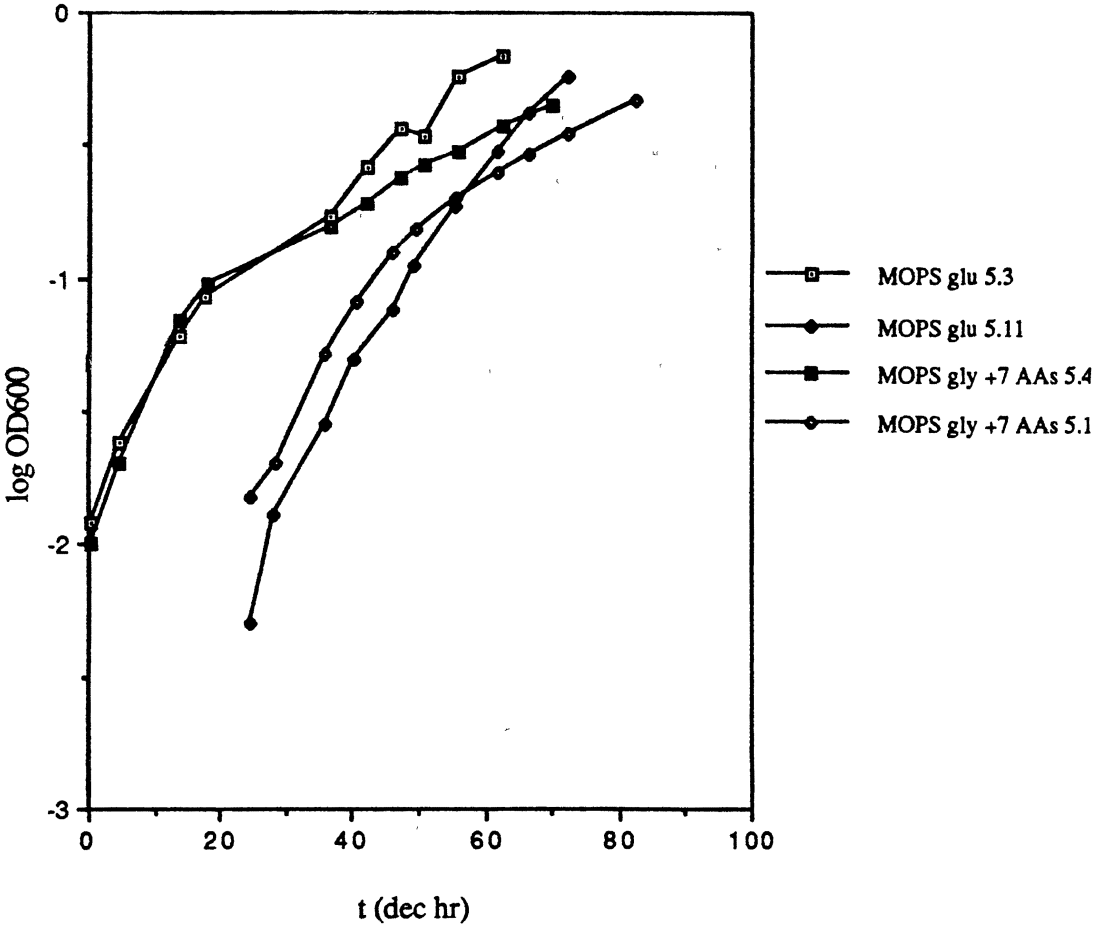


Figure 22. Growth Curve Set 5, Comparison of MOPS MM1 with Glucose or Glycerol and Seven Amino Acids. *Xcm* H was grown in MOPS medium with glucose or glycerol and the seven amino acids (alanine, arginine, asparagine, histidine, isoleucine, threoinine, and phenylalanine). Time (t) is reported in decimal hours.

TABLE 6

GROWTH RATE DETERMINATIONS

Determ.	race	Medium	C-source	AA additives		Lin reg slope	R^2	Doubl. time g 1/(hr)
GC1.9	S	KB	137 mM gly	peptone		0.089	0.997	3.4
GC2.1	"	"	"	type I		0.109	0.988	2.8
GC3.16	"	"	"	"		0.078	0.968	3.9
GC4.12	"	"	"	"		0.103	0.99	2.9
GC5.1a	H	"	"	"		0.125	0.999	2.4
GC5.1b	"	"	"	"		0.081	0.951	3.7
GC5.9a	"	"	"	"	2 pts	0.15	1	2
GC5.9b	"	"	"	"		0.091	0.953	3.3
GC1.3	S	Stanier's	137 mM gly			ND		
GC1.4	"	"	"	2 mM P		ND		
GC1.1	"	"	10 mM glu			ND		
GC1.2	"	"	"	2mM P		ND		
GC5.5	H	MOPS MM2 a	137 mM gly			0.014	0.996	22.1
GC5.13	"	"	"			0.016	0.99	19.3
GC1.7	S	MOPS MM1 a	137 mM gly		early	0.05	0.967	6
					late	0.029	0.995	10.3
GC3.1	"	"	"			0.032	0.996	9.4
GC4.1	"	"	"			0.02	0.985	15.3
GC5.2	H	"	"			0.015	0.969	19.9
GC5.10	"	"	"			0.025	0.942	12.1
GC1.5	S	MOPS MM1	10 mM glu			0.036	0.996	8.3
GC5.3	H	"	68.5 mM glu		early	0.048	0.988	6.3
					late	0.023	0.951	12.9
GC5.11	"	"	"		early	0.064	0.929	4.7
					late	0.034	0.992	8.8
GC1.6	S	MOPS MM1	10 mM glu	2 mM P		0.042	0.994	7.1
GC1.8	S	MOPS MM1	137 mM gly	2 mM P	early	0.04	0.996	7.6
					late	0.023	0.995	12.8
GC2.2	"	"	"	"		0.019	0.991	16.2
GC3.2	"	"	"	"		0.029	0.996	10.5
GC4.2	"	"	"	"		0.031	0.993	9.7
GC3.3	"	MOPS MM1	137 mM gly	10 mM P		0.033	0.977	9.1
GC3.9	S	MOPS MM1	137 mM sor	2 mM P		0.011	0.994	28.5
GC2.3	"	"	137 mM gly, sor	"		0.028	0.994	10.9
GC3.6	"	"	"	"		0.048	0.969	6.3

TABLE 6 (Continued)

Determ.	race	Medium	C-source	AA additives		Lin reg slope	R^2	Doubl. time g 1/(hr)
GC3.4	S	MOPS MM1	137 mM gly	0.57 mM A, H		0.034	0.988	8.8
GC3.5	"	"	"	1.17 mM A, H, I, T		0.035	0.959	8.6
GC4.3	"	"	"	5mM E		0.035	0.986	8.7
GC4.6	"	"	"	9.69 mM 10 AAs b		0.038	0.988	7.9
GC4.5	"	"	"	9.39 mM 9 AAs c		0.025	0.978	12.1
GC4.4	"	"	"	2.39 mM 7 AAs d		0.047	0.999	6.4
GC5.4	H	"	"	"	early	0.062	0.999	4.9
					late	0.013	0.994	22.6
GC5.12	"	"	"	"	early	0.049	0.995	6.2
					late	0.015	0.986	19.4
GC4.7	S	MOPS MM1	137 mM gly	11.52 mM NH4Cl		0.023	0.951	12.9
GC4.8	"	"	"	14.52 mM NH4Cl		0.02	0.993	15
GC4.9	"	"	"	13.91 mM NH4Cl		0.02	0.967	14.9
GC4.10	"	"	"	19.21 mM NH4Cl		0.021	0.988	14.6
GC4.11	"	"	"	21.21 mM NH4Cl		0.022	0.985	13.9
GC5.6	H	Tris MM1	137 mM gly		early	0.016	0.998	19.3
					late	0.008	0.991	36.4
GC5.14	"	"	"			0.014	0.982	20.9
GC2.4	S	"	"	2 mM P		0.03	0.992	10.2
GC3.11	"	"	"	"		0.026	0.974	11.4
GC5.7	H	Hepes MM1	137 mM gly			0.02	0.996	15.4
GC5.15	"	"	"			0.012	0.945	24.4
GC2.5	S	"	"	2 mM P		0.004	0.956	70.7
GC3.13	"	"	"	"		0.028	0.992	10.6
GC5.8	H	Pi MM1	137 mM gly			0.017	0.997	17.3
GC5.16	"	"	"			0.016	0.967	19.1
GC2.6	S	"	"	2 mM P		0.007	0.972	44.2
GC3.14	"	"	"	"		ND		
GC2.7	S	No buf MM1	137 mM gly	2 mM P		0.013	0.988	22.4
GC3.15	"	"	"	"		0.012	0.981	24.8

a MM1: Basal salts without NaCl or H3BO3
MM2: Basal salts with NaCl or H3BO3

b 10 AAs: A,R,N,C,E,H,I,T,F,P

c 9 AAs: A,R,N,E,H,I,T,F,P

d 7 AAs: A,R,N,H,I,T,F

increase in growth comparable to that seen for *Xcm* H in the presence of glucose and no added amino acids (Figures 17 and 22).

Although set 4 suffered slight contamination from the ubiquitous *Bacillus* sp. in at least the KB(I) sample, contamination did not seem to have seriously interfered with the growth curve analysis since only small amounts of white cells were found in a sample centrifuged in a 1.5 ml centrifuge tube using a microcentrifuge. It is clear that the effect of amino acid addition is not solely one of the availability of N since NH_4SO_4 supplied to the media at the same levels as N in the AAs did not lead to a stimulation of growth (Figure 18).

Fluctuations in growth constants and doubling times obtained from OD measurements reported in Table 6 illustrate that the data are far from perfect. Fluctuations in rates can be attributed to strain or medium variations or errors in readings. Trends are observable in spite of these diffculties, which included instances of biphasic growth for curves using OD_{600} as the dependent variable. Measurements of CFU/ml for time points for the growth curves in Figures 19 through 22 resulted in faster rates of growth calculated than use of OD measurements for the exponential phase of the Jr. Coleman when the OD_{600} was 0.1 or greater (data not shown). The CFU/ml vs. time curves did not exhibit biphasic growth, although too few points may have been taken for this to be apparent.

Perhaps, OD_{600} is a simplistic gauge of growth, since cell size in *Xcm* could vary throughout exponential phase unlike *E. coli* (Ingraham, Maaloe, and Neidhardt, 1982). Alternatively, the wavelengths 590 and 600 nm may carry too high of a dependence on the absorbance of the yellow–brominated pigments, xanthomonidins. Addition of Br to the media in trace amounts may be able to enhance the production of xanthomonidin throughout growth, if xanthomonidin becomes a limiting factor to measuring growth for the media combinations used in this study. Further medium supplements which could be tested are the amino acid levels found in cotyledon intracellular wash fluids from cotton infected with *Xcm* (Essenberg, unpublished). Levels analogous to those the bacteria see *in*

planta may well support growth better than the amino acid levels used herein which were determined for *E. coli* (Davis *et. al*, 1980).

Summary

In conclusion, the defined medium chosen for further use was Neidhardt's medium, MOPS MM1, buffered with MOPS at pH 7.2 without NaCl or H_3BO_3 using 137 mM glycerol as the C–source and supplemented with seven amino acids (alanine, arginine, asparagine, histidine, isoleucine, phenylalanine, threonine). This medium has found considerable usage within the laboratories of Dr. Dean Gabriel and Dr. Margaret Essenberg. Although considerable effort could still be spent at refining the concentrations of medium components, it is satisfying that both medium component concentrations and amino acid concentrations defined for *E. coli* can support growth of *Xcm* with few modifications.

CHAPTER IV

MOLECULAR BIOLOGICAL ANALYSIS OF AVIRULENCE IN *XCM*

Introduction

The goal of this work was, initially, to characterize an avirulence gene of *Xcm* by transposon mutagenesis. This involved transfer of a transposon from *E. coli* using a plasmid carrier which would be unstable in *Xcm*. Instability of the carrier vector would allow the selection of Tn-mutants simply by the selection of *Xcm* mutants which carried the Tn antibiotic resistance marker. Since problems were encountered while attempting Tn-mutagenesis directly into *Xcm*, and the goal of identifying presumptive avr genes had been accomplished (Gabriel *et al.*, 1986), I decided instead to attempt Tn-mutagenesis of one of the avr gene-carrying clones in *E. coli* in order to delineate the region of the cloned insert in the cosmid pUFA809 which contained the avr gene.

The clone chosen for these latter experiments was pUFA809, one of four avr gene-carrying clones reportedly conferring avirulence to *Xcm* N when screened on Ac B_5 plants. We hoped that a study of pUFA809 would help to define the mechanism whereby four non-homologous presumptive avr cosmid clones could all confer incompatibility to a plant line thought to posess a unique resistance gene (a more detailed description of experiments designed to confirm the genetics of Ac B_5 follows in Chapter 5). The method chosen to partially characterize clone pUFA809 was Tn-mutagenesis by Tn*3*-HoHo1 mutagenesis. The use of Tn*3*-HoHo1 would also allow analysis of transcriptional direction and promoter strength.

Problems were also encountered with this approach and led to the attempt to localize the avr gene on the insert of pUFA809 by restriction fragment linked polymorphism

mapping among a group of *Xcm* strains. Included in this group were several spontaneous race change mutants of *Xcm* H which were isolated from a segregant of Ac B_5 that appeared to carry the R gene corresponding to the avr gene contained within pUFA809. The isolation of the spontaneous race change mutants is described in Chapter 5, while hybridization patterns of pUFA809 to these mutants and attempts at complementation of the mutation by pUFA809 are described below. Some of these results were reported at meetings of the American Phytopathological Society (McNally *et. al*, 1986 and 1987).

Materials and Methods

Bacterial Strains and Media

The bacterial strains used in this study are described in Table 2 for *Xcm* and Table 7 for *E. coli*. *Xcm* was grown in KB containing type I or type III peptone, Difco nutrient broth or in MOPS MM1 supplemented with glycerol and various combinations of amino acids as described in chapter 2. *E. coli* was grown in LB medium (Maniatis *et. al*, 1982). Antibiotic selection levels for *Xcm* were 7 to 50 µg/ml kanamycin, 50 to 200 µg/ml streptomycin, 75 to 100 µg/ml rifampicin, 3 to 10 µg/ml tetracycline, 100 µg/ml trimethoprim, 50 µg/ml nalidixic acid, and 50 µg/ml chloramphenicol. For *E. coli* either the same amount was used as for *Xcm* (*e.g.*, in suicide plasmid or triparental conjugations) or 25 to 50 µg/ml were used. *Xcm* was cultured at 30° C with moderate shaking at 150 rpm while *E. coli* was grown at 37° C with vigorous shaking at 200 or more rpm.

General Molecular Genetics Techniques

Large scale plasmid preparations were made from *E. coli* cells using the chloramphenicol amplification procedure followed by nonidet P40 (a non-ionic detergent, Sigma Chemicals) lysis (Davis *et al.*, 1980 as modified by Gabriel, unpublished) or the

TABLE 7

ESCHERICHIA COLI STRAINS AND PLASMIDS

E. coli strain or plasmid	genotype	reference
HB101	F$^-$, *hsd*S20(r_B^-, m_B^-), *rec*A13, *ara*-14, *pro*A2, *lac*Y1, *gal*K2, *rps*L20(Sm^r), *xyl*-5, *mtl*-1, *sup*E44, λ^-	Boyer and Roulland-Dussoix (1969)
C600	F-, *thi*-1, *thr*-1, *leu*B6, *lac*Y1, *ton*A21, *sup*E44, λ^-	Appleyard (1954)
C2110	Nal^r, *pol*A1, *rha, his*	Leong *et al.* (1982)
MC1061	D(*lac*IPOZY)(*ara,leu*), *gal*U$^-$, *gal*K$^-$, *hsr*$^-$, *hsm*$^+$	Casadaban and Cohen (1980)
pAS8Rep-1	pAS8 Tc^S, *rep*-1::Tn7	Sato *et al.* (1981)
pUW964	pRK2013 *kan*::Tn7,:*xyz*:Tn5	Weiss *et al.* (1983)
pXL2	pRK2013::Tn5	N. Panopoulos
pSa::Tn10	IncW, tra$^+$, mob$^+$, Ap^r,	C. Kado
pSUP1011::Tn5	Mob(RP-4), Cm^r	Simon *et al.* (1983)
pUFA809	pSa747 with *Xcm* H insert	Gabriel *et al.* (1986)
pSa747	*Inc*W, tra$^+$, cos$^+$, Kn^r, Sp^r	Tait *et al.* (1983)
pSa322	*col*E1, tra$^+$, mob$^+$, Ap^r	Tait *et al.* (1983)
pHoHo1	*col*E1, *tuf*B-*lac*ZYA, *tnp*R, *bla*	Stachel *et al.* (1985)
pSShe1	*col*E1, *tnp*A, Cm^r	Stachel *et al.* (1985)
pUCD800	pUCD5, *sac*B, *sac*R	Gay *et al.* (1985)

alkaline-SDS technique (Maniatis *et. al*, 1982). Genomic DNAs were prepared from *Xcm* using the technique of Gabriel *et al.* (1986). Small scale preparations of both *E. coli* and *Xcm* DNA were done using the alkaline SDS procedure. Nick translations were carried out using the procedure of Melcher *et al.* (1981). Southern transfers and hybridizations were performed using the technique of Southern (1975), and washing steps were carried out as follows: 2X SSC with 0.1 % SDS for 30 min at room temperature performed twice, followed by 0.1 X SSC with 0.1 % SDS at 65° C performed twice. Restriction endonuclease digests were performed using the conditions suggested and buffers supplied by the manufacturer.

Field-inversion Gel Electrophoresis

Field-inversion gel electrophoresis (FIGE) was performed with 1.6 sec forward current and 0.6 sec reverse current at a voltage gradient of 10 volts/cm of gel with 1.0% agarose slab gels using TAE buffer at 4° C (Carle *et al.*, 1986 and Bostock, 1988). The FIGE apparatus consisted of a Commodore Vic 20 computer and tape drive attached to a digital to analog converter. A BASIC computer program ran the converter controlling a double pole/double throw switching relay (P.B. Grover, unpublished). Molecular sizing was done using standard n-degree polynomial regressions using a program written in Apple-soft BASIC and designed by Richard C. Essenberg (unpublished). Molecular size standards were λ phage HindIII restriction fragments. Migration distances from three gels were obtained, fit to a polynomial, and the resulting molecular sizes were averaged.

Suicide Plasmid Conjugations

Transposon mutagensis of *Xcm* was attempted by conjugation of *E. coli* strains harboring various Tn-loaded plasmids. Routinely, 10^9 donors and recipients were used in the conjugations. Two methods for loading the cell mixtures onto a filter for mating were

used: either a low vacuum was drawn on a Millipore filter unit with an aspirator as the cell mixture was pipetted onto it, followed by a wash with sterile KB, or the cell mixure was blotted onto the filter by capillary action using sterile filter paper and paper towels for wicking. The first technique was used until lower numbers of surviving *Xcm* than expected were noted. The second technique was used thereafter. The filter containing the cell mixture was incubated at 30° C from 4 to 48 hours on KB(I) or KB(type III peptone) media which lacked exogenous Mg^{++} or Ca^{++} where specified. After incubation at 30° C, the bacteria were resuspended from the filter by trituration or gentle shaking, dilutions were made of the cell mixture in sterile 0.9% NaCl, and both selective and non-selective plates were spread. Selective plates were either rich media containing antibiotic counterselections against both *Xcm* and *E.coli* or the defined MOPS minimal medium for auxotrophic selection against *E. coli* with antibiotic counterselection against non-recipient *Xcm*. The mating plates were then incubated at 30° C for 3 to 7 days before colonies on them were counted.

Triparental Matings

Triparental matings were performed by modification of the technique of Ditta *et al.* (1980): 10^9 cfu *Xcm*, 10^6 to 10^7 cfu *E. coli* donors, and 10^6 cfu *E. coli* helpers were mixed, centrifuged, and resuspended in 50 ml sterile 0.9% (w/v) NaCl, and blotted onto sterile membrane filters by capillary wicking. The filters were placed on MOPS MM1 containing glycerol and peptone (Gabriel *et al.*, 1986) for 24 hours without antibiotic selection. The filters were then transferred to MOPS minimal medium with 35 µg/ml kanamycin for an additional 48 hours incubation. The bacterial mass was resuspended, diluted, and then plated on MOPS minimal plates with the same level of antibiotic. *Xcm* colonies were picked from each mating, streaked onto LB plates with kanamycin, and

restreaked onto MOPS minimal medium with kanamycin. Individual colonies were picked and cultured in liquid MOPS minimal medium for plant inoculations to test virulence.

Results and Discussion

Attempted Transposon Mutagenesis in *Xcm*

Many attempts were made to achieve transposon mutagenesis in *Xcm*. Table 8 shows the vectors used, the *Xcm* isolates that were the intended recipients, the mating techniques employed, and whether any transconjugants were observed. Of the 49 mating attempts described in Table 8, only three matings appeared to be successful in that *Xcm* colonies grew in the presence of the antibiotic selection for Tn incorporation. Other matings attempted using pUW964 and pXL2 in an alternate host, which were failures, are not reported in this table.

Often, *Xcm* colonies would appear on the selective plates but at a frequency too close to the frequency of spontaneous mutants to antibiotic resistance for that particular mating attempt (*i.e.*, the frequencies differed by at most 10^2). Only the instances where the number of spontaneous antibiotic-resistant *Xcm* was at least a hundredfold lower than the number of *Xcm* transconjugants obtained after growth on selective media are reported here as successes. When colonies did appear on selective plates, they were usually found to be unstable, *i.e.* they could not be subcultured onto medium containing the same level of selective antibiotic. Perhaps one difficulty with the selection process was the use of antibiotic concentrations barely above levels at which wild-type *Xcm* can grow (for Tn5 the level of kanamycin used was 7 μg/ml while for Tn10 it was 3 μg/ml). The concentrations were thought necessary since it was not known to what level an antibiotic resistance gene from *E. coli* would function in *Xcm*, and a low level of antibiotic would not completely overwhelm the *Xcm* cells prior to expression of the antibiotic resistance.

TABLE 8

FREQUENCIES OF TN-MUTAGENESIS IN *XCM*

E. coli donor	*Xcm* recipient	Selection	Matings attempted		Conjugants/Recipients
			Total	Successful	
pAS8::Tn*7* in HB101	race 18	KB Rf, Sm	7	1	3×10^{-6} Sm^r, Rf^r
pUW964 in HB101	*Xcm* S,	MOPS MM1 Kn	7	1	1×10^{-7} Kn^r
(Tn*5*, Tn*7*)	pool[a], A, B, E, H		6	0	
	D		1	1	3×10^{-7} Kn^r
pSa::Tn*10* in C600	*Xcm* S, pool, E, H	MOPS MM1 Tt	17	0	(1×10^{-5} Tt^r Gabriel, unpublished)
pSup1011	race 18,	KB Rf, Sm	2	0	
(Tn*5*)	*Xcm* S, pool	MOPS MM1 Kn	2	0	
pXL2 in HB101 (Tn*5*)	*Xcm* S, pool	MOPS MM1 Kn	7	0	
			49	3	

[a] Pool consisted of a mixture of *Xcm* A, B, D, E, F, G, H, I, J, N, O, Q, R, S, T.

A further problem encountered was that when conjugants were observed, the numbers obtained from a conjugation were quite low. Hundreds, if not thousands, of separate and unique Tn-mutants would have been needed to assure the success of screening *in planta* of putative *avr*::Tn mutants. Possible explanations for the failure of these mating attempts are 1) that the vector was not mobilized into *Xcm*, 2) that the vector was mobilized but the extensive host restriction/modification system of three type I and one type II restriction endonucleases allowed *Xcm* to degrade the carrier vector prior to transposition from the suicide plasmid onto a replicon of *Xcm*, or 3) that stable maintenance of the transposon did not occur within *Xcm*.

Table 9 summarizes results from other labs working on different *Xc* pathovars. Of the published results, stable and random mutants were obtained when the Tn used was a construct (Shaw *et al.*, 1988). Turner *et al.* (1984) found that the transposons Tn*7* and Tn*5* inserted either at a single hot spot or were unstable. Both of these groups worked with *Xanthomonas campestris* pv. *campestris*. Other workers have had little success in attempting transposon mutagenesis into a variety of pathovars. The results of Turner *et al.* provided part of the impetus for abandoning Tn mutagenesis as a viable molecular tool for analyzing the genetics of avirulence in *Xcm*.

Tn*3*-HoHo1 Mutagenesis of pUFA809

While direct Tn-mutagenesis was being attempted, avr gene-carrying clones were reportedly identified by a shotgun cloning and screening approach (Gabriel *et al.*, 1986). At that point, I decided to attempt characterization of one of these avr gene-carrying clones using the Tn*3*-HoHo1 transposon of Stachel *et al.* (1985). Tn*3*-HoHo1 is a modified Tn*3* carrying a promotorless *lac*ZYA cassette from *E. coli*. Since its *tnp*A gene coding for the primary transpositional protein is disrupted by the cloned *lac*ZYA, transposition of Tn*3*-HoHo1 is only possible in the presence of an intact *tnp*A gene expressed *in trans*; therefore,

TABLE 9

ATTEMPTED TN-MUTAGENESIS OF VARIOUS *X. CAMPESTRIS* PATHOVARS

Investigator	pathovar	transposon	results
Turner *et. al* (1984)	*campestris*	Tn*5*, Tn*7*	obtained Tn mutants at hot spots, mutants were unstable
Shaw*et al.* (1987)	*campestris*	Tn*4431*	stable, random mutants (modified Tn1721)
Mellano and Cooksey (1988)	*translucens*	Tn*5*	transconjugants were stable
J. Leach (personal communication, 1987)	*oryzae*	Tn*5*	no success
D. Gabriel (personal communication, 1990)	*citrumelo translucens*	Tn*5*	stable, random mutants
	malvacearum	Tn*5*	no success with "methylase" helper

timing of the transpositional event can be strictly controlled through the use of a helper plasmid. The presence of the *lac*ZYA genes downstream of the left terminal repeat allows assay of both 1) transcriptional direction when transposition occurs into a gene in either orientation and 2) promoter strength upon insertion into a gene downstream of a promoter. A major drawback to the use of Tn*3*-HoHo1 is its large size (14.3 Kbp), so that the efficiency of transfer of any replicon harboring it to some other organism is expected to be lower. An advantage of this approach is marker-exchange between the chromosome of the recipient bacterium and the Tn*3*-HoHo1-mutated DNA insert, cloned into a non-*col*E1 plasmid, from the bacterium can occur in bacteria for which there are no (or poor) cloning vectors.

Figure 23 shows the structure of the two plasmids needed for the use of Tn*3*-HoHo1, pHoHo1 and pSShe. pHoHo1 carries the Tn*3*-HoHo1 construct while pSShe supplies an intact *tnp*A gene in *trans*. Figures 24 a to c, depict the steps undertaken to use this system for characterizing pUFA809. The first step was transformation of both pHoHo1 and pSShe into the target strain HB101 carrying pUFA809. After verification that transformants possessed all 3 plasmids, a triparental mating from HB101 of all three plasmids to the *E. coli* strain C2110, which is *pol*A1, with the helper plasmid pSa322 mobilizing pUFA809 was performed. Since both pHoHo1 and pSShe are *col*E1 replicons and require DNA polymerase I for replication, their presence is selected against in this step with transposition of Tn*3*-HoHo1 occurring in C2110 at the same time. The third step is transfer of the Tn-mutants to the strain MC1061 in order to analyze induction of β-galactosidase production. MC1061 carries a complete deletion for both the regulatory and coding regions of β-galactosidase. If any Tn*3*-HoHo1 mutant expresses β-galactosidase in MC1061, then the production is due to the *lac*ZYA genes of Tn*3*-HoHo1 under the control of a promoter upstream of the insertion site. The final step in the protocol is the transfer of mutated clones from MC1061 to *Xcm* H for marker-exchange mutagenesis with the chromosome of *Xcm* H.

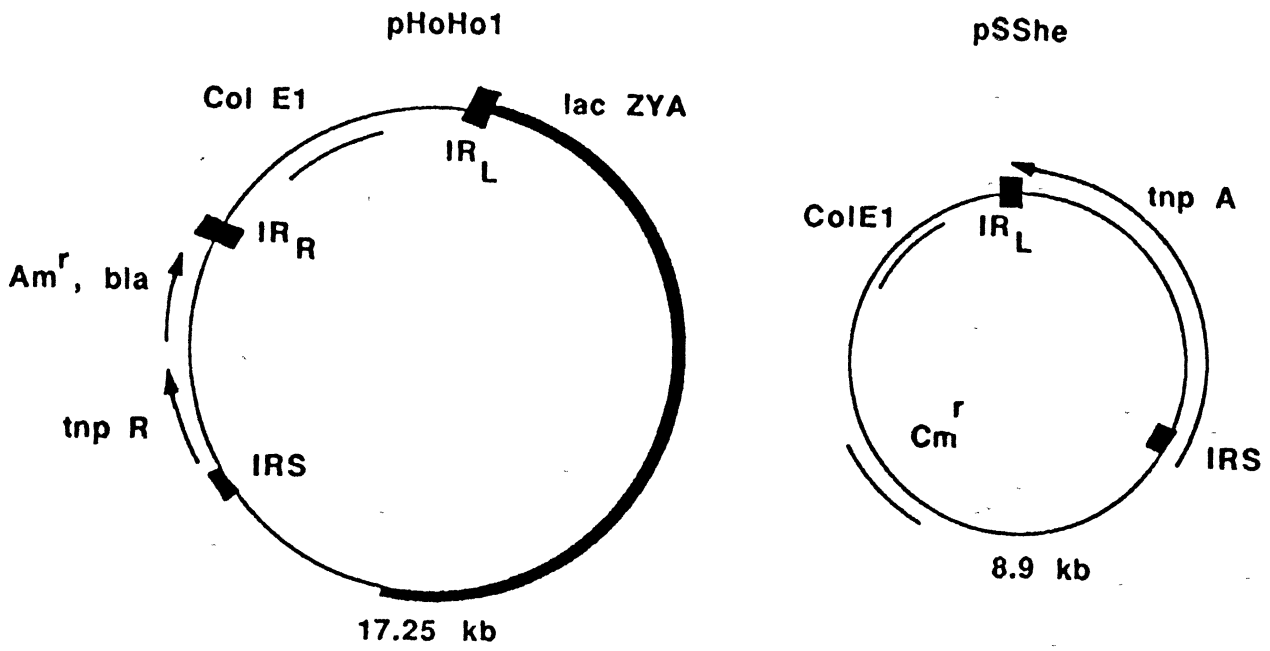


Figure 23. Structure of pHoHo1 and pSShe.

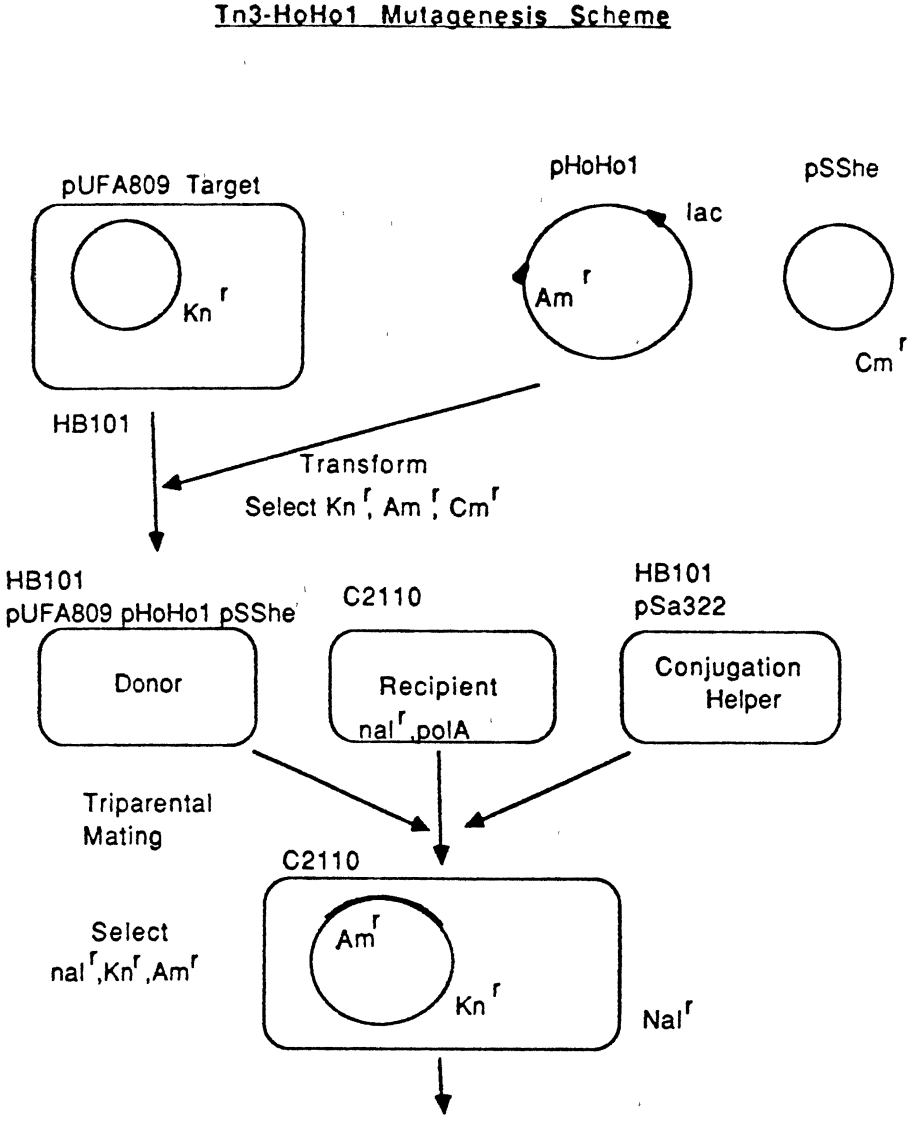


Figure 24a. Experimental Scheme for Tn*3*–HoHo1 Mutagenesis, Part 1. pHoHo1 and pSShe are transformed into HB101 carrying the target plasmid pUFA809 with transformants selected for resistance to kanamycin, ampicillin, and chloramphenicol. A transformant then serves as the donor in a triparental mating to the strain C2110 using the helper plasmid pSa322. Since C2110 is *pol*A, transposition of Tn*3*–HoHo1 occurs from pHoHo1 via its helper pSShe to the target plasmid pUFA809. Transconjugants are selected for kanamycin, ampicillin, and nalidixic acid resistance.

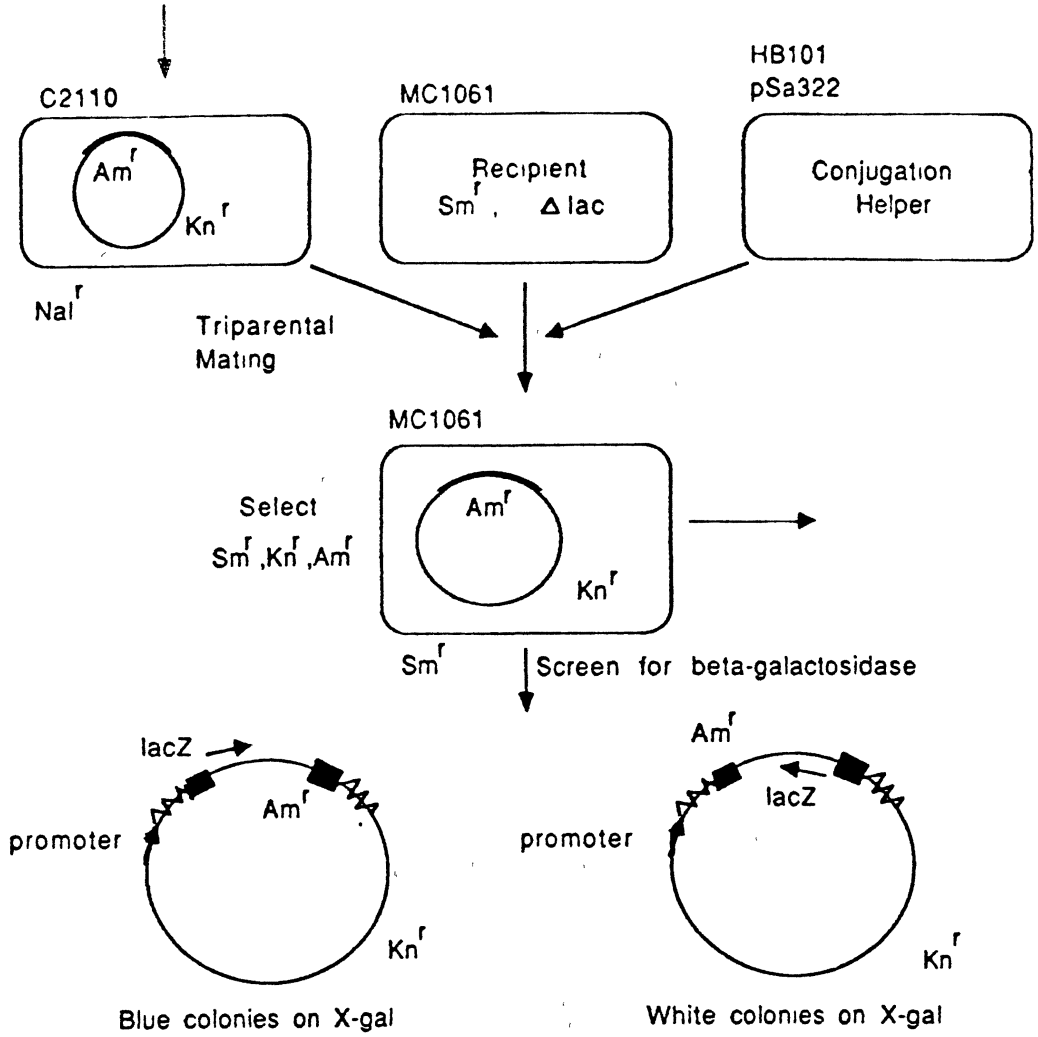
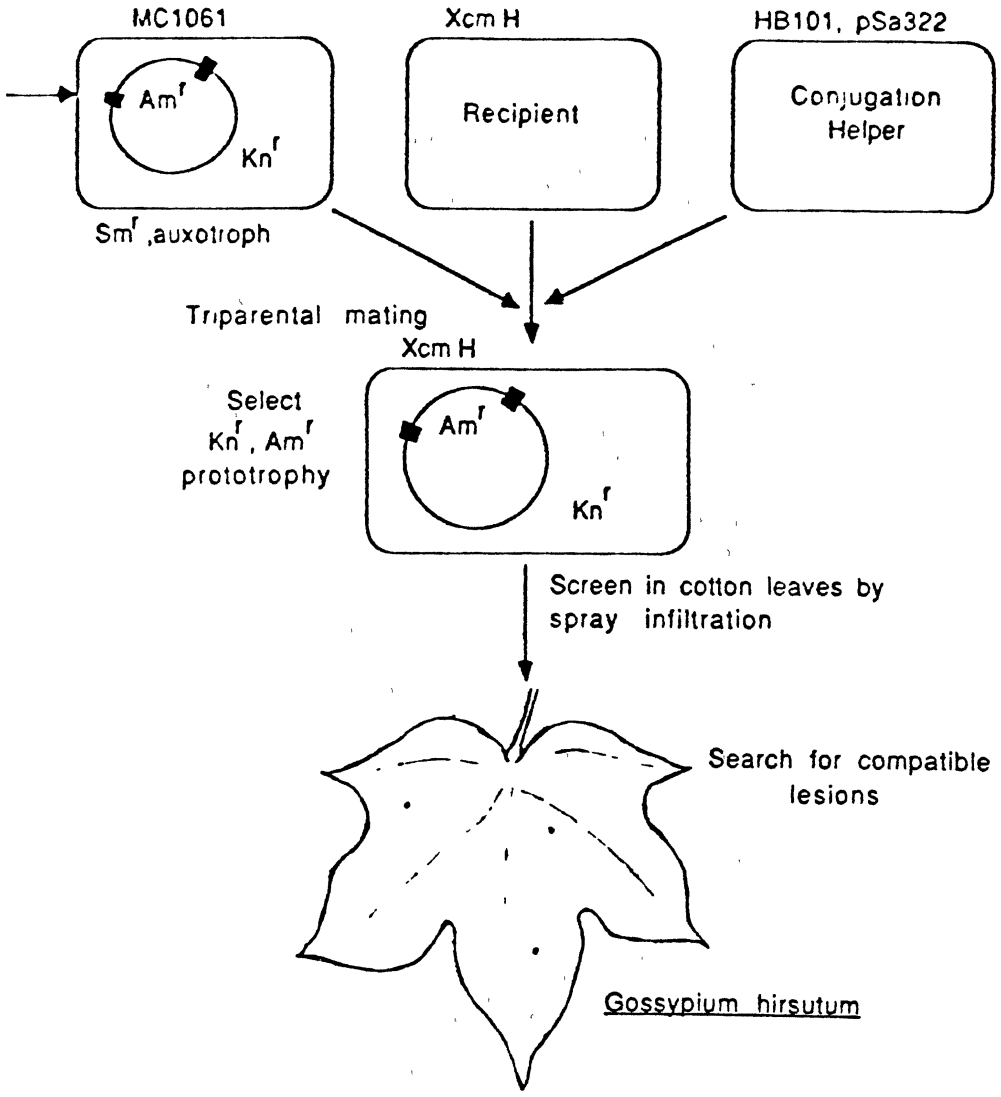


Figure 24b. Experimental Scheme for Tn*3*–HoHo1 Mutagenesis, Part 2. C2110 transconjugants containing the Tn*3*–HoHo1 mutated pUFA809 DNA serve as donors in a triparental mating to the strain MC1061 via the conjugation helper pSa322. Since MC1061 carries a deletion of the lactose operon, β–galactosidase production may be screened in this strain. Insertion of Tn*3*–HoHo1 downstream of a promoter on the target DNA leads to production of β–galactosidase in one orientation and not in the other.



Figrue 24c. Experimental Scheme for Tn*3*–HoHo1 Mutagenesis, Part 3. Tn*3*–HoHo1 mutated pUFA809 is transferred from MC1061 to *Xcm* H via the helper pSa322. If marker exchange of the mutated *Xcm* H insert DNA of pUFA809 with the homologous region of the *Xcm* H recipient chromosome and the mutated gene is the avirulence gene, compatible lesions would be observable when the population is screened on the cotton line containing the corresponding R gene.

In the initial transformation step, many transformants were obtained. Several transformants were screened by mini-prep analysis, verifying the presence of three replicons within HB101. pUFA809 was then mobilized from HB101 to C2110 using the helper plasmid pSA322. A mixed pool of over 10,000 transconjugants from this mating was collected. This mixed pool of C2110 containing pUFA809::Tn*3*-HoHo1 served as the donor cells for the triparental mating to MC1061 via the helper plasmid pSa322. Two separate matings were done, and when 157 colonies were screened for b-galactosidase production on plates containing 5-bromo,4-chloro-3-indoxyl β-D-galactoside, 56% of the colonies were blue and 44% faint blue to white. The predicted ratio of blue to white colonies would be 50% each, assuming random insertion into both strands independent of orientation. Since the observed ratio is close to that predicted, we took this as a good sign of random and stable insertion of Tn*3*-HoHo1 into pUFA809.

A mixed population of MC1061 transconjugants served as the donor cells for a mating with *Xcm* H via pSa322. This conjugation was successful, with a large number of transconjugants obtained. The *Xcm* H pUFA809::Tn*3*-HoHo1 transconjugants were pooled and inoculated by spray infiltration onto prescreened Ac B5 plants from the J4307 seed lot. Virulent lesions were observed to occur at a rate of 1 in 40 of the population of bacteria inoculated, whereas spontaneous conversion to virulence on Ac B5 was observed at a rate of 1 in 10^4 of *Xcm* H inoculated. A total of 37 isolates from the mixed inoculum of *Xcm* H pUFA809::Tn*3*-HoHo1 were recovered from both virulent and avirulent lesions on highly resistant plants. Thirty-six of the 37 retained resistance to 35 μg/ml kanamycin, and 23 of 37 were sensitive to 50 μg/ml ampicillin. Since the vector pSa747 is highly unstable in *Xcm* with less than 1% retention after 12 generations (De Feyter *et. al*, 1990), these results appeared to indicate retention of pUFA809 and less frequently Tn*3*-HoHo1 by a single integrative recombination event. Marker exchange requires two recombination events and a loss of the vector; the high frequency (1 in 40) of mutation to increased virulence of *Xcm* H was therefore not the result of marker-exchange.

When isolates were rescreened by spot-inoculation at a high inoculum concentration, all 37 isolates were virulent at a disease rating of 3 on the cotton line Ac 44 while all but one of 18 avirulent isolates retested as avirulent at a rating of 0 on the cotton line Ac B5 51B. Genomic DNA was prepared from 7 isolates which came from compatible lesions and 14 isolates which came from incompatible, all lesions were from Ac B5 51B leaves (one of the 14 isolates, 66.6, coming from an incompatible lesion type retested as being compatible on Ac B5 51B). These DNAs were cut with *Eco*RI or *Bam*HI, separated on gels, and blotted to nitrocellulose. Genomic DNA from *Xcm* H, N, and KM1 (a spontaneous race change mutant from avr to vir isolated on Ac B5 51B) served as controls on these blots. Figure 25 shows a composite of lanes from a blot of 13 isolates. Intact, nick-translated pHoHo1 served as the probe for detecting the presence or lack of Tn*3*-HoHo1.

In every case, pHoHo1 hybridized both to the controls and to the isolates. pHoHo1 hybridized to two *Eco*R1 and two *Bam*H1 fragments of the controls and most isolates. One isolate, 66.6, had two additional *Eco*R1 and *Bam*H1 bands in digests hybridizing to pHoHo1. The interpretation of the blot was complicated by pHoHo1 hybridization to the controls, perhaps resulting from homology between Tn*3*-HoHo1 to *Xcm* DNA. The lack of additional hybridizing bands in the other virulent transconjugants suggests that Tn*3*-HoHo1 was unstable when residing in *Xcm* and confirms that marker-exchane is not responsible for the observed change in virulence of *Xcm* H. The observed instability could also have been due to the long period *in planta* required for macroscopic lesions to develop from single cell infection sites and the fact that during this time the transconjugants were no longer under selective pressure for maintenance of the vector or Tn*3*-HoHo1. Stachel *et al.* (1985) state that when the target plasmid for Tn*3*-HoHo1 mutagenesis was a cosmid greater than 40 kbp a majority of the transductants obtained after *in vivo* packaging contained deletions. Perhaps, the large size (about 64 kbp) of a

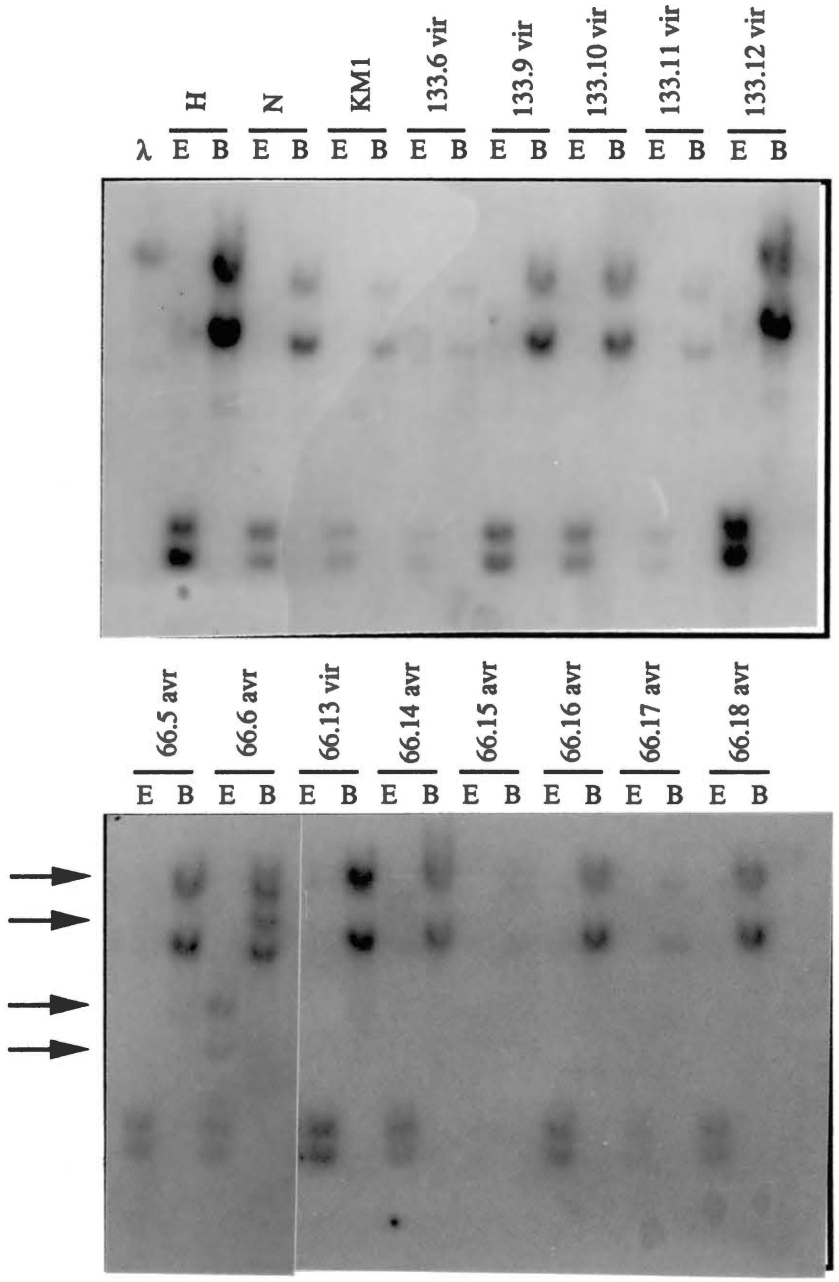


Figure 25. Hybridization of pHoHo1 to *Xcm* H, N, and KM1 and to *Xcm* H(pUFA809::Tn*3*-HoHo1) virulent and avirulent transconjugants. Total DNAs from each strain or isolate were cut with either *Eco*RI (E) or *Bam*HI (B), run on an agarose gel, transferred to nitrocellulose, and then hybridized with labelled pHoHo1 DNA. Arrows point to extra bands present in the avirulent (avr) isolate 66.6 of *Xcm* H(pUFA809::Tn*3*-HoHo1) and no other.

pUFA809::Tn*3*-HoHo1 mutant allowed a similar deletion process to occur during conjugation of mutants from *E. coli* to *Xcm.*

Restriction Mapping of pUFA809

The cosmid clone pUFA809 was mapped with the restriction enzymes *Eco*RI and *Bam*HI. Table 10 gives fragment lengths for complete, partial, and double digests determined from migration distances in FIGE gels fit to λ *Hind*III molecular weight markers by polynomial regression. Figure 26 shows one of the gels from which migration distances were taken. Errors for experimentally determined λ *Hind*III fragment lengths ranged from 0.3 to 7.0 % (data not shown).

Partial digest fragments were assigned complete digest fragments such that the sum of their lengths approximated that of the partial digest fragment. The order of restriction fragments was determined to be v, c, b, e, a, d for *Eco*RI and v, b, c, a, d for *Bam*HI. pUFA809 was also mapped in Gabriel's laboratory by Robert De Feyter (personal communication). His assignments of fragment order were v, c, b, e, a, d for *Eco*RI and v, d, a, c, b for *Bam*HI. In order to verify which of the orderings was correct, both sets of data were analyzed using the algorithm of Schroeder and Blattner (1978) implemented by Richard C. Essenberg for the Apple IIE. This algorithm derives restriction maps from ordered cutsite data. Table 10 gives double digest data which have been adjusted to conform to a best fit by the imposed order. The total squared deviation and root mean square deviation for my determination was 421.8 and 1.2, respectively, while those for De Feyter's map were 318.0 and 1.0. These values are not sufficiently different to indicate one map over the other. De Feyter's analysis of subcloned fragments (personal communication) shows that his map is the correct one. Both maps are shown in Figure 27. These maps were drawn using the *Eco*RI/*Bam*HI double digest data from Table 10.

TABLE 10

RESTRICTION FRAGMENT LENGTHS FOR PUFA809

*Eco*RI complete avg 2 gels	*Eco*RI partial 1 gel	assign-ments	*Bam*HI complete avg 2 gels	Bam part partial 1 gel	assign-ments	*Eco*RI/*Bam*HI complete adjusted McNally's	*Eco*RI/*Bam*HI complete adjusted De Feyter's
28.6		v,c,d	33.7		v,b,c,d		
25.4		v,c	30.6		v,b,d	15.4	16.5
21.5		a,b	24.8		a,b,c,d	9.8	8.9
18.7		v,d	22.8		v,d	4.8	5.0
17.4	17.2	v	20.3	20.1	v	4.1	4.0
14.4		a,d,e	17.6		a,c,d	3.7	3.8
12.2		a,e	13.5		a,d	2.5	2.3
12.0	11.5	a	12.4	11.9	a	2.0	2.1
9.9	9.8	b	7.3	7.4	b	1.9	1.9
7.8	8.0	c	3.7	3.8	c	1.6	1.6
2.5	2.5	d	1.6	1.7	d	0.3	0.3
0.3	0.3	e				0.3	0.3
49.9			45.3			46.9	46.8

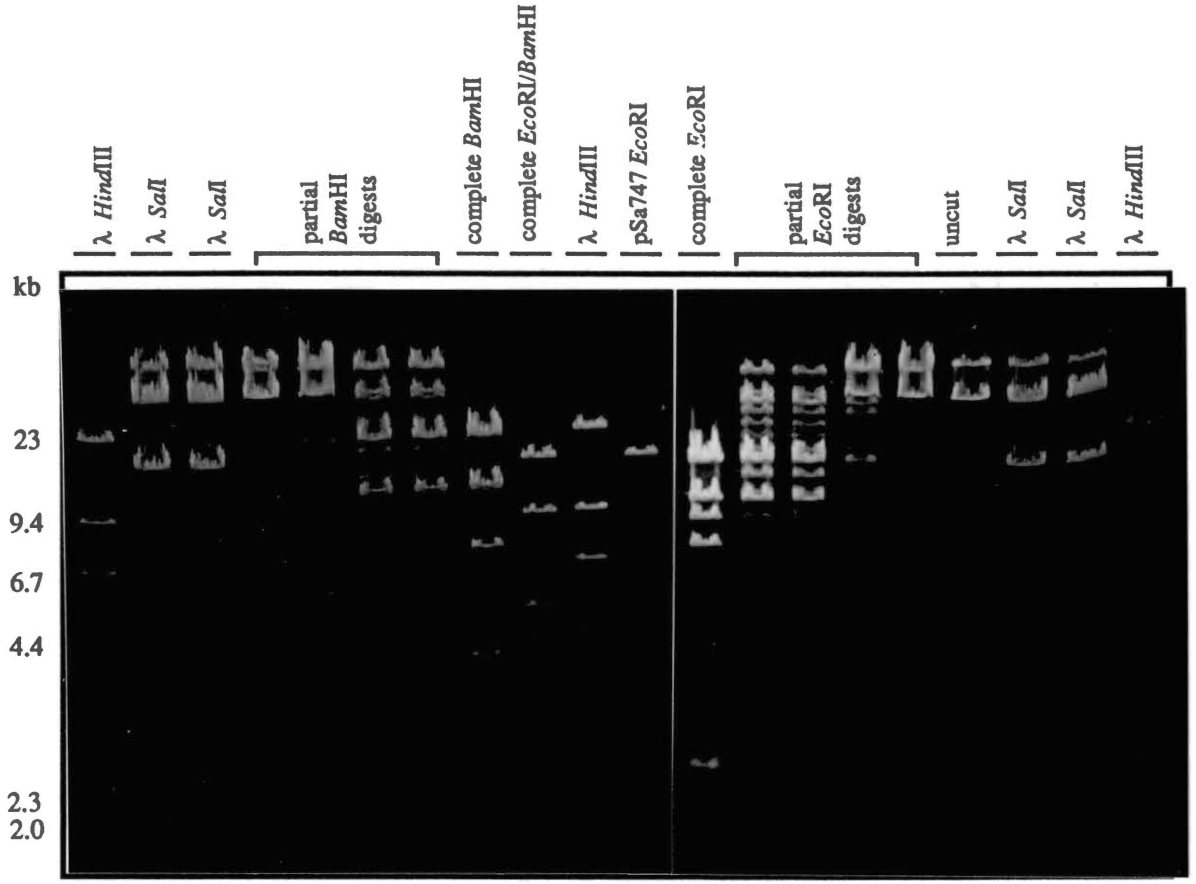


Figure 26. Agarose Gel Electropheresis of pUFA809 DNA Cut with *Eco*RI and *Bam*HI. Sizes given are for a *Hind*III digest of λ. Unless otherwise noted, DNA loaded onto the gel was pUFA809 plasmid DNA.

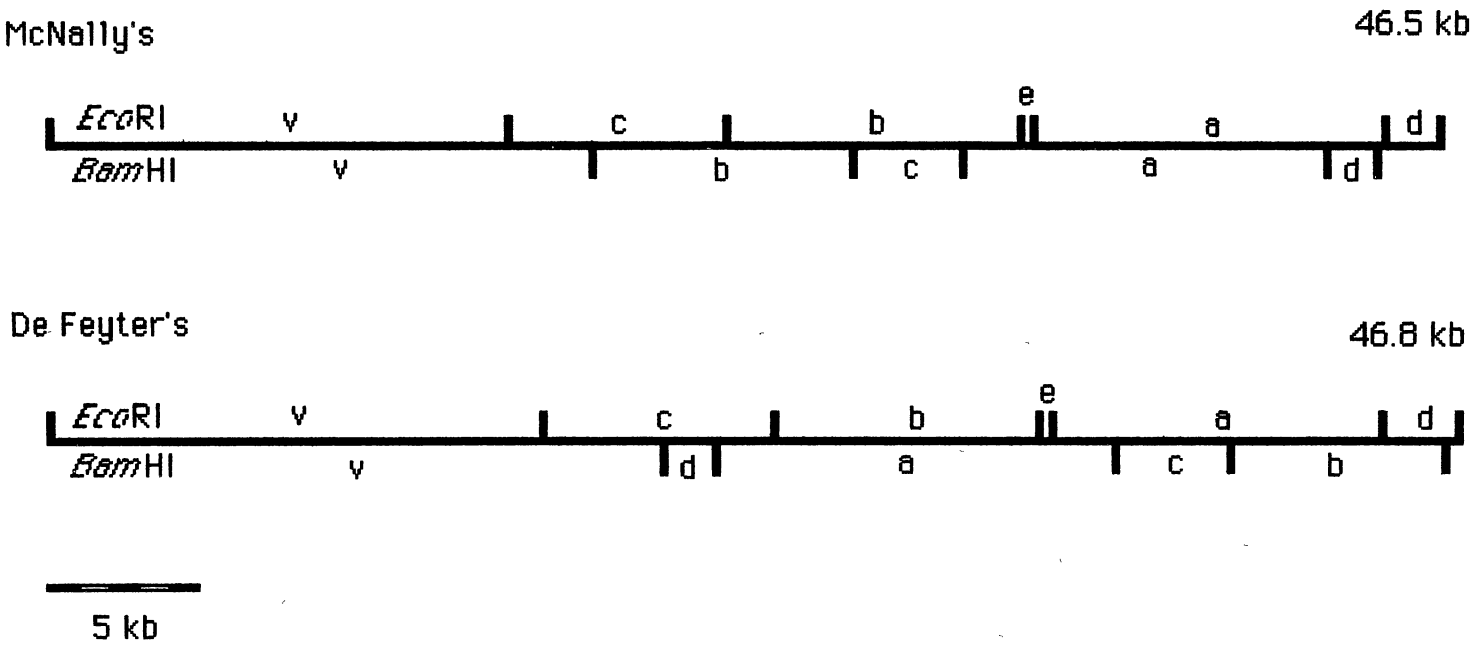


Figure 27. pUFA809 Restriction Maps

Complementation of *Xcm* N by pUFA809 in Oklahoma

In contrast to the report of Gabriel *et al.* (1986), cosmid pUFA809 failed to convert *Xcm* N to avr when either transconjugants sent by Gabriel or transconjugants obtained from conjugations I performed were screened. These transconjugants were tested on plants under two sets of conditions, one developed in Stillwater to differentiate clearly among races (Appendix 1) and the other used by Gabriel's laboratory. The conditions differ in night temperatures, 23° C for my test versus 30° C for Gabriel's, and in the inoculum concentrations, 5 x 10^6 cfu/ml versus 1 x 10^9 cfu/ml.

With the Stillwater conditions, strain N inoculated on Ac B5 plants had an average disease rating of 2, corresponding to low virulence, while Gabriel's transconjugants and mine had a disease rating of 1.5 on the same Ac B5 plants. The transconjugants of N caused a slightly lower amount of water soaking than N but complete conversion to incompatibility was not seen. With Gabriel's conditions used here, strain N inoculated on Ac B5 had a disease rating of 4, *i. e.* high virulence, while his transconjugants also had a disease rating of 4. Gabriel *et al.* (1986) report the virulence of strain N to have a disease rating of 4 (similar to the virulence of strain H on the fully susceptible line Ac 44 found by both of us) while their strain N(pUFA809) transconjugants are reported to have a disease rating intermediate between 4 and 0, the avirulence of *Xcm* H on Ac B5. Subsequent work in Gabriel's laboratory has also failed to confirm earlier work with the clone pUFA809 (Gabriel, personal communication).

Strain N water soaked the fully susceptible line Ac 44 at the high inoculum concentration and 23° C night temperature with a disease rating of 4. While this disease rating was greater than that for the lower inoculum, a disadvantage was the predominance of dried tissue in the center of lesions with only the edges remaining water-soaked. Since one of the problems with these tests was the lower virulence given by strain N with our conditions except when inoculated at high concentrations, and since I wanted to avoid

using high concentration inoculums so a disease rating would not be limited to that for a narrow ring surrounding a dry lesion center, I decided to attempt complementation of spontaneous race change mutants rather than complementation of *Xcm* N by the reported avr gene-carrying clone pUFA809.

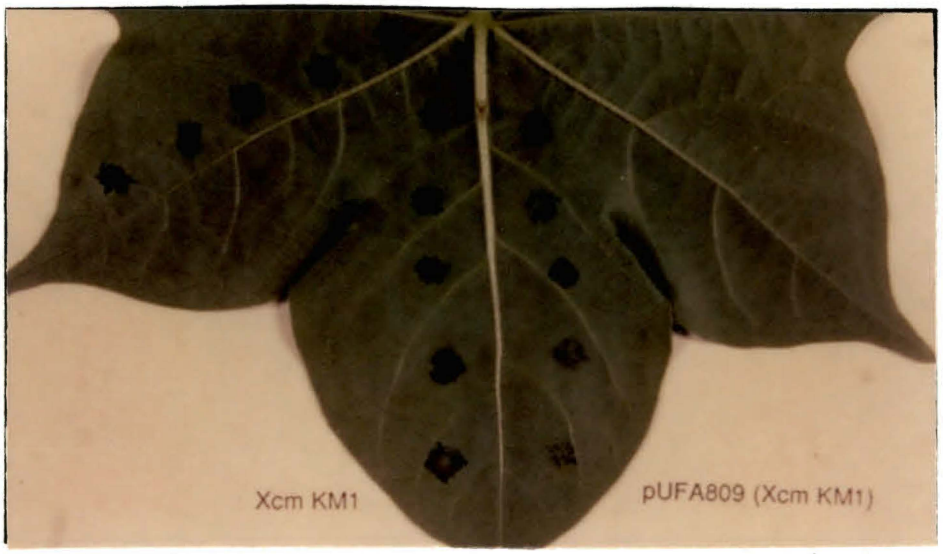
Complementation of SRCMs by pUFA809

Spontaneous race change mutants KM1, KM2, KM6, KM13, and KM21 were isolated as virulent mutants of the widely avirulent strain H on plants of Ac B_5 51B, a highly resistant segregants of Ac B_5 (Chapter 5). The frequency for appearance of these race change mutants was about 1 mutant for 1 x 10^4 parent cells screened. Complementation of such race change mutants would provide an alternative host to strain N for testing avirulence gene activity. Since the mutants are derived from strain H, which served as the source for the cloned DNA in the avr gene-carrying cosmids, the background of the mutants is similar. I hoped that the reactions given by a reported avr clone in a SRCM would be less ambiguous than strain N transconjugants since the virulence of the SRCM was consistent (described in Chapter 5).

Transconjugants of pUFA809 into KM1, *i.e.*, colonies which were resistant to kanamycin at 35 μg/ml, were obtained from four mating attempts, two matings into KM2, and one mating each into KM6, KM13, and KM21. Figure 28 shows the results of virulence analysis for a KM1 transconjugant obtained from mating 49 inoculated on Ac 44 and Ac B_5 51B leaves (with the latter being from either plant 5 or 6 of the July 1987 test). The control strains H and KM1 and the KM1(pUFA809) transconjugant gave compatible interactions on Ac 44 (Figure 28 panel a). While H and the transconjugant gave incompatible interactions on Ac B_5 51B, the race change mutant KM1 was compatible on this leaf (Figure 28 panel b). It appeared from these results that pUFA809 carries an avirulence gene specific for the resistance gene carried by Ac B_5 51B.

a) Ac 44 leaf 9 days post-inoculation

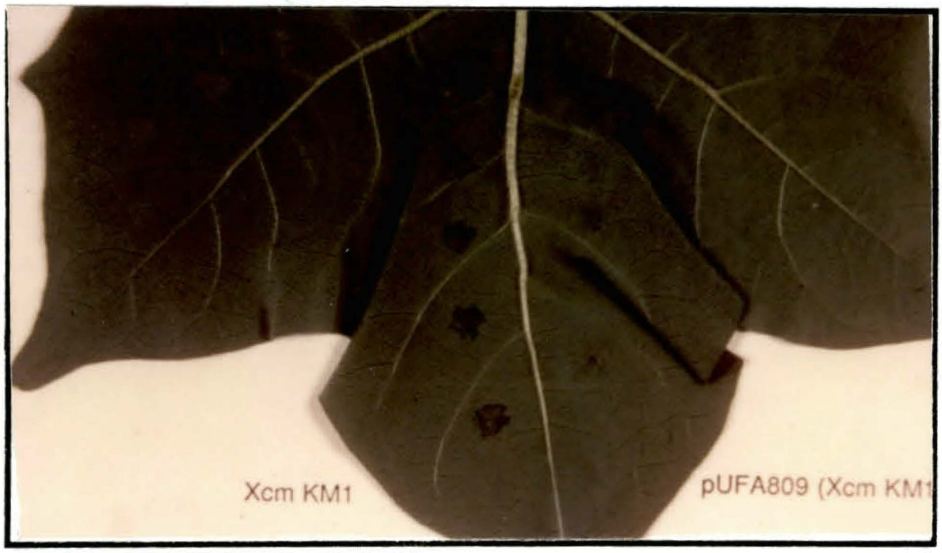
Xcm H



Xcm KM1 *Xcm* KM1(pUFA809)

b) Ac B_5 51B leaf 9 days post-inoculation

Xcm H



Xcm KM1 *Xcm* KM1(pUFA809)

Figure 28. *Xcm* KM1(pUFA809) Complementation Assay, Ac 44 and Ac B_5 51B Leaves.

Table 11 gives virulence scores for transconjugants tested in both the Conviron chamber and in the CERL chamber (conditions for these chambers appear in Figure 32 of Chapter 5). While two screenings of KM1(pUFA809) transconjugant 49 on Ac B5 51B, the one described above and an earlier test of March 1987, resulted in incompatible lesion types, KM1(pUFA809) transconjugant 49 on Ac B5 51B in the August 1988 test and KM1(pUFA809) transconjugant 43 in the March 1987 test gave weakly compatible lesions for the screenings carried out using the Conviron chamber.

For most of the transconjugants listed in Table 11, virulence scores obtained by screening in the Conviron chamber were lower on Ac B5 51B than on Ac 44 and also lower than their parent SRCM on Ac B5 51B. Notable exceptions were KM1(pUFA809) transconjugant 93 and KM2(pUFA809) transconjugant 94 which scored as virulent on Ac B5 51B as on Ac 44. Reactions for Ac B5 51B plants obtained at the same time in the CERL chamber were usually more virulent than reactions obtained in the Conviron chamber: a total of ten incompatible or weakly compatible reactions for strains and conjugants were scored on Ac B5 51B in the Conviron chamber versus eight for Ac B5 51B plants scored in the CERL chamber. Tests carried out in both chambers cast doubt on whether or not pUFA809 carried an avr gene since conditions under which complementation was observable were not easily controlled.

Hybridization Analysis of SRCM Genomic DNA to pUFA809

Genomic DNAs from *Xcm* strains H, N, KM1, KM2, KM6, KM13, KM21, RS4, and TS5.1 were prepared, cut with various restriction endonucleases, separated on an agarose gel, transferred to nitrocellulose, and hybridized with pUFA809 as the probe. Figure 29 shows a blot of H, KM1, and N genomic DNAs cut with a number of restriction

TABLE 11

REACTIONS OF PUFA809 TRANSCONJUGANTS

		Conviron Chamber				CERL Chamber							
date	strain or transconjugant	Ac 44E	51B	51A	62-3	Ac 44E	51B	51A	44-21	65-7	55-18	65-31	Ac B4
Aug-88	H	+	-	-	+/-	+	-	-	-	+/-	+	-	-
Aug-88	KM1	+	+	-	+/-	+	+	-	+	+	+	-	+
Mar-87	KM1(pUFA809) 43	+	+/-	-									
Aug-88	" 43	+	+/-	-	+/-	+/-	+/-	-	+	+/-	+/-	+/-	+
Mar-87	" 49	+	-	-									
Jul-87 plants 5, 6	" 49	+	-										
Jul-87 plants 7, 8	" 49	+	+/-										
Aug-88	" 49	+	+/-	-	+/-	+/-	+/-	-	+	+/-	+/-	+/-	+
"	" 69	+	-	-	+/-	+	+/-	-	+	+/-	+/-	-	+
"	" 93	+	+	-	+/-	+	+/-	-	+	+/-	+/-	-	+
Aug-88	KM2	+	+	-	+	+	+	-	+	+	+	-	+
"	KM2(pUFA809) 94	+	+	-	+/-	+	+	-	+	+/-	+/-	-	+
"	" 95	+	+/-	-	+/-	+	+	-	+	+/-	+	-	+
"	KM6	+	+	-	-	+	+	-	+	+/-	+/-	-	+
"	KM6(pUFA809) 97	+/-	-	-	-	+	+/-	-	+/-	-	-	-	+
"	KM13	+	+/-	-	-	+	+	-	+	+/-	+/-	-	+
"	KM13(pUFA809) 98	+/-	-	-	+/-	+	+/-	-	+/-	+/-	+/-	-	+/-
"	KM21	+	+/-	-	+/-	+	+	-	+	+/-	+	-	+
"	KM21(pUFA809) 101	+	-	-	-	+	+/-	-	+	+/-	+	-	+

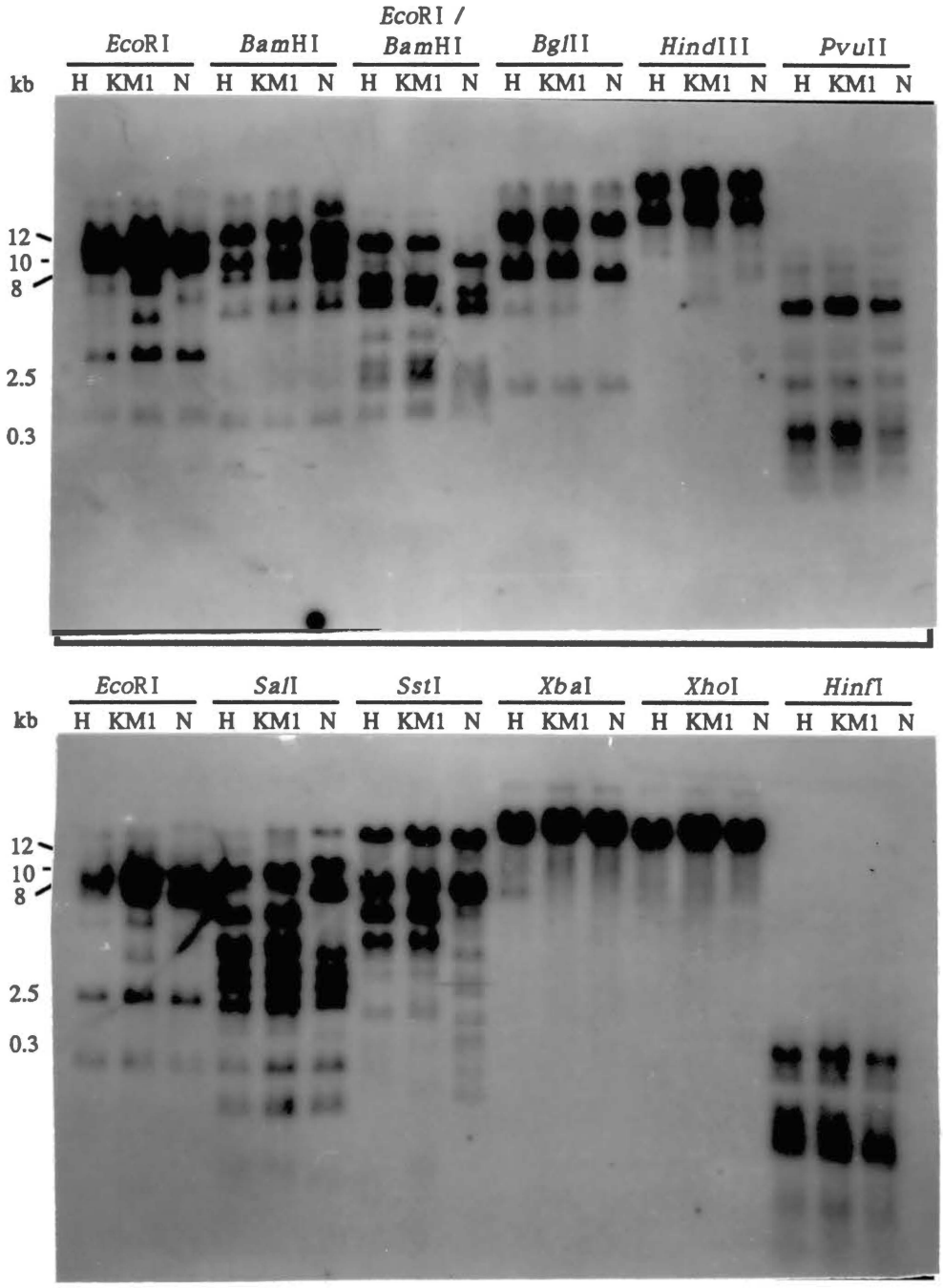


Figure 29. pUFA809 DNA Hybridized to *Xcm* H, KM1, and N Genomic DNAs digested with Ten Restriction Endonucleases. Total genomic DNAs from *Xcm* H, KM1, and N were digested with *Eco*RI, *Bam*HI, *Bgl*II, *Hind*III, *Pvu*II, *Sal*I, *Sst*I, *Xba*I, *Xho*I, or *Hinf*I. The digested DNA was electropheresed on an agarose, transferred to nitrocellulose, and hybridized to labelled pUFA809 DNA. Size standards are the bands in *Xcm* H which hybridize to pUFA809 (the same fragments as contained in the insert of pUFA809).

endonucleases while Figure 30 shows a blot of DNAs from all of the strains given above cut with *Eco*RI and *Bam*HI.

Strains H, N, and KM1 have considerable differences among their banding profiles. For these strains and the *Eco*RI digests shown in Figures 29 parts a and b and in Figure 30, relative to strain H, mutant KM1 had a new major band at about 4 Kbp while N was missing the 10 Kbp band but had a doublet at about 8 Kbp. For the corresponding *Bam*HI digests, the 12 Kbp band in mutant KM1 had a slower mobility than it had in strain H while it had a greater mobility in N. Strain N appeared to harbor a deletion of about two Kbp while KM1 appeared to contain an insert of about four Kbp. As shown in Figure 31, both the deletion and insertion mapped to the same general seven Kbp region of the insert DNA in pUFA809 where the Eco RI fragment b and Bam HI fragment a overlap (fragment designations are for De Feyter's map). The other enzymes used for the digests shown in Figure 29 yielded essentially identical patterns for strains H and KM1 with strain N having either fewer major or minor bands (*Bgl*II), having fewer major bands and more minor bands (*Sst*I), or less major and minor bands (*Sal*I) digest. Regardless, these results are consistent with the genomes of strains KM1 and N differing from that of H over the region detected by pUFA809. Complete interpretation of the hybridization profiles in Figure 29 requires the availability of a corresponding pUFA809 restriction map including these enzymes. Further work with fragments of pUFA809 subcloned into a stable vector has revealed that the cloned *Xcm* H DNA is consistently converted to DNA which gives a fragmentation pattern like that of *Xcm* N; the *Xcm* H DNA clone was never recovered from an *Xcm* N transconjugant in 14 different attempts (Gabriel, personal communication).

Strains KM1 and KM2 appear to be quite similar in their hybridization patterns (Figure 30) while KM6, KM13, and KM21 seem to be almost identical to H. Particularly for the *Bam*HI digests, the differences between H and KM1 for the blot in Figure 30 were difficult to see. When the *Bam*HI digests of H and KM1 are side by side, as in Figure 29 part a, small shifts can be seen in the mobilities of bands hybridizing to pUFA809.

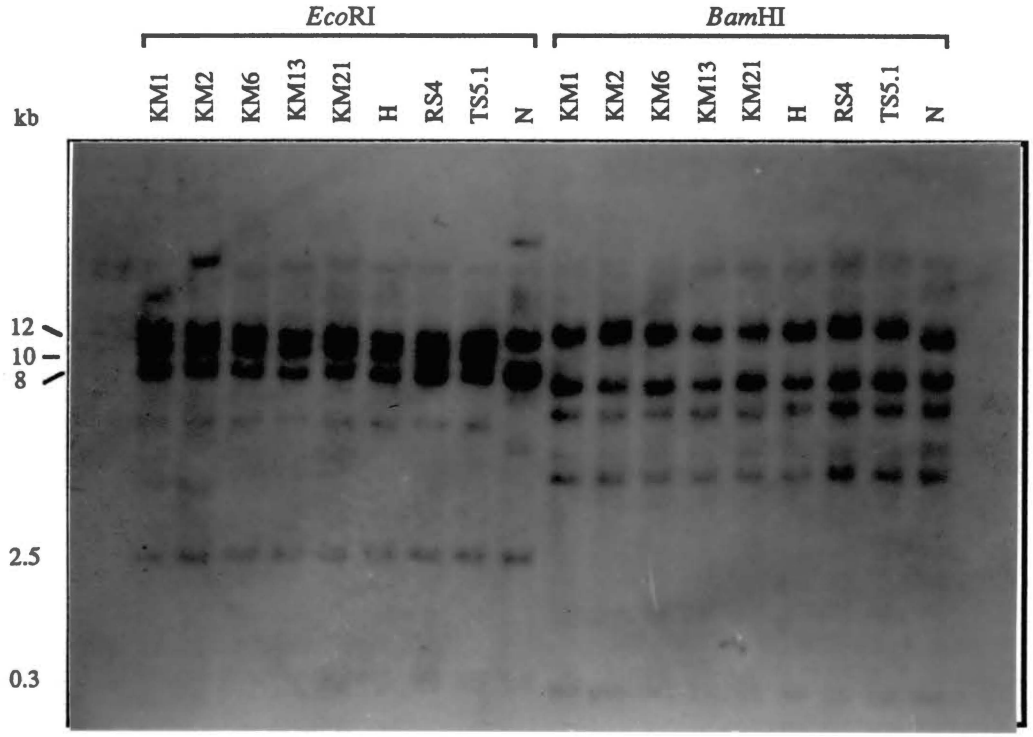
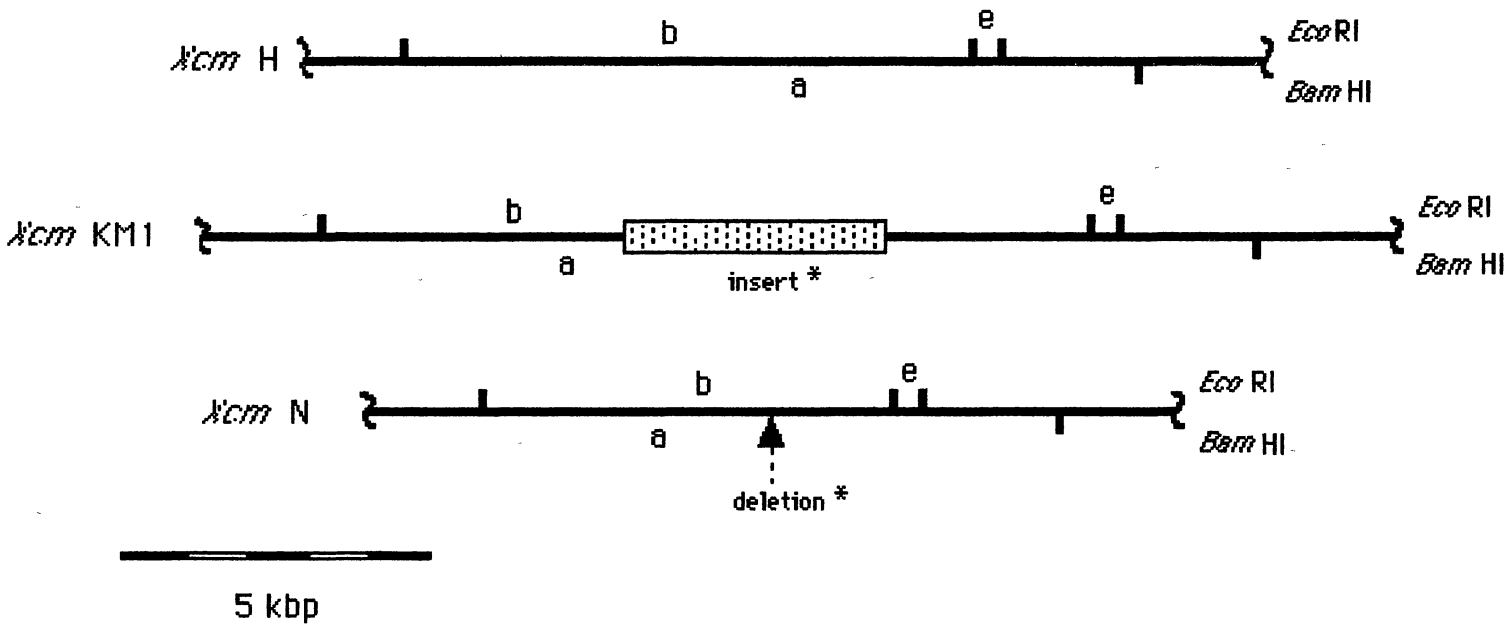


Figure 30. pUFA809 Hybridized to Genomic DNAs from Six Independent SRCMs and Three *Xcm* Strains. Genomic DNA was purified from SRCMs KM1, KM2, KM6, KM13, KM21, and TS5.1 and from *Xcm* H, RS4, and N. The DNAs were digested with the endonucleases *Eco*RI and *Bam*HI, separated on an agarose gel, transferred to nitrocellulose, and hybridized with pUFA809 as the probe. Size standards are the bands in *Xcm* H which hybridize to pUFA809 (the same fragments as contained in the insert of pUFA809).



* Both the insert and deletion size and position are approximate.

Figure 31 Predicted Maps for H, KM1, and N

Strain TS5.1 was isolated as a virulent mutant of a streptomycin and rifampicin resistant isolate of *Xcm* R3, RS4, on Ac B_5 51B by Sun (1987). The third largest major band in the *Eco*RI digest of RS4 (at about 8 kbp) is about twice the intensity of the first and second largest bands (of about 12 and 10 kbp) while for TS5.1 the first and second largest bands are about twice the intensity of the third largest band. Thus, TS5.1 appears to contain about 14 kbp in additional bands hybridizing to pUFA809 than does RS4 (or ((2*12)+(2*10)+8) = 52 kbp for TS5.1 minus (12+10+ (2*8)) = 38 kbp for RS4, with a maximum of 14 kb extra in TS5.1 assuming the hybridization intensities are double for the bands multiplied by two). These differences are less apparent in the *Bam*HI digest lanes although the largest major band does differ between the two. If pUFA809 is detecting hybridization to DNA from these strains which is distant from the region of *Xcm* H that is carried on pUFA809, then analysis of differences between the strains is difficult to view as happening in the region analogous to the DNA of Xcm H which is the insert region of pUFA809. Differences in minor bands which are too faint to adequately compare between RS4 and TS5.1 could decrease the apparent size of the new DNA in TS5.1.

The small differences seen for the phenotypically identical race change mutants KM6, KM13, and KM21 as compared to KM1 and KM2 are more difficult to interpret unless gross changes associated with the phenotype occurred but are not detectable with pUFA809, *i.e.*, the change in avr phenotype in these mutants is not detectable with the cloned DNA from strain H contained in pUFA809 since the cloned DNA in pUFA809 may not be associated with the change in avirulence phenotype. If the mutation associated with conversion of phenotype from avirulence to virulence on Ac B_5 51B in KM6, KM13, KM21 was within the region of their chromosomes detectable using pUFA809 as a hybridzation probe, then the mutations occurring are point mutations.

Finally, five bands are predicted to hybridize to strain H cut with *Eco*RI (Table 10, excluding the vector band), but six or more major and minor bands are detected when pUFA809 hybridizes to strain H in both Figure 29 parts a and b and in Figure 30 indicating

the presence of a repeated sequence. Also, the total length of the major *Sal*I bands hybridizing to pUFA809 was somewhat larger than that predicted from the restriction map for the insert DNA from strain H in pUFA809. Consequently, a sequence contained within the insert DNA of pUFA809 may be a dispersed repetitive sequence in the H genome.

Attempted Trapping of an Insertion Sequence in *Xcm*

Since spontaneous race change mutant KM1 appeared to contain an insert and mutants having the same phenotype as KM1 were recovered at a high frequency (Chapter 5 Table 12), a possible mechanism accounting for these observations is transposition of an endogenous insertion sequence (IS) element into the genome fragments corresponding to the *Eco*RI band b and the *Bam*HI band a of pUFA809. To test this hypothesis, the transposon trapping vector pUCD800, used to isolate an endogenous IS element from *Agrobacterium tumefaciens*, was chosen (Gay *et al.*, 1985). pUCD800 contains the regulatory and coding regions for levansucrase from *Bacillus subtilis*. Levansucrase is induced by growth of transconjugants on sucrose plates. The sucrose is hydrolyzed with the glucose being used and the fructose converted to levan polymers toxic to the cells. Any cell surviving this treatment will have undergone a mutational change within the levansucrase gene or its regulatory region.

Gay *et al.* (1985) found that growth of pUCD800 transconjugants of *E. coli, A. tumefaciens,* and *Rhizobium meliloti* on 5% sucrose was sufficient to inhibit cell growth or lyse the cells. Transconjugants of pUCD800 of strain H which were resistant to kanamycin were obtained and plated on 5% sucrose plates. However, *Xcm* strain H grew vigorously on 5% sucrose with the colonies on the inverted plates dripping xanthan gum onto the lid of the dish. Two possibilities can explain the absence of lysis: either the levansucrase genes were not induced in *Xcm* or, perhaps, the level of sucrose used in the test was too low to overwhelm the anabolic potential of *Xcm* for sucrose use.

Conclusions

The molecular experiments designed to help characterize the molecular basis of avirulence in *Xcm* were complicated by numerous problems. The attempts at transposon mutagenesis both in *Xcm* and in *E. coli* were frustrated by failure of the vector to conjugate at even moderate frequencies or by unstable maintenance of the transposon after conjugation. The complementation experiments were plagued by lack of reproducibility in conjugation and variability of the phenotype of the reaction when conjugants were inoculated onto plants. Dr. Gabriel's group encountered great difficulty subcloning and transferring even small fragments from the reported avr gene-carrying cosmid clones including pUFA809 (Gabriel, personal communication).

As previously mentioned, the cosmid pSA747, the vector used for the construction of the *Xcm* H clone bank from which pUFA809 and the other reported avr gene-carrying clones, were not stable when conjugated into *Xcm* (De Feyter *et. al*, 1990). The instability of the vector allowed integration of some clones into the genome, including pUFA809 (Gabriel, personal communication). This integration event is assumed to have disrupted genes necessary for regulation or expression of avirulence. New vectors have been constructed by Gabriel's group which maintain of intact vector/insert clones in *Xcm* (De Feyter *et al.*, 1990). Transfer of genes to *Xcm* was also facilitated by isolation of clones for the methylase genes of *Xma*I, *Xma*III, and possibly *Xma*II (De Feyter and Gabriel, unpublished).

Most of the *Xcm* strain H inserts contained in the reported avr gene-carrying clones, including pUFA809, reported by Gabriel *et al.* (1986) failed to convert the phenotype of *Xcm* N from virulence to specific avirulence when subcloned in the new vector, pUFR027 (Gabriel, personal communication). De Feyter and Gabriel have constructed a new clone bank of *Xcm* DNA in a cosmid derivative of pUFR027, pUFR043. Screening of this new clone bank for avr gene-carrying clones has resulted in

the identification of a multi-gene family of at least eleven *avr* genes which have been confirmed by subcloning and/or by sequencing (De Feyter and Gabriel, unpublished). None of the genes is homologous to those originally reported, but several different avirulence genes have been found to react with the same R gene locus and several individual avirulence genes react with a number of different cotton lines (De Feyter and Gabriel, 1990). A given *Xcm* strain may evidently provide more than one avirulence gene which reacts with a given R gene locus, *i.e.* the *Xcm*/cotton interaction is not completely gene-for-gene specific according to De Feyter and Gabriel (1990).

However, *Gossypium hirsutum* is an allotetraploid in which multiallelism could occur (Fehr, 1987). It has not been shown by classical genetics that the R genes present in the cotton lines used by De Feyter and Gabriel a given R gene has a single chromosomal locus. Two other possibilities may occur for cotton: (1) an R gene is a locus of tightly linked genes behaving as though it is a single gene when crosses within the same background are performed or (2) an R gene has multiple alleles distributed among both chromosome sets of the A and D genomes in *Gossypium hirsutum* or *barbadense*. Crosses between species, such as the cross of *G. barbadense* Grenadine White Pollen which contained Knight's B_5 to *G. hirsutum* cultivar Ac 44, would mix the two genetic backgrounds and allow chromosomal translocations to occur between the homologues of the A and D genomes of each. Linkage of R gene clusters would be broken, and a population of plants would segregate for multiple resistance genes, possibly as was observed for the cotton line Ac B_5 (Chapter 5).

Though the changes seen in the hybridization patterns of strains KM1, KM2, and N in relation to H when probed with pUFA809 are not directly associated with a specific avirulence gene once thought to be carried by pUFA809, they are still interesting since hybridization by pUFA809 detected changes which were strain specific, *i.e.* independent virulent mutants KM1 and KM2 appeared to possess inserted DNA in the same pUFA809-homologous sequence, and *Xcm* N appears to have a deletion in the same general region.

Recent results from De Feyter in Gabriel's laboratory indicate that the region of H absent in N (Figure 30) is a repetitive element that hybridizes to many other fragments in each of the strains. This observation clarifies the complicated hybridization profiles (Figure 29 parts a and b and Figure 30) where many faint bands appear when pUFA809 is used as the probe. The insertion seen in KM1 and KM2 could well be due to an insertion sequence element like those found in *Xc.* pv. *vesicatoria* (Kearney *et. al*, 1988) and *citri* (Tu *et. al*, 1989).

In conclusion, the results I presented above, which appeared to support the report that pUFA809 carried an avr gene, can be explained as artifacts of the instability of the cosmid vector pSa747 or of constructs made from it, and possibly of recombination of the *Xcm* DNA carried on pUFA809. Now it can be said that certain of these experiments would never have worked in the manner expected. The identification of the insert in *Xcm* KM1 which could be an endogenous IS element by hybridization with pUFA809 was fortuitous. One possibility for further work would be to attempt trapping of endogenous IS elements, and then to ascertain their involvement in spontaneous race-change mutations and, possibly, in the regulation of avirulence genes.

CHAPTER V

IDENTIFICATION OF MULTIPLE RESISTANCE GENES IN THE SEGREGATING *GOSSYPIUM HIRSUTUM* LINE ACALA B5

Introduction

Ac B5 was developed by Hunter and Brinkerhoff (1961) by crossing Knight's B5 line with Ac 44. Knight's B5 was in the cultivar Grenadine White Pollen of *G. barbadense* (Table 1). The female parent of the cross was *G. hirsutum* cv. Ac 44. Progeny were selected by Hunter and Brinkerhoff having resistance to race 1 of *Xcm*. The resistant plants were backcrossed by Brinkerhoff to Ac 44 at least once to yield the line he labelled "Ac B5". Periodic increases of Brinkerhoff's Ac B5 led to seed lot J4301 which served as the source of plants in a breeding program initiated by Essenberg, who showed that Ac B5 was segregating for multiple resistant phenotypes when single progeny rows were screened with *Xcm* race 1 during the summer of 1984. Subsequent screenings, individual plant selections, and backcrosses of resistant plants to the recurrent parent Ac 44, resulted in the lines used in the following experiments undertaken to identify lines containing single resistance genes in the segregating Ac B5 population.

Xcm race 1, a widely avirulent race, was used in the field to screen for resistance in these backcrosses. *Xcm* race 1 cannot distinguish between resistances contributed by different R genes unless the resistances expressed by the genes are quantitatively different. Essenberg showed that some plants were highly resistant while others were resistant but at

a lower, intermediate level. The intermediate level of resistance could be due to either (1) an R gene in a heterozygous state which when in the homozygous state would contribute a high level of resistance or (2) a resistance gene which contributes an intermediate level of resistance when present as either a homozygote or a heterozygote. Since the intermediate level resistance of one single progeny row from the Ac B5 J4301 seed lot (row 51B62-3) was uniform, Ac B5 might contain multiple R genes.

To determine whether the resistance carried by two plants is due to different R genes, races of the pathogen which differ in avirulence to the R genes of these plants, presumably due to inactivation or lack of the corresponding avr genes, must be available. Since R genes cannot be identified unless differential races are used for screening and since only *Xcm* race 1 was used to screen the plants used in the Ac B5 backcross program through the third backcross by Essenberg, the resistant plants produced in the Ac B5 program possessed an unknown number of R genes.

Since results obtained in Gabriel's laboratory while at Oklahoma State University, as well as after his move to University of Florida, indicated that *Xcm* H carries more than one *avr* gene capable of conferring recognition of Ac B5 (Gabriel *et. al*, 1986), I attempted an isolation of the predominant resistance genes in the segregating population. Because the identification of an R gene requires a race that differs from other races by exhibiting differential virulence toward the R gene, a search for new races of *Xcm* was concomitantly undertaken. Once a set of cotton lines that contained single R genes and a set of differential races were in hand, I hoped to test the hypothesis of Gabriel (1986) that the set of *avr* B5 genes constitutes a gene-for-genes system, *i. e.*, one in which several avr genes confer recognition by a single R gene. As a consequence of the difficulties with pUFA809 and the other reported *avr* B5 gene clones, this latter hypothesis remains to be tested.

Materials and Methods

Cotton Lines and Growth Conditions

The cotton lines used in this study are listed in Table 12. This table includes Ac B_5. Dr. Brinkerhoff's Ac B_5 was maintained by selfing resulting in the seed lot J4301 at the start of these experiments. Later experiments involved plants from first, second, or third backcrosses to Ac 44 performed by Essenberg. De Feyter and Gabriel have isolated an *Xcm avr* gene-carrying clone that confers avirulence toward Essenberg's Ac B_{In} and Ac B_N lines (listed in Table 12) and toward 1-10B, but not toward 20-3 (Gabriel, personal communication). Since 1-10B and 20-3 are official differentials containing B_{In} and B_N, respectively, this indicates that both Essenberg's Ac B_{In} and Ac B_N lines actually possess only B_{In}.

Cotton plants used in growth chamber experiments were grown in 6-in clay or plastic pots in Jiffy Mix Plus soil/peat/vermiculite mixture (Jiffy Products of America, Inc., West Chicago, IL). Plants used in greenhouse experiments were grown in flats of Jiffy Mix Plus until they had three or four fully expanded leaves and were four weeks old, when they were transplanted into 8-in plastic pots containing a 1:1:1 mixture of soil:sand:peat moss. Plants used in field experiments were planted at the Oklahoma State University field nursery at Perkins, OK, in sandy loam/clay soil in rows four feet apart with individual plants spaced at least 6-in apart. Growth chamber and greenhouse plants were fertilized once weekly with Stern's Miracle-Gro at the rate of 1.5 g dry wt / L tap water.

The conditions for the Conviron E-15 controlled environment chamber in Biochemistry (Figure 32 part a) consisted of ramped temperature from a 19° C nighttime low to 30° C daytime high with lighting from none to full increased step-wise over the 3 hour "sunrise" and decreased step-wise over the 3 hr "sunset" with a full photoperiod of 14 hr. At 24 cm below bulb level, the photosynthetic photon flux density was determined to be 6.3 x 10^2 $\mu mol/m^2se$: (Sun *et al.*, 1989). The chamber conditions for the Controlled

TABLE 12

PLANT PEDIGREES

Plant	Line	Pedigree	Source
Ac44	82-LB-677	Not available	Brinkerhoff
Ac44E	V87-020	Ac44 82-LB-677 sel.	Essenberg
AcB_5	BJ1348	1+? Ac44 X (Ac44 X Knight's B_5F_2)F_2	Brinkerhoff & Johnson
	J4301	AcB_5 BJ1348F_2 or F_3	
	62-3	AcB_5 51B062-3 (J4301 sel.)	This work
	51B	AcB_5 51B063-1 or 2 (J4301 sel.)	"
	51A	AcB_5 51A065-2 (Ac44 X AcB_5J4301F_2 sel.)	"
	43-32	AcB_5 J4301 selection	"
	44-6, 13, 15, 21, 23	Ac44 X AcB_5J4301F_2 sel.	"
	55-18	2Ac44 X AcB_5J4301F_2 sel.	"
	65-3, 4, 5, 7, 8, 9, 10,11, 12, 19, 20, 24, 27, 28, 29, 30, 31, 34, 35, 36, 37, 40, 41, 42, 43, 44, 45, 46	3Ac44 X AcB_5J4301F_2 sel.	"
	66-25, 26, 38, 39	Ac44 X AcB_5V84-004-2F_2 sel.	"
AcB_1	V87-001	Ac44E X (3Ac44 X AcB_1F_2)F_2	Essenberg
AcB_2	V87-003	3(Ac44 X AcB_2V83-002-5F_2)F_2	"
AcB_3	V87-004	Ac44E X (2Ac44 X AcB_3 V83-003-7F_2)F_2	"
AcB_4	V87-006	(Ac44E X 3Ac44 X AcB_4F_2)F_2	
AcB_6	V87-007	(Ac44E X 3Ac44 X AcB_6F_2)F_2	"
AcB_7	V87-009	(Ac44E x 2Ac44 X Acb_7V83-001-5F_2)F_2	"
AcB_N	V87-00	(Ac44E X 2Ac44 X AcB_N41A66F_2)F_2	"
AcB_{In}	V87-012	(Ac44E X 3Ac44 X "AcB_{In}"F_2)F_2	"
Sto2B-S9	J4254	Not available	Johnson
Gregg	J4256	Not available	Johnson
Im216	J4258	(Im216/BJ544) increase	Brinkerhoff & Johnson

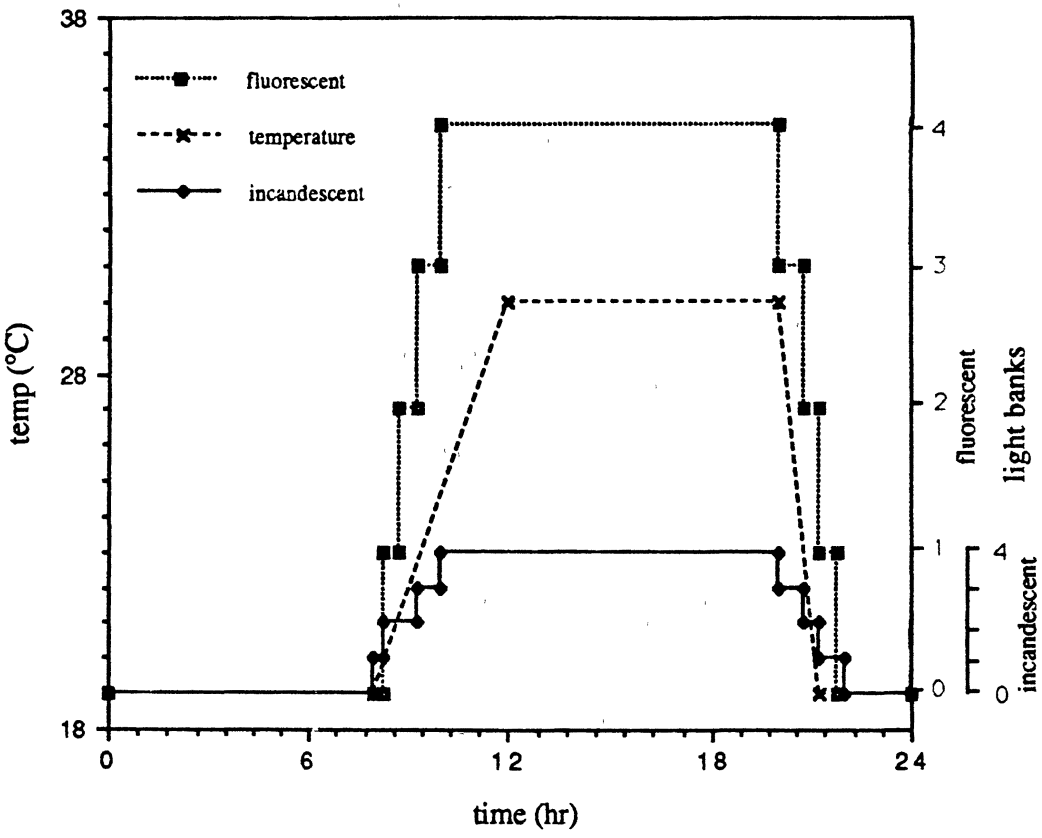


Figure 32a. Growth Chamber Conditions for the Conviron Chamber.

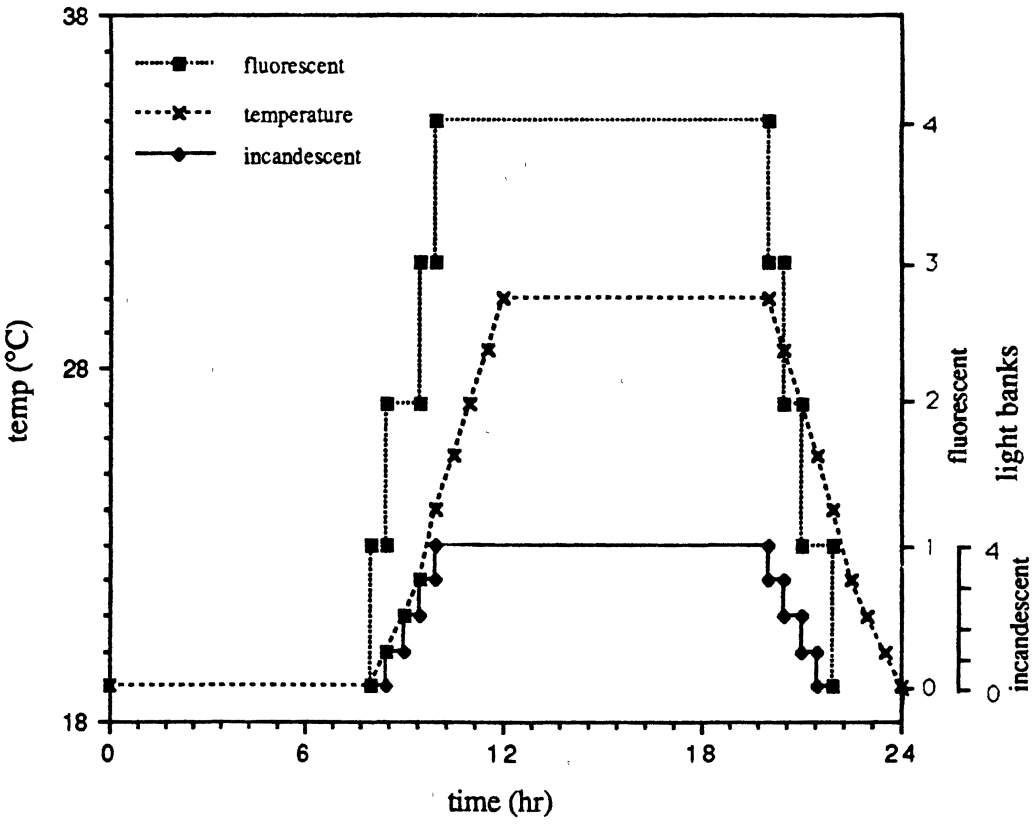


Figure 32b. Growth Chamber Conditions for the CERL Chamber.

Environment Research Laboratory (CERL) chamber (Figure 32 part b) used in the tests of Ac B_5 segregants versus SRCMs mimicked the program for the Conviron chamber as closely as possible. The major difference between programs for the two chambers was a stepwise increase of temperature for the CERL chamber instead of a ramping increase.

Greenhouse temperatures ranged from a maximum high of 43° C to a minimum low of 12° C, with an average of 22° C over the summer 1987 experiment. Field temperatures from the Agronomy Field Station at Perkins ranged from a maximum of 40° C to a minimum of 11° C with an average daily temperature of 27° C during the summer 1988 experiment while during the summer of 1989, the maximum temperature was 37° C, the minimum 11° C, and the daily average 24° C.

Plant Inoculations and Reaction Grading Scale

To determine whether *Xcm* strains were incompatible on given cotton plants, leaves were infiltrated with 10^6-10^8 cfu/ml in sterile saturated calcium carbonate. Infiltration of small leaf areas was done by applying pressure to a needleless syringe against the abaxial surface of the leaf. Leaves which were fully expanded and less than 5 nodes away from the apical meristem were used for the bioassays. Transparent plastic bags were routinely placed over leaves 1 to 2 hours before inoculation to encourage stomatal opening in response to the increased humidity. Field inoculations were done with a sprayer (Brinkerhoff *et. al*, 1984).

The grading scale for disease reactions from plants inoculated in either the greenhouse or growth chamber was the following: (a) No visible browning or water soaking, considered a null reaction, scored as -1, (b) a hypersensitive reaction, *i. e.* rapid necrosis, indicated by browning of the inoculated tissue, 0, (c) several to many pinpoint water-soaked flecks but not covering the infiltrated area, 1, (d) pinpoint or larger water-soaked lesions that cover the inoculated region, 2, (e) water-soaked patches with some

confluent areas, 3, (f) confluent water-soaked lesion spreading beyond area of original inoculation, the maximum value or 4. Disease grades were typically scored on days 7, 9, or 11 post-inoculation. The grading scale used for field inoculations was that used by Brinkerhoff *et al.* (1984).

Screening Method for Isolation of Spontaneous Race Change Mutants

The method used to screen for SRCMs was the following: A single colony of an avirulent streptomycin resistant parent *Xcm* was grown overnight in NB at 30° C. The culture was diluted to 5 x 10^3 to 1.5 X 10^4 cfu/ml in sterile saturated calcium carbonate. Fully expanded cotton leaves were inoculated by spray infiltration using an air-pressurized paint sprayer at 100 psi. Approximately 2 to 4 weeks post infiltration small, pinpoint water-soaked lesions were sometimes found after careful observation. An 18 gauge blunt ended stainless steel needle was then used to remove several small disks of leaf tissue containing pinpoint lesions. The disks were placed on the surface of a NB plate. After *Xcm* had grown out of the leaf disks to form colonies, bacteria were streaked onto NA Str 50 µg/ml plates to obtain single colonies. To test their virulence phenotype, several of colonies were grown in NB and syringe inoculated onto plants at an inoculum level of about 5 x 10^6 cfu/ml.

Plant Segregant Screenings

Plants originating from the Ac B_5 seed lot J4301 and F_2 plants from each of Essenberg's backcross generations were screened for resistance to *Xcm* H and KM1. Strains H and KM1 were then inoculated at low concentrations onto resistant plants, as described in the above section. The plants were screened for the presence of compatible, water-soaked lesions. Bacteria were isolated from the lesions. The set of plants was

screened with a subset of race change mutants. The interactions resulting from this test was analyzed by the statistical method of cluster analysis (Lebeda and Jendrulek, 1987) in order to determine groupings of plants which contain the same R genes and groups of races distinguishing among the R genes. A subset of each of the plant segregants and the race-change mutants, which represented major groupings in both detected by cluster analysis, was retested. Plant selections following this test were selfed to provide seed of the F_1 generation post-selection to serve as the male parents in backcrosses of the selections which contained independent, newly identified R genes.

Cluster Analysis of Race/Cultivar Interactions

The statistical tool of cluster analysis provides a method to distinguish natural groupings within data (Romesburg, 1984). If the data in question are from a set of mutant bacterial races and a set of plant segregants from which they were purified, then it should be possible to discern both between plant lines with different resistance genotypes and between races possessing different avirulence genotypes.

Cluster analysis depicts the reactions between 31 bacterial mutants and 42 plant segregants as points in 42-dimensional space when considering the bacterial mutants as the attributes and the segregants as the objects and as points in 31-dimensional space when considering the segregants as attributes and the bacteria as the objects. A dissimilarity coefficient of Euclidean distance (Wilkinson, 1987) was calculated to measure the separation between two objects for all pairwise combinations of objects mapped in the n-dimensional space, where n is the number of attributes. Once the distances are calculated, then a statistical test of the closeness between all pairwise combinations of distances is calculated. If the two distances are judged to be similar or close, then the two distances are "linked" or clustered together. A distance is then calculated between that cluster and all remaining distances or clustered distances.

Four different methods for evaluating the statistical closeness, or linkage, between groups are the average, complete, median and centroid methods. For the average linkage method, an average of the Euclidean distances between members of a cluster is calculated. For the complete method, the the distance between two clusters is taken to be the distance between two objects, one from each cluster, which furthest separates the clusters. The median linkage method uses the median of the distances in a group to represent the distance for that cluster of groups while the centroid method uses the distance of the object most near to the center of the group to represent the distance for the cluster.

Cluster analysis was applied to the data with the SYSTAT package of programs for the MacIntosh (SYSTAT, Inc., Evanston, IL) using the average, complete, centroid, and median clustering methods and an Euclidean distance metric. Both bacterial mutants and plant segregants, *i.e.* the rows and columns of the matrix, were clustered simultaneously. The nearness of two mutants in a table (vertical separation) or two plants in a table (horizontal separation) is representative of the distance between them.

Results and Discussion

Widely Avirulent *Xcm* H Changes Race at High Frequency

Xcm H is a widely avirulent strain which exhibits the phenotype of race 1 (Brinkerhoff, 1970). Strain H is avirulent toward 10 differential cotton lines (Gabriel *et al.*, 1986). De Feyter and Gabriel have identified and cloned eleven plasmid-borne nonhomologous avirulence genes from *Xcm* H (Gabriel, personal communication).

Xcm H mutants, virulent toward lines carrying specific resistance genes, are readily obtained. Spray infiltration at inoculum levels of about 1 x 10^4 cfu/ml through open stomata allowed populations of at least 50,000 bacteria to be screened in 10 leaves of about 100 cm^2 each. The inoculum level was such that about 50 bacterial colonies developed per

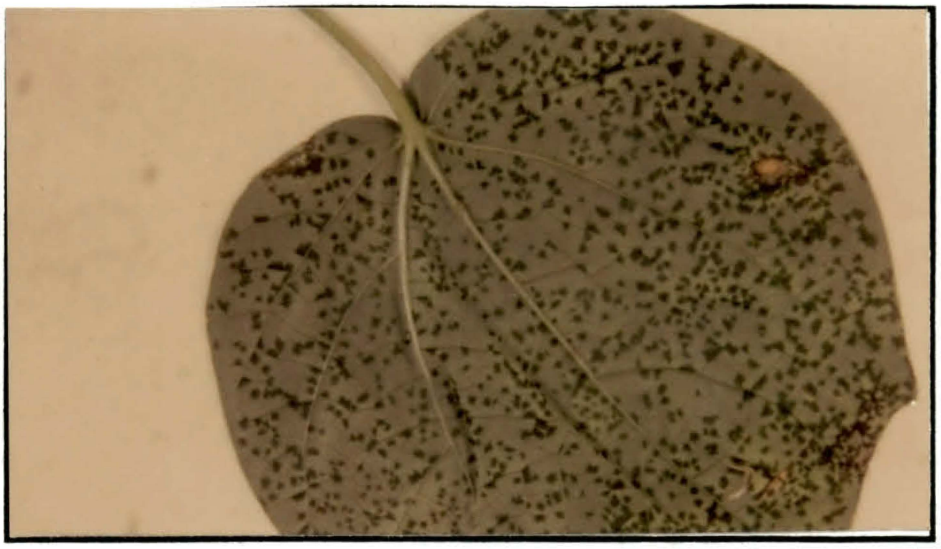
cm^2 leaf area. This density spaces individual colonies approximately 240 palisade cell widths apart (Essenberg *et. al*, 1979a). Resistant reactions of cotton leaf tissue to avirulent colonies of *Xcm* are localized (Essenberg *et al.*, 1979a). At the spacing obtained, host reactions to colonies were sufficiently independent that virulent mutants in the inoculum produced macroscopically visible, water-soaked lesions.

Segregant 51B was uniformly resistant to *Xcm* H in the 116 51B plants and progeny from self-pollinated plants screened. Two independent spontaneous race-change mutants from H, KM1 and KM2, were isolated on Ac B_5 51B. Figure 33 shows two leaves, one of the susceptible line Ac 44 and one of the Ac B_5 segregant 51B, inoculated with strain H at 3 weeks post-inoculation. In the Ac 44 leaf, the compatible, water-soaked lesions have begun to merge such that the inoculum concentration appears to be lower than the 1×10^4 cfu/ml used. Arrows point to two water-soaked lesions which appeared on the Ac B_5 51B leaf.

The spray inoculation described above was performed on two occasions. Mutants virulent on segregant 51B were observed at frequencies of 0.0001 and 0.009 per bacterial cell inoculated, where the frequency is given by the number of compatible lesions divided by the number of cfu sprayed into the leaves at an inoculum concetration sufficiently dilute that lesions develop from single bacterial cells. Race-change mutants KM1 and KM2 were isolated from water-soaked, compatible lesions of the respective experiments. For comparison, the numbers of *Xcm* with antibiotic resistance in populations plated were on the order of 1 in 10^7 to 1 in 10^9 (estimated by multiple platings). The race changes associated with KM1 and KM2 were stable. Table 13 gives approximate frequencies of mutants in cultures of various *Xcm* strains tested on several different Ac plant lines by Brinkerhoff and by members of Essenberg's research group. These frequencies are not measure of mutation rate, which would require fluctuation analysis.

Self-pollinated progenies of the segregants 51B and 62-3 from the cotton line Ac B_5 J4301 exhibited uniform high and intermediate levels, respectively, of resistance to

a) Ac 44 leaf 3 weeks post-inoculation



b) Ac B_5 51B leaf 3 weeks post-inoculation

Figure 33. SRCM Selection Assay, Ac 44 and Ac B_5 51B Leaves Infiltrated at Low Inoculum. Arrows in panel b point to two separate virulent lesions like those from which SRCM KM1 was isolated.

TABLE 13

FREQUENCIES OF SPONTANEOUS RACE-CHANGE MUTATIONS IN *XCM*

Xcm	cotton line	Technique[a]	Approximate frequency[b]	reference
R1	b7 B_N	clonal, syringe	0.06	Brinkerhoff (unpublished)
R3	b7	clonal, syringe	0.007	Pierce (unpublished)
	b7	clonal, syringe	0.02	Hall (unpublished)
RS4	B_1, B_2, B_3, B_4, B_5 51B, B_6, b7, B_N, Gr, St	mixed or clonal, spray	0.0025 to 0.00033	Sun (1987)
H	B_5 51B	mixed, spray	0.0001	this work
	B_5 51B	clonal, spray	0.009	"

[a]Clonal refers to use of a culture which was obtained from a single colony while mixed refers to use of one that was not. Syringe refers to an inoculation done at high inoculum concentration of individual colonies while spray refers to an inoculation done at low bacterial concentration.

[b]Frequency is the number of compatible lesions divided by the number of incompatible lesions for the syringe technique and by cfu sprayed into the leaves for the spray technique.

Xcm H under field conditions. Segregant 51B is resistant to *Xcm* H and susceptible to *Xcm* KM1 and KM2. Segregant 62-3 exhibited intermediate resistance to *Xcm* H, KM1, and KM2. Their resistance phenotypes were confirmed in the growth chamber. Segregant 51A was a single plant selection from the first backcross of Ac B_5 J4301 to Ac 44 and was resistant to *Xcm* H, KM1, and KM2 under greenhouse and growth chamber conditions. *Xcm* N was virulent when inoculated on the segregants 51B, 62-3, and 51A under growth chamber conditions. Table 14 shows the race/cultivar interactions for these strains and plant lines and indicates that SRCMs KM1 or KM2 could distinguish among the segregants. The observation that a SRCM selected on a specific plant segregant could differentiate between that segregant and others provided the framework for the summer 1987 experiment.

Combined Race Change Mutant and Segregant Search

Screening for Mutants. Approximately 800 plants from the original Ac B_5 seed lot J4301, self-pollinated individual plant selections from this seed lot, or self-pollinated F_2 generation plants from a first, second, or third backcross of resistant plants to susceptible Ac 44 were planted in flats and screened for resistance to *Xcm* H and KM1 in the greenhouse. Eighty-five plants possessing resistance to strain H and/or KM1 were infiltrated with suspensions from either four independent *Xcm* H single colony isolates or three independent KM1 single colony isolates.

If water soaked, compatible lesions were observed on a plant, discs containing the lesions were removed. This screening resulted in 120 bacterial isolates stored for further study from 40 of the 85 plants inoculated with bacterial suspensions of H or KM1. Plants from which putative SRCMs were isolated were maintained in the greenhouse and self-pollinated for increase. This maintenance involved thrip and white fly control and pruning prior to later screenings.

TABLE 14

EARLY SEGREGANTS FROM AC B5

strain	Ac 44	Ac B5 segregants		
		62-3	51B	51A
H	+	+/-	-	-
KM1	+	+/-	+	-
N	+	+	+	+

+ refers to a compatible interaction while +/- and - refer to an intermediate and an incompatible interaction, respectively.

The plants maintained for rescreening were moved to a CERL growth chamber in the winter of 1987-1988. Thirty-one of the 120 isolates were inoculated onto the maintained plants and onto Ac 44, and Ac B5 51B. The table of interactions between these *Xcm* isolates and plant lines was subjected to cluster analysis (described in the following section) in order to identify a subset of isolates and plants for retesting.

Second Screening of Lines and Putative Mutants: Cluster Analysis. Table 15 contains the reactions observed for 29 of the 120 putative SRCMs isolated from the screening described above and *Xcm* H and mutant KM1 when screened on 39 independent plant selections and Ac 44 and Ac B5 51B. The values, highly compatible (++), compatible (+), incompatible ( ), or inconsistent (i), were scored on days 6, 7, and 9 post-inoculation. The most compatible score on any of these days is reported in Table 15, with the scores normalized to the compatibility seen on the fully susceptible line Ac 44. The values in this table were assigned numerical equivalents of 4.0 for highly compatible, 2.0 for compatible, 1.75 for inconsistent, and 0.0 for incompatible. This simplified scaling was designed to somewhat mimic the grading scale used customarily for scoring disease reactions *in planta*, Materials and Methods, with the exception that the inconsistent reactions were weighted towards compatibility.

Tables 16, 17, 18, and 19 give the results of cluster analysis by average, centroid, complete, and median linkage, respectively, each using the Euclidean distance metric. The cluster analysis was performed on both the rows (bacterial mutants) and the columns (plant selections) simultaneously. Data for Ac 44 was left out of the analysis. Perhaps, inclusion of this data would have provided a root to the hierarchical clustering. With every clustering method multiple groups were observed: these are the boxed in areas (Tables 16, 17, 18, and 19). The criterion for assignment as a box was at least five reactions in an adjacent three by three square of reactions (Table 19). Once again, this is a somewhat arbitrary designation and is valid only if every possible R gene was present as a single R gene in at

TABLE 15

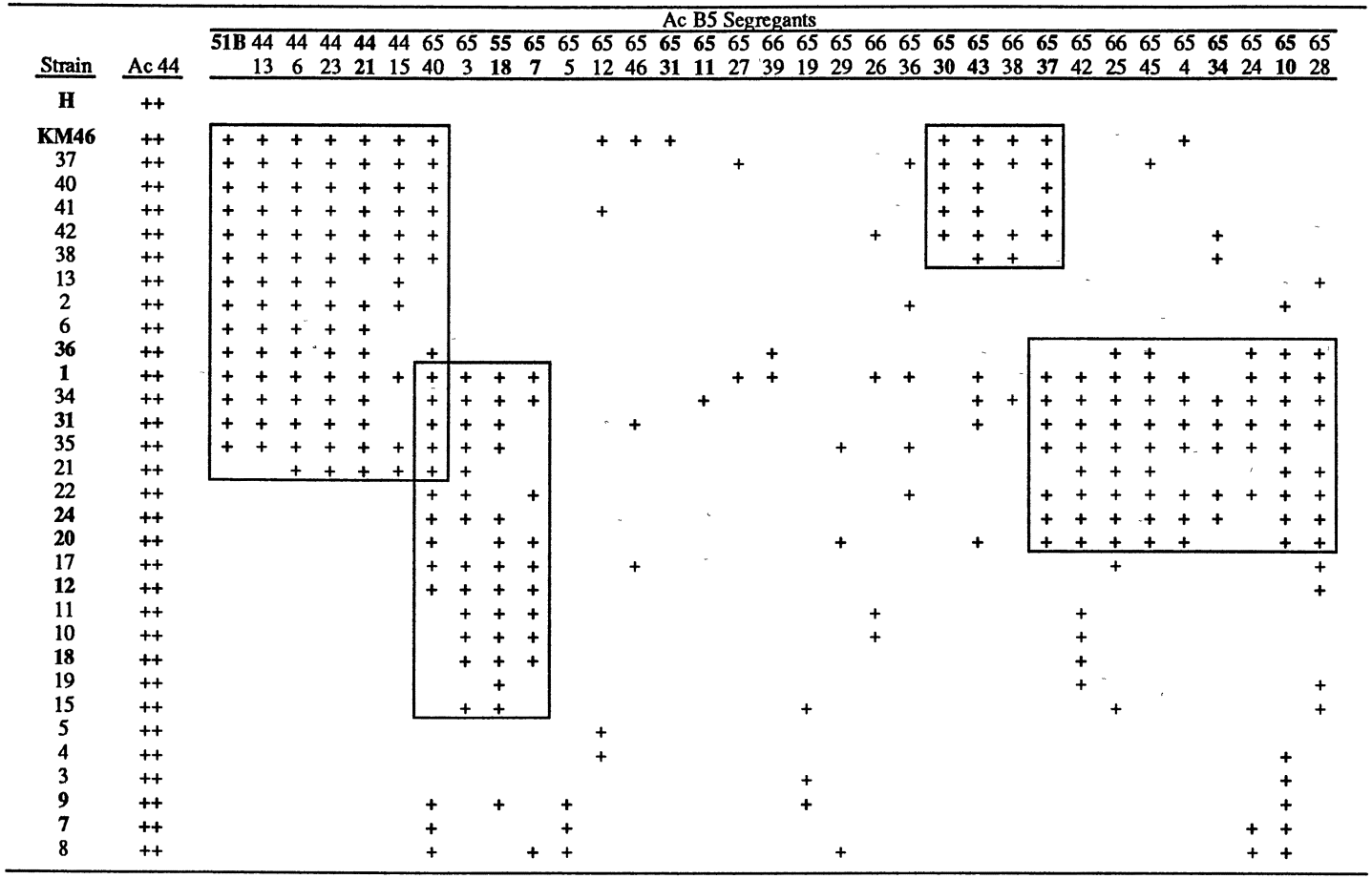
SPONTANEOUS RACE-CHANGE MUTANTS/AC B5 SEGREGANT REACTIONS

			Ac B5 Segregants																																							
		Ac 44	51B	65 3	65 4	65 5	44 6	65 7	65 8	65 9	65 10	65 11	65 12	44 13	44 15	55 18	65 19	65 20	44 21	44 23	65 24	66 25	66 26	65 27	65 28	65 29	65 30	65 31	J43 32	65 34	65 35	65 36	65 37	66 38	66 39	65 40	65 41	65 42	65 43	65 44	65 45	65 46
parents	H	++		i							i					i					i		i	i	i							i				i			i		i	
	KM1	++	+	i	i		+	i			i			+	i	i			+	+	i	i	i	i	i							i	i		i	i		i	i		i	
H mutants	2	++	+				+				+			+	+				+	+												+										
	3	++									+						+																									
	4	++									+		+																													
	5	++											+																			+										
	6	++	+				+							+					+	+																						
	7	++				+					+										+															+						
	8	++				+		+			+										+					+										+						
	9	++				+					+					+	+																			+						
	10	++		+				+								+							+															+				
	11	++		+				+								+							+															+				+
	12	++		+				+								+									+											+						
	13	++	+				+							+	+					+					+																	
	15	++		+												+	+					+			+																	
	17	++		+				+								+						+			+											+						
	18	++		+				+								+																						+				
	19	++														+									+													+				
	20	++			+			+			+					+						+			+	+							+			+		+	+		+	
	21	++		+			+				+				+				+	+		+			+											+		+			+	
	22	++		+	+			+			+										+	+			+					+		+	+			+		+			+	
	24	++		+	+						+					+						+			+					+			+			+		+			+	
KM1 mutants	31	++	+	+	+		+				+			+		+			+	+	+	+			+								+			+		+	+		+	+
	34	++	+	+	+		+	+			+	+		+		+			+	+	+	+			+					+			+	+		+		+	+		+	
	35	++	+	+	+		+				+			+	+	+			+	+	+	+				+				+		+	+			+		+			+	
	36	++	+				+				+			+					+	+	+	+			+										+	+					+	
	37	++	+				+							+	+				+	+				+			+					+	+	+		+			+		+	
	38	++	+				+							+	+				+	+										+				+		+			+			
	40	++	+				+							+	+				+	+							+						+			+			+			
	41	++	+				+						+	+	+				+	+							+						+			+			+			
	42	++	+				+							+	+				+	+			+				+			+			+	+		+			+			
	46	++	+		+		+						+	+	+				+	+							+	+					+	+		+			+			+

Reactions were scored as ++ for highly compatible, + for compatible, " " for incompatible, and i for inconsistent.

TABLE 16

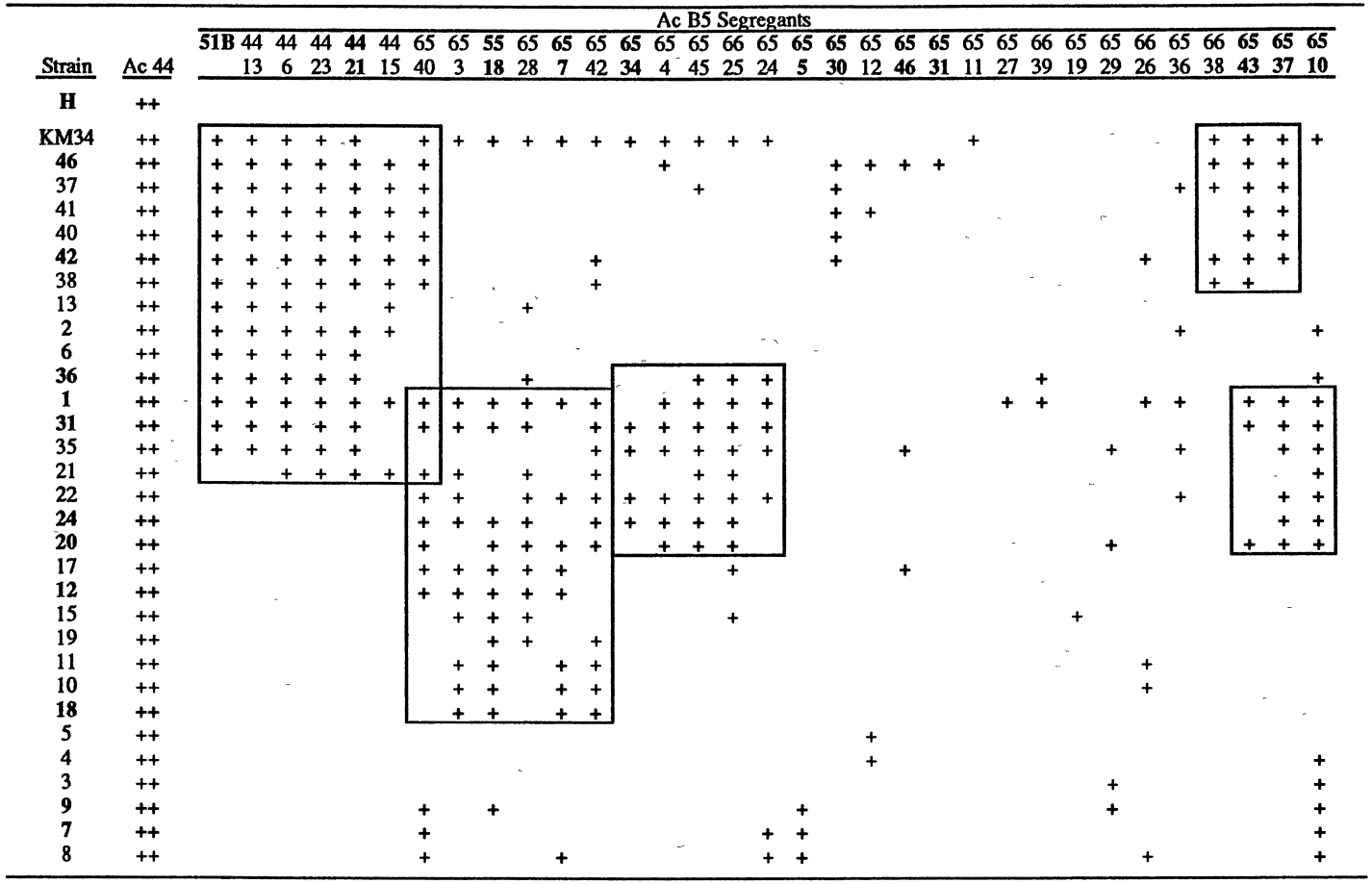
CLUSTER ANALYSIS BY AVERAGE LINKAGE



Reactions were scored as ++ for highly compatible, + for compatible, and " " for incompatible.

TABLE 17

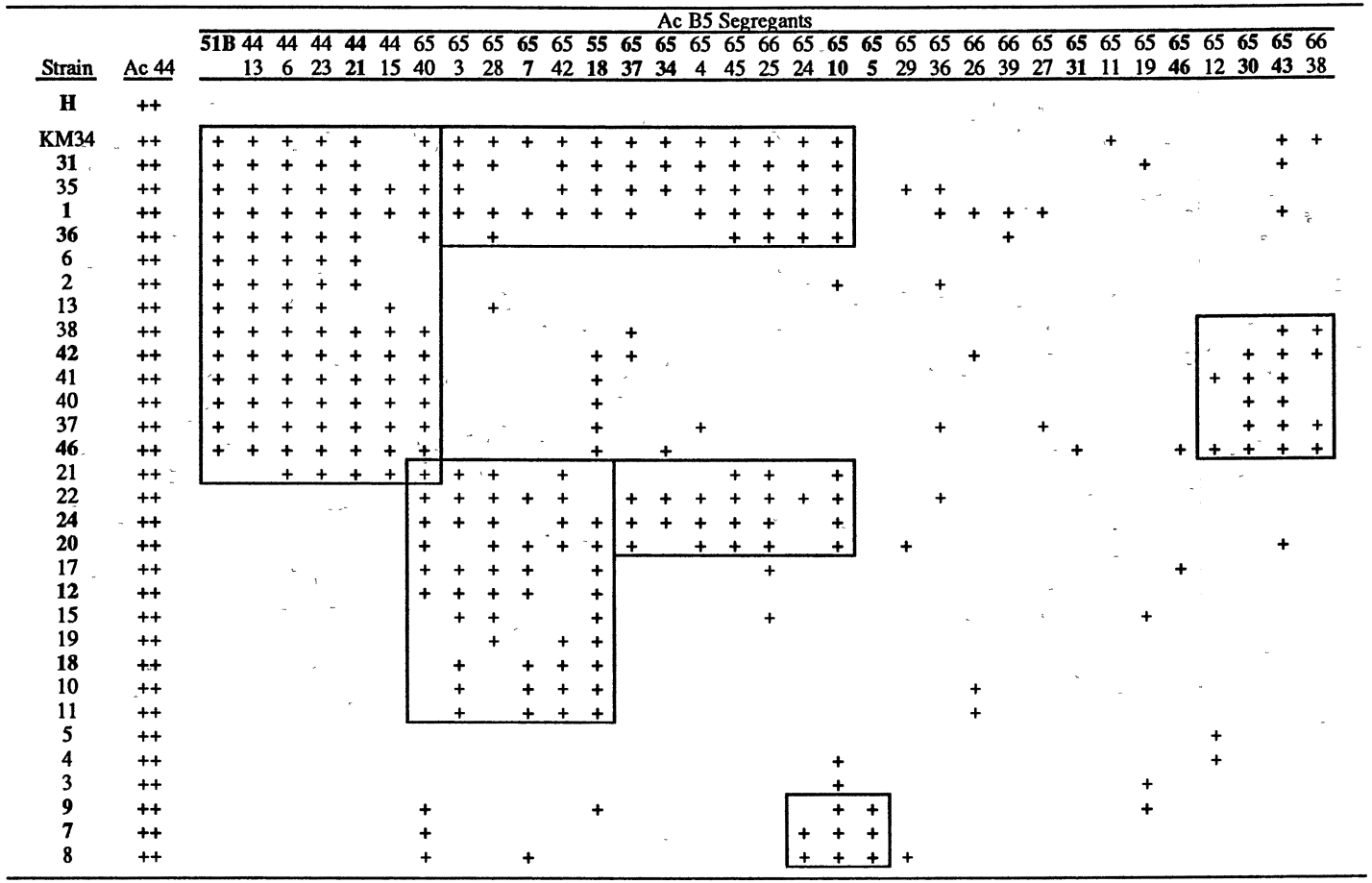
CLUSTER ANALYSIS BY CENTROID LINKAGE



Reactions were scored as ++ for highly compatible, + for compatible, and " " for incompatible.

TABLE 18

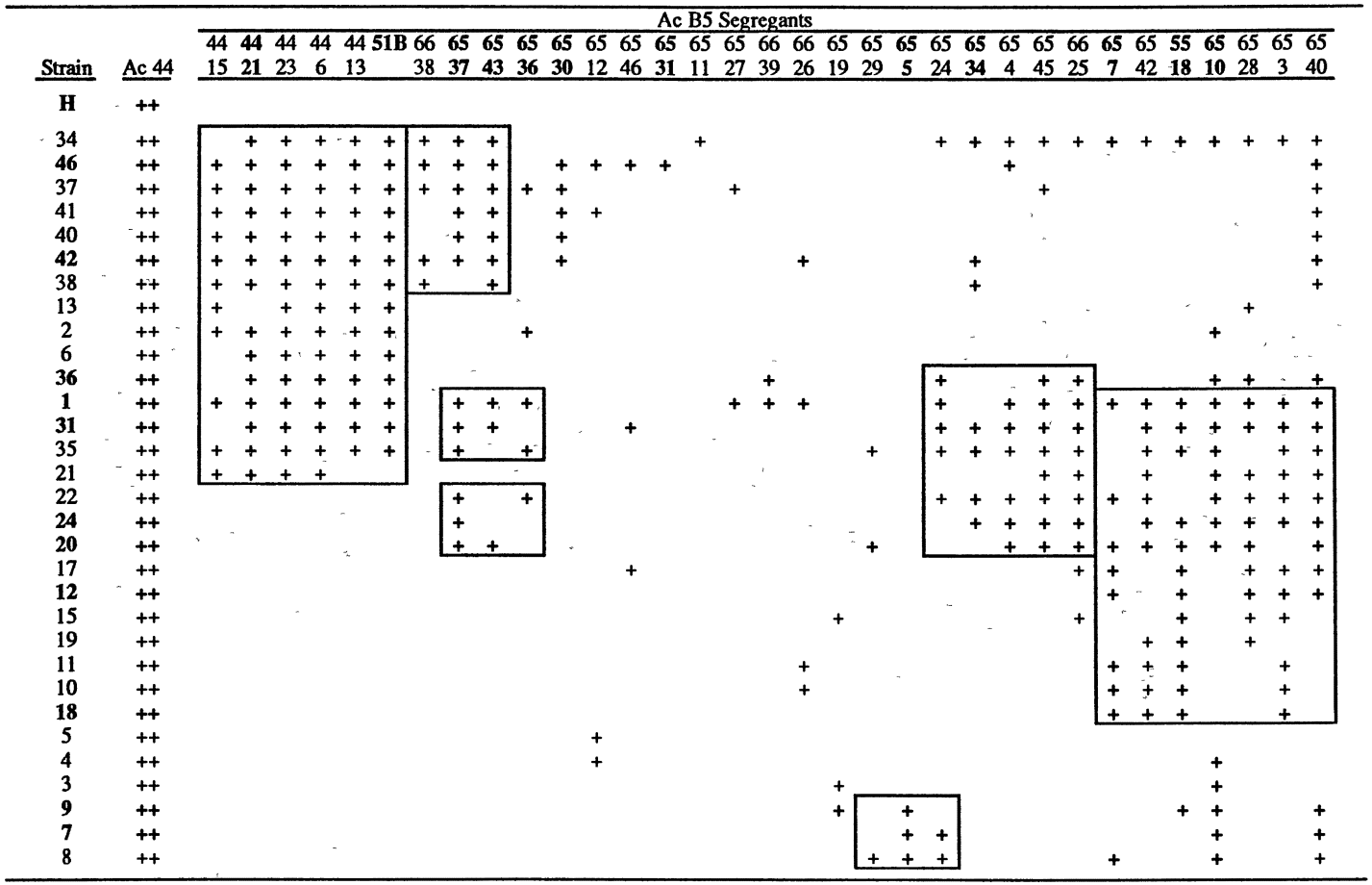
CLUSTER ANALYSIS BY COMPLETE LINKAGE



Reactions were scored as ++ for highly compatible, + for compatible, and " " for incompatible.

TABLE 19

CLUSTER ANALYSIS BY MEDIAN LINKAGE



Reactions were scored as ++ for highly compatible, + for compatible, and " " for incompatible.

least three lines and if there was also a corresponding differential race (Person, 1959 and Lebeda and Jendrulek, 1987).

The average clustering method resulted in four groupings being found while the centroid method had five, the complete method six, and the median method seven. If a natural grouping corresponds to a single avr gene/R gene interaction, then the Brinkerhoff's Ac B5 line could have from four to seven separate and unique resistance genes:

> Earlier work by Essenberg (unpublished) on the segregation ratios of segregants 51B, 62-3 and 51A suggested that at least two genes were present in Ac B5. 51B was uniform for high resistance and remained uniform through the F_2 and F_3 generations whereas segregant 62-3 remained uniform for intermediate resistance through the F_2 generation post-selection, when both were screened in the field with *Xcm* race 1.
>
> On the other hand, the F_2 generation post-selection for segregant 51A screened for several resistance phenotypes, but not in a manner easily interpretable from the number of plants screened. 51A appeared to contain at least two R genes. Consistent with that hypothesis is the fact that spontaneous race change mutants were not recovered on any 51A plants screened (data not shown). The hypothesis of multiple resistance genes in 51A predicts that spontaneous mutation in at least two avr genes are required to recover a SRCM and double mutants would probably not be found in the small population screened. Mutants KM25, KM26, KM38, and KM38, were recovered from F_2 plants from a backcross of Ac B5 51A to Ac 44 (Table 15 lines 66-25, 66-26, 66-38, and 66-39). Segregation occurred during the backcross which resulted in these lines, separating at least two genes conditioning a high level of resistance or separating one gene conditioning a high level of resistance and at least one gene

conditioning an intermediate level. The latter possibility seems plausible, especially if the R genes in 51A are codominant, conditioning a higher level of resistance than expected when present together than they do separately.

Third Screening: Subsets of Plant Lines and Bacterial Strains. A set of 10 bacterial mutants and a set of 11 plant selections were chosen from the major groups in Tables 16, 17, 18 and 19. Both the bacterial mutants and plant selections are highlighted in each table in bold typeface. The members of these sets were chosen by inspection with an attempt to be unbiased.

Seed from a subset of 11 self-pollinated plants chosen by cluster analysis was planted in the chamber during the summer of 1988. A differential series of lines including Ac 44E, 51B, 51A, 62-3, Im 216, Gregg, Sto. 2B-S9, and the available near-isogenic blight resistance lines as F_2 generations from their backcross programs were also planted. Since seed from most of the 11 plants and the single gene blight resistance lines were not expected to be homozygous for resistance, they were prescreened for resistance to *Xcm* strains H and KM1. Some of the resistant plants were transferred to the field after prescreening while others were kept in the chamber for screening with the subset of 11 SRCMs. The bacterial mutants were inoculated onto resistant plants of the selfed F_1 generation of the plant selections with the resulting reactions shown in Table 20.

Xcm strains H and KM1 were included in the inoculations, and F_2 plant selections from backcrosses of the other Ac blight resistant lines and some non-Acala cultivars, Gregg, Stoneville 2B-S9, and Im 216, were included. All bacterial mutants reacted as predicted from the earlier test (Table 15) on Ac44E, and on segregants 51B, 62-3, and 44-21. Mutants KM7, KM36, KM42, and KM46 were predicted to be incompatible on 55-18 but in this test scored as weakly compatible. KM12 was predicted to be weakly compatible on 65-5 but scored as incompatible. KM9 was predicted to be incompatible on 65-7 but tested as being weakly compatible. Other bacterial mutant/plant selection reactions were

TABLE 20

TEST RESULTS OF SEGREGANT AND MUTANT SUBSETS INTERSECTING MAJOR GROUPS IN CLUSTER ANALYSIS SCHEMES

		Ac B5 Segregants														Acala cultivars								non-Ac		
Strain	Ac 44E	51B	51A	62 3	65 5	65 7	65 10	55 18	44 21	65 30	65 31	65 34	65 37	65 43	65 46	B1	B2	B3	B4	B6	B7	BN	Bln	Gr	Pg	Im 216
H	+			+/-				+/-												+/-						
KM1	+	+		+/-				+/-	+										+	+/-						
7	+			+/-				+/-								+/-				+/-						
9	+			+/-		+/-		+/-								+/-				+/-						
12	+			+/-				+/-								+/-	+/-			+/-						
18	+			+/-				+/-								+/-				+/-						
20	+			+/-				+/-								+/-				+/-						
24	+			+/-				+/-								+/-	+/-			+/-						
31	+	+		+/-				+/-	+							+/-			+	+/-						
36	+	+		+/-				+/-	+										+	+/-						
42	+	+		+/-				+/-	+								+/-		+	+/-						
46	+	+		+/-		+/-		+/-	+		+/-								+	+/-		+	+			

Reactions were scored as + for compatible, +/- for weakly compatible, and " " for incompatible

also not as predicted with incompatible interactions now weakly compatible and weakly compatible interactions now incompatible. Since (1) this test was done in the CERL chamber, (2) reactions were found to vary widely between that chamber and the Conviron chamber (Table 11), and (3) the screening depicted in Table 15 was done in the greenhouse with little control over temperature, the fluctuation of reactions was expected. Yet, a further subset of plant selections and of bacterial mutants could be identified for further tests. These bacterial mutants and plant selections are highlighted in bold typeface (Table 20).

Table 21 shows the results of screening the new bacterial mutant subset of the earlier subset (bacterial mutants KM1, KM2, KM6, KM13 and KM21) plus *Xcm* H and N on the chosen plant selections, the other Acala blight resistant lines, and the non-Acala lines used previously. From the interactions in this table, a prediction was made of the number of R genes in each segregant. Plant selections 51B, 62-3, 65-7, 55-18, 44-21, and 65-31 appear to contain single R genes while 51A may have at least 2. This conclusion is based on (1) spontaneous race change mutants were identified having altered avirulence that were compatible or weakly compatible and (2) the segregation ratios for the small numbers (< 20 plants screened) of the F_1 selfed generation post-selection (data not shown).

Fourth Screening in the Field of Further Subsets. Resistant plants from the F_1 selfed generation post-selection of segregants 44-21, 55-18, 65-7, and 65-31 were used as the male parents for backcrossing to Ac44 in the field during the summer of 1988 by Essenberg. Screenings done by Essenberg in the field in 1989 identified selection 44-21 as possessing one R gene with a ratio of 3:1 resistant to susceptible plants. The ratios for segregants 65-7, 55-18, and 65-31 were less clear (Essenberg, unpublished). The mutant set KM1, KM9, KM20, and KM46 was also used by Essenberg and myself to screen a second selfed generation (F_2 post-selection) of these segregants in the field. Table 22 gives a summary of the test in the chamber (1988) and the field (1989). The reactions of

TABLE 21

PUTATIVE DIFFERENTIAL ACALA CULTIVAR SERIES AND DISTINGUISHING RACE CHANGE MUTANTS

		Ac B5 Segregants							Acala cultivars								non-Ac		
Strain	Ac 44E	51B	51A	62 3	65 7	55 18	44 21	65 31	B1	B2	B3	B4	B6	B7	BN	BIn	Gregg	BSm	Im 216
H	+	-	-	+/-	+/-	+/-	-	-	+/-	+/-	+/-	-	(+)	-	-	-	-	-	-
KM1	+	+	-	+/-	+/-	+/-	+	-	+/-	+/-	+/-	+	(+)	-	-	-	-	-	-
2	+	+	-	+/-	+/-	+/-	+	-	+/-	+/-	+/-	+	(+)	-	-	-	-	-	-
6	+	+	-	-	-	-	+	-	-	-	-	+	(+)	-	-	-	+/-	+/-	-
13	+	+	-	-	-	-	+	-	-	-	-	+	(+)	-	-	-	-	-	-
21	+	+	-	+/-	-	+	+	-	+/-	+/-	-	+	(+)	-	-	-	-	-	-
KM9	+	-	-	+/-	-	-	-	+/-	+/-	+/-	+/-	-	(+)	-	-	-	-	-	-
KM20	+	-	-	+/-	+/-	-	-	-	+/-	+/-	-	-	(+)	-	-	-	-	-	-
KM46	+	+	-	+/-	+	+	+	+	-	+/-	-	+	(+)	-	+	+	-	-	-
N	+	+	+	+	+/-	+	+/-	+	+/-	+	+/-	+/-	(+)	+	+/-	+/-	+/-	+/-	+/-
# of genes		1	>2	1	1	1	1	1	1	1	1	1	1	1	1	1	1	>2	3^
same		*					*												
similar				?		?													

^Im216 contains B3, B7, BN, and probably BSm (Brinkerhoff et al , 1984)

TABLE 22

SERIES OF AC B5 SEGREGANTS AND DISTINGUISHING RACES TESTED IN THE FIELD

		Ac B5 J4301 Segregants			
Races	Ac 44E	55-18	65-7	65-31	44-21
H	+	(+, –)	(–, –)	(–, –)	(–, –)
KM1	+	(+/–, –)	(–, –)	(–, –)	(+, +)
KM9	+	(+/–, –)	(–, –)	(–, –)	(–, –)
KM46	+	(+, –)	(–, –)	(–, –)	(+, +)

Field reactions are given for selfed progeny of segregants and the F2 of Ac44E X segregants as the first and second members in parentheses. The + refers to reractions with disease grades of 3 or greater, +/– refers to disease grades from 2 to 2.9, and – refers to disease grades up to 1.9

Xcm H and KM1 on 65-7 in the field were weakly compatible but incompatible in the CERL chamber. Weather conditions in the field during the summer of 1989 were very mild. Perhaps 65-7 contained an R gene which is highly temperature sensitive and a further test in the Conviron chamber would confirm its resistance.

Conclusions

By screening a large population of plants from backcross progenies and by identifying new races of *Xcm* which were also spontaneous race change mutants, several possibly new resistance genes were identified. These are contained in the plant selections 44-21, 55-18, 65-7, and 65-31. Mutants KM1, KM9, and KM46 and *Xcm* H, when screened on those plant selections, constitute a set of differential *Xcm* races under growth chamber conditions while not under field conditions. It remains to be shown if the genes within these segregants are unique genes and whether they constitute a multiple allelic set of B5 resistance genes.

CHAPTER VI

CONCLUSIONS

The original goal of this work was to identify, clone, and analyze an avr gene. However, as described in Chapter IV, work toward that goal encountered far more difficulty than was anticipated. The vector commonly used for introduction of transposons into plant pathogenic prokaryotes (Turner *et. al*, 1984), pRK2013, failed to work. Also, when suspected Tn-mutants were obtained (Table 8), they were not stable, nor was the transposon Tn*3*-HoHol (Figure 25). The method of Tn*3*-HoHol mutagenesis was used in an attempt to partially characterize a reported *avr* gene-carrying clone of *Xcm* H identified by Gabriel *et. al* (1986). This clone, pUFA809, has since been shown to possess no avr gene (De Feyter and Gabriel, unpublished). Noel Keen has reviewed the current status of cloned *avr* genes now identified (Keen, 1990) from *Pseudomonas syringae* pvs. *glycinae* and *tomato* and *Xanthomonas campestris* pvs. *vesicatoria, oryzae,* and *malvacearum.* Some of the genes have been sequenced but as yet the biochemical role they play has not been determined (Keen, 1990).

By searching for spontaneous race change mutants, three new races of *Xcm* KM1, KM9, and KM46, allowed four R genes to be identified within the segregating AcB_5 population. These are the genes present in segregant selections 44-21, 65-7, 65-31, and 55-18. It is not clear, though, how or where these genes arose, if Knight's B_5 line in fact possessed one R gene. If instead, it was a set of tightly linked R genes in the *G. barbadense* D genome, perhaps transfer of the resistance to the *G. hirsutum* cv. Acala background and recurrent backcrossing allowed linkage among the group of R genes to be broken by mixing of the A and D genomes (similar to the explanation Barrow *et al.* (1973)

gave for their observation of viriscent twinning in *G. hirsutum*). This event is hypothetical and could be tested by (1) repeating Hunter and Brinkerhoff's original cross (1963) and (2) following resistant progeny from generation to generation by screening with the differential set of *Xcm* strains H, KM1, KM9, and KM46. This test requires, of course, that all four strains of the differential *Xcm* set test as avirulent on Knights' B5 *G. barbadense* Grenadine White Pollen.

The observations that (1) spontaneous race change mutants occur frequently and (2) that massive DNA rearrangements occur in these mutants, i.e. rearrangements that can be detected by a cosmid clone which does not possess any avr gene, is fascinating. pUFA809 may well contain a repetitive element (Chapter IV, Fig. 29 and De Feyter and Gabriel, unpublished). A likely explanation is that if it does carry a repetitive element, it is an endogenous insertion element. Trapping of this element by pUCD800 in *Xcm* or in *E. coli* would conclusively answer this. Having an endogenous IS element from *Xcm* would allow construction of a Tn suitable for use in *Xcm* for identification of further genes by Tn-tagging. Figure 34 gives a cartoon depiction of several steps through which transposition of endogenous IS elements could lead to new races of the *Xcm*. Perhaps this is the mechanism by which the new races KM1, KM9, and KM46 arose.

Finally, a defined minimal media useful for the growth of *Xcm* was identified. This medium has been used by Gholson *et. al* (1988), Sun *et. al* (1988), and Gabriel *et. al* (1986), with further modifications. MOPS minimal medium allowed growth of *Xcm* to proceed with a shorter generation time and with little or no lag time (Chapter III) and may prove useful for other xanthomonads as well.

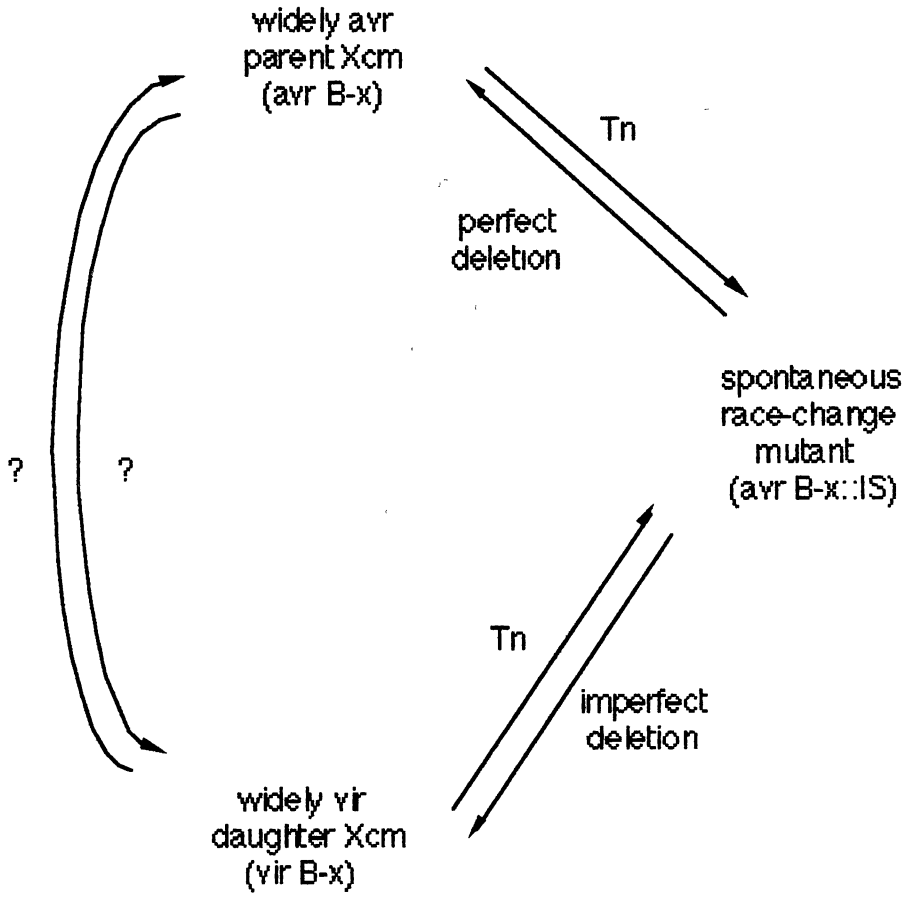


Figure 34. Model for Race/Change Conversions

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APPENDIXES

APPENDIX A

COMPARISON OF 30°C DAY WITH 23°C OR 19°C NIGHT FOR DIFFERENTIAL REACTIONS DISTINGUISHING *XCM* N FROM OTHERS

Xcm H, N, R2 and R4 were grown in NB as per Materials and Methods Chapter III. Inocula at 5 X 10^6 cfu/ml in sterile saturated $CaCO_3$ were prepared as per Materials and Methods Chapter V. These inocula were screened on the cotton lines Ac 44, Ac B_2, Ac B_3, Ac b_7 and Ac B_N (Table 12 for pedigrees) using replicate sets of plants, one set each for the 30°C day/23°C night and 30°C day/19°C night conditions. The programs for the Conviron chambers differed only in the minimum night temperature reached (Figure 32a, Chapter V, gives the program for the 19°C night). Reactions were scored on days 5, 6, 7, 8, 10, and 12 post-inoculation. Plots of disease grades (Materials and Methods, Chapter V) are shown for each strain and line combination (Figures 35 to 39).

From these plots, the following conclusions may be drawn: (1) Strain N is more compatible at a 23°C night than 19°C on Ac 44, Ac b_7, Ac B_3, Ac B_N and possibly Ac B_2, and N reaches a higher level of compatibility sooner, i.e., it is more aggressive at 23°C night than at 19°C night. (2) Race 4 gives better H_2O soaking and earlier soaking with a 23°C night on Ac B_N than at 19°C. (3) Race 2 and Race 4 are slightly more distinct from one another on Ac B_N on Day 6 at 23°C than at 19°C. From these conclusions, in order to distinguish strain N from the others, a 23°C night temperature is preferable to a 19°C night. Brinkerhoff and Presley (1966) found that disease expression appeared to be enhanced in Ac 44 when plants were maintained using 25.5°C day and 19°C night temperatures. In this case, disease expression, *i.e.* compatibility, was favored when a 23°C night was used.

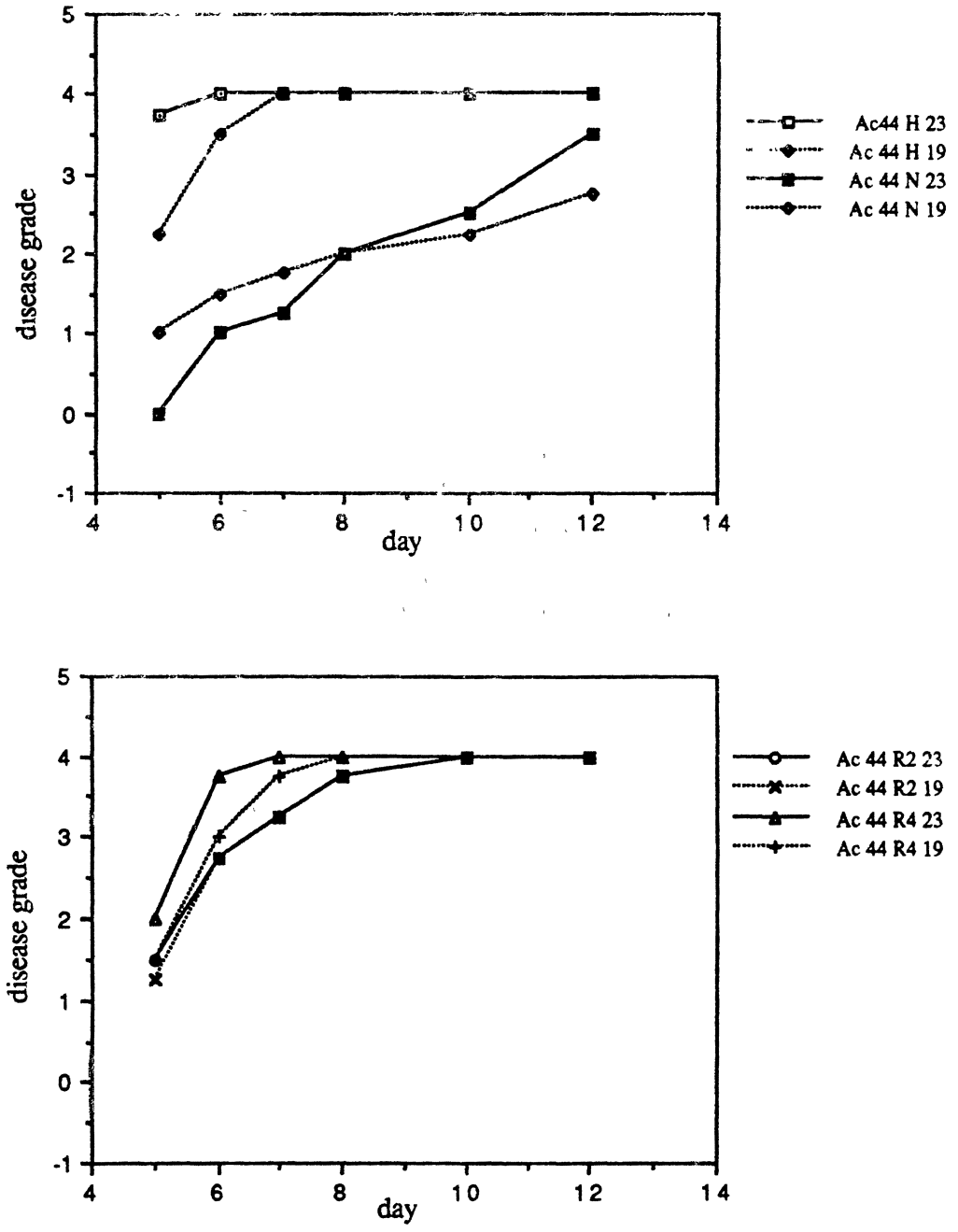


Figure 35. *Xcm* H, N, R2 and R4 Inoculated on Ac 44. *Xcm* were inoculated by syringe infiltration onto Ac 44 plants at 30°C day and 23°C or 19°C night.

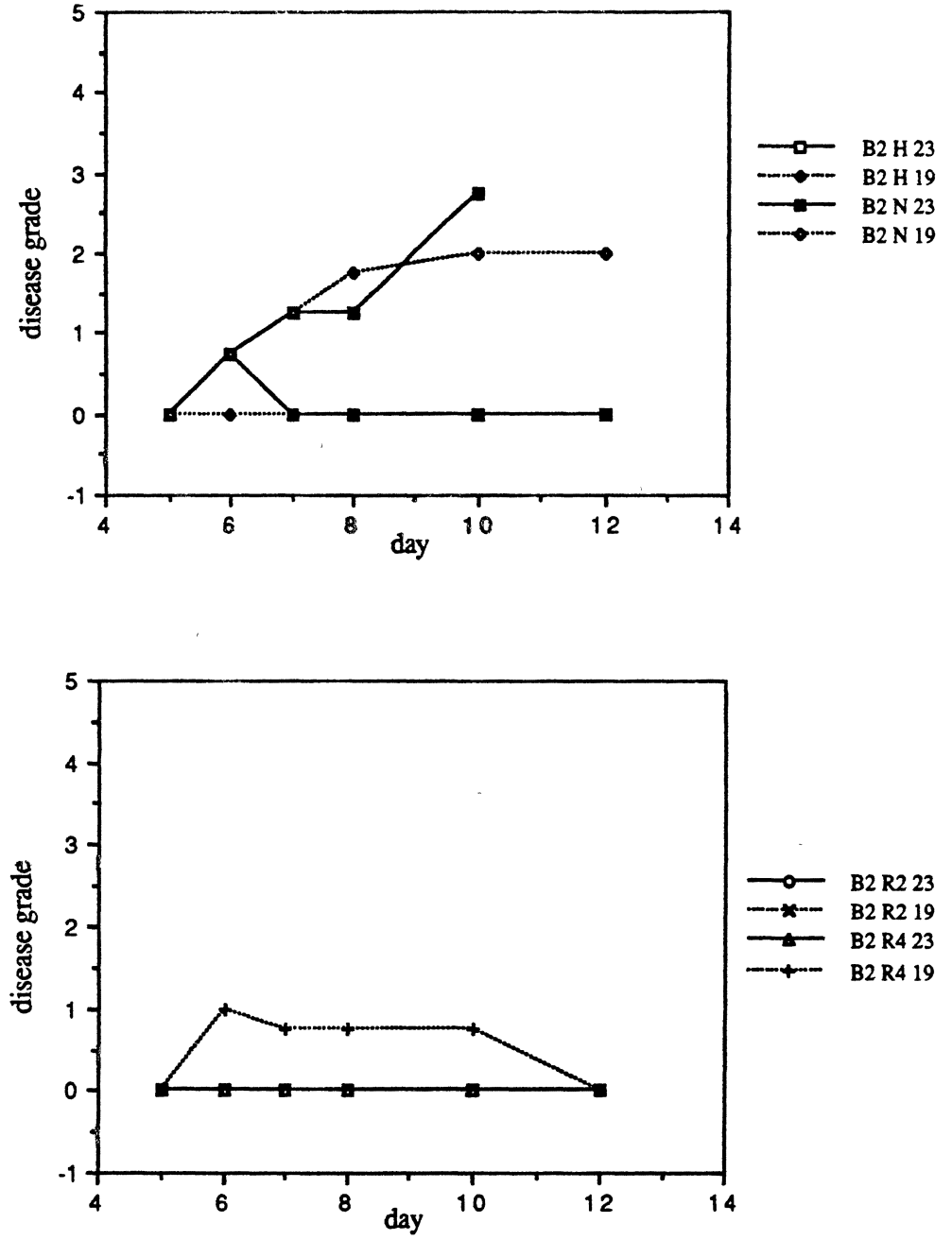


Figure 36. *Xcm* H, N, R2 and R4 Inoculated on Ac B_2. *Xcm* were inoculated by syringe infiltration onto Ac B_2 plants at 30°C day and 23°C or 19°C night.

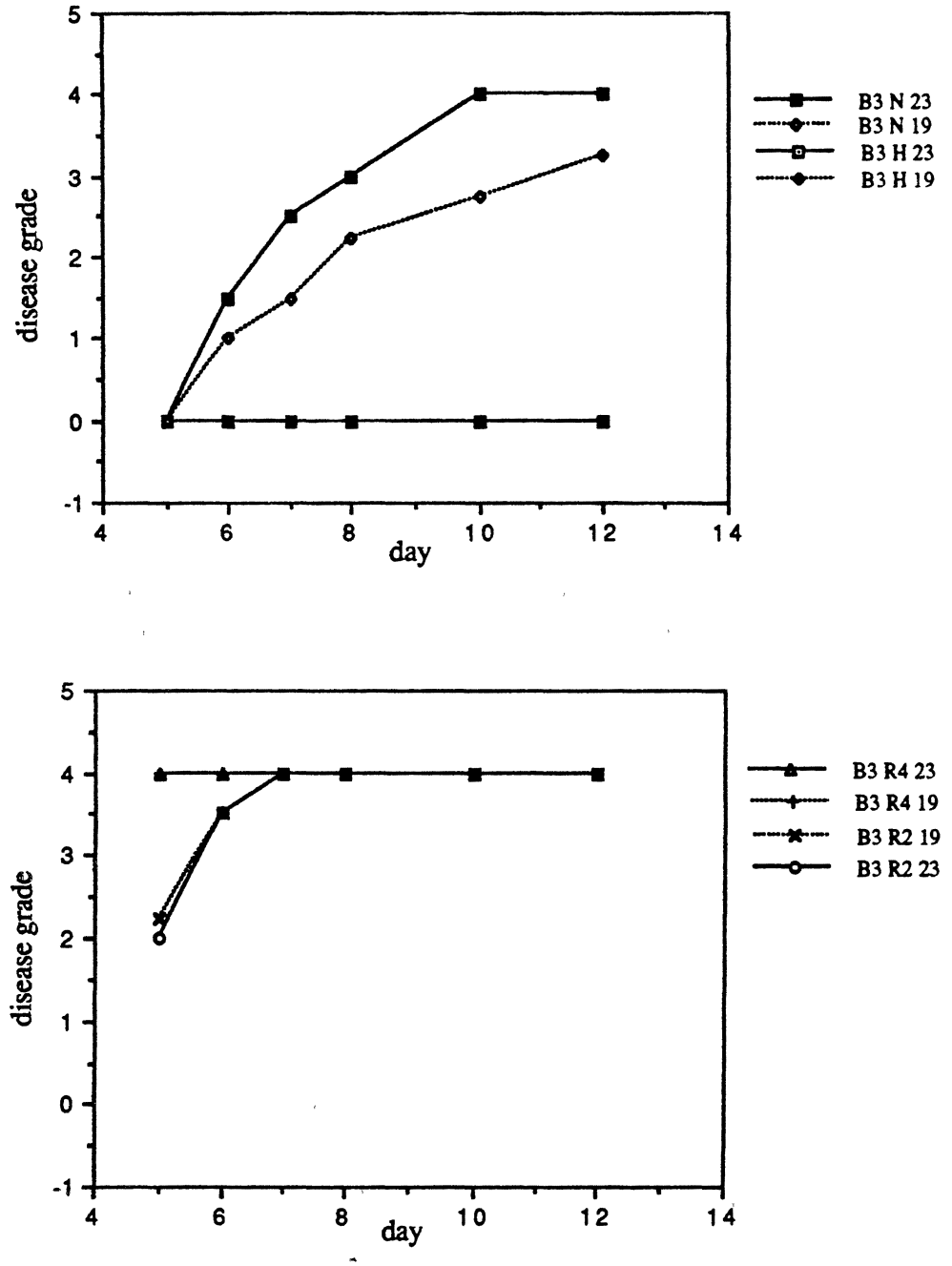


Figure 37. *Xcm* H, N, R2 and R4 Inoculated on Ac B_3. *Xcm* were inoculated by syringe infiltration onto Ac B_3 plants at 30°C day and 23°C or 19°C night.

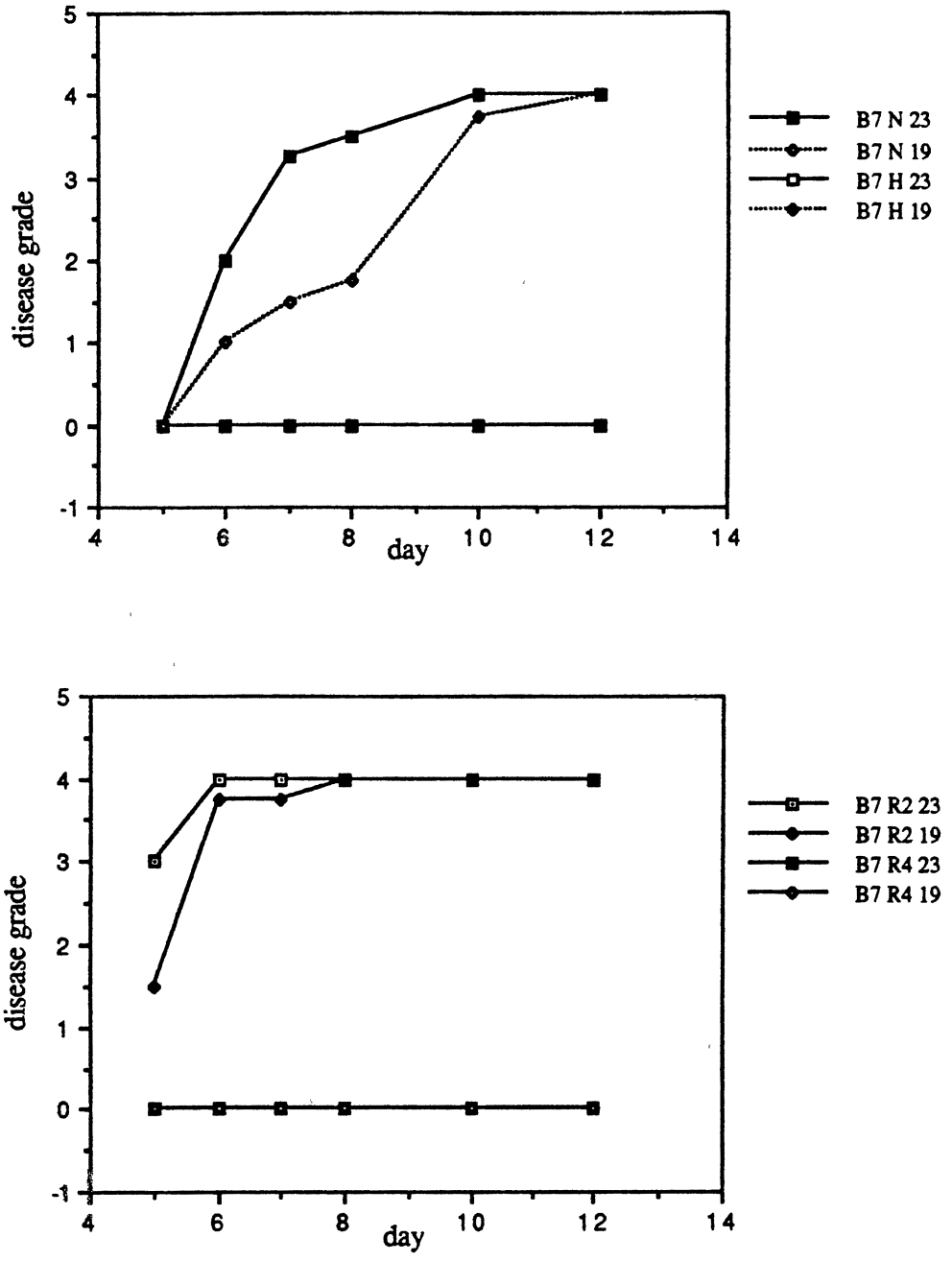


Figure 38. *Xcm* H, N, R2 and R4 Inoculated on Ac b7. *Xcm* were inoculated by syringe infiltration onto Ac b7 plants at 30°C day and 23°C or 19°C night.

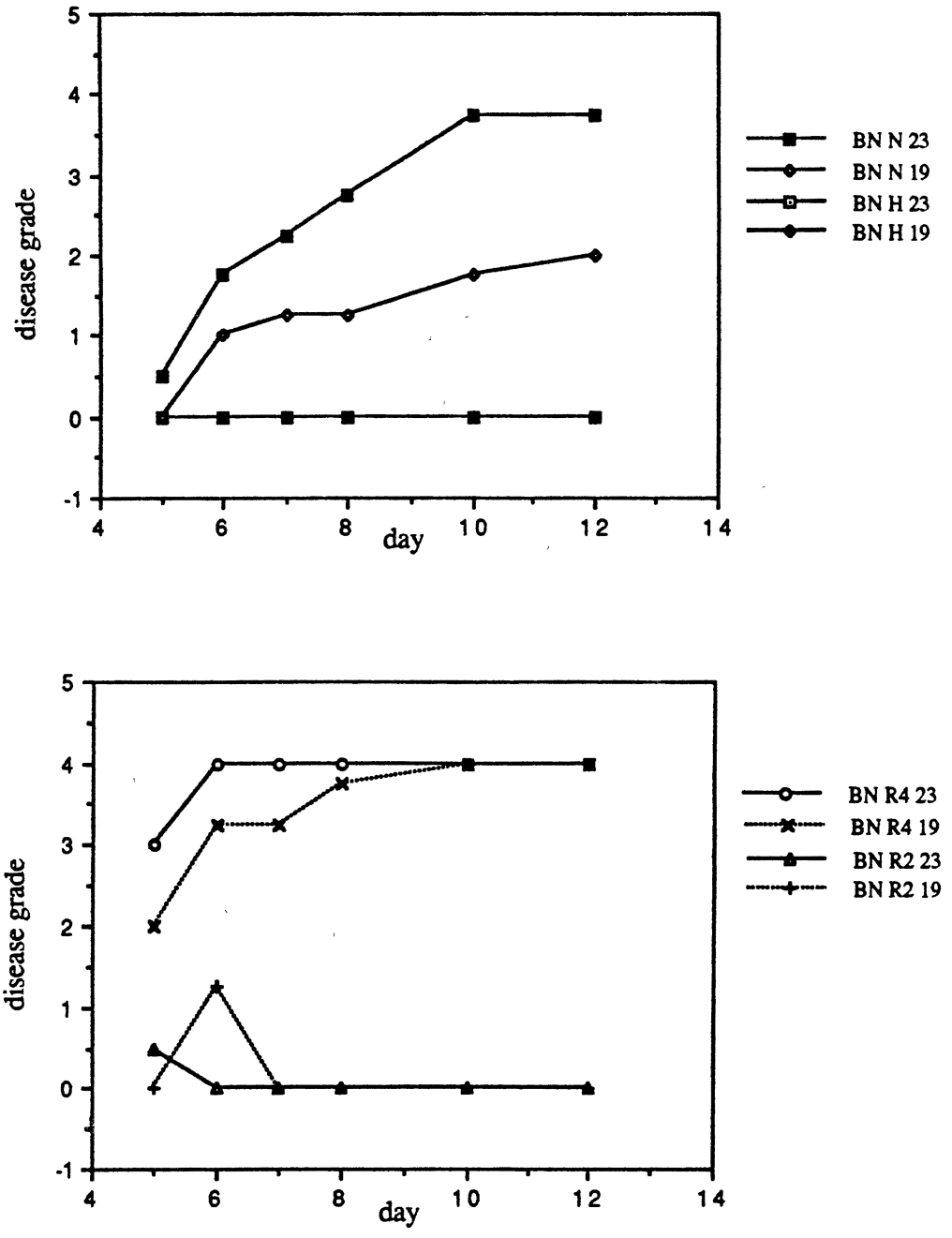


Figure 39. *Xcm* H, N, R2 and R4 Inoculated on Ac B_N. *Xcm* were inoculated by syringe infiltration onto Ac B_N plants at 30°C day and 23°C or 19°C night.

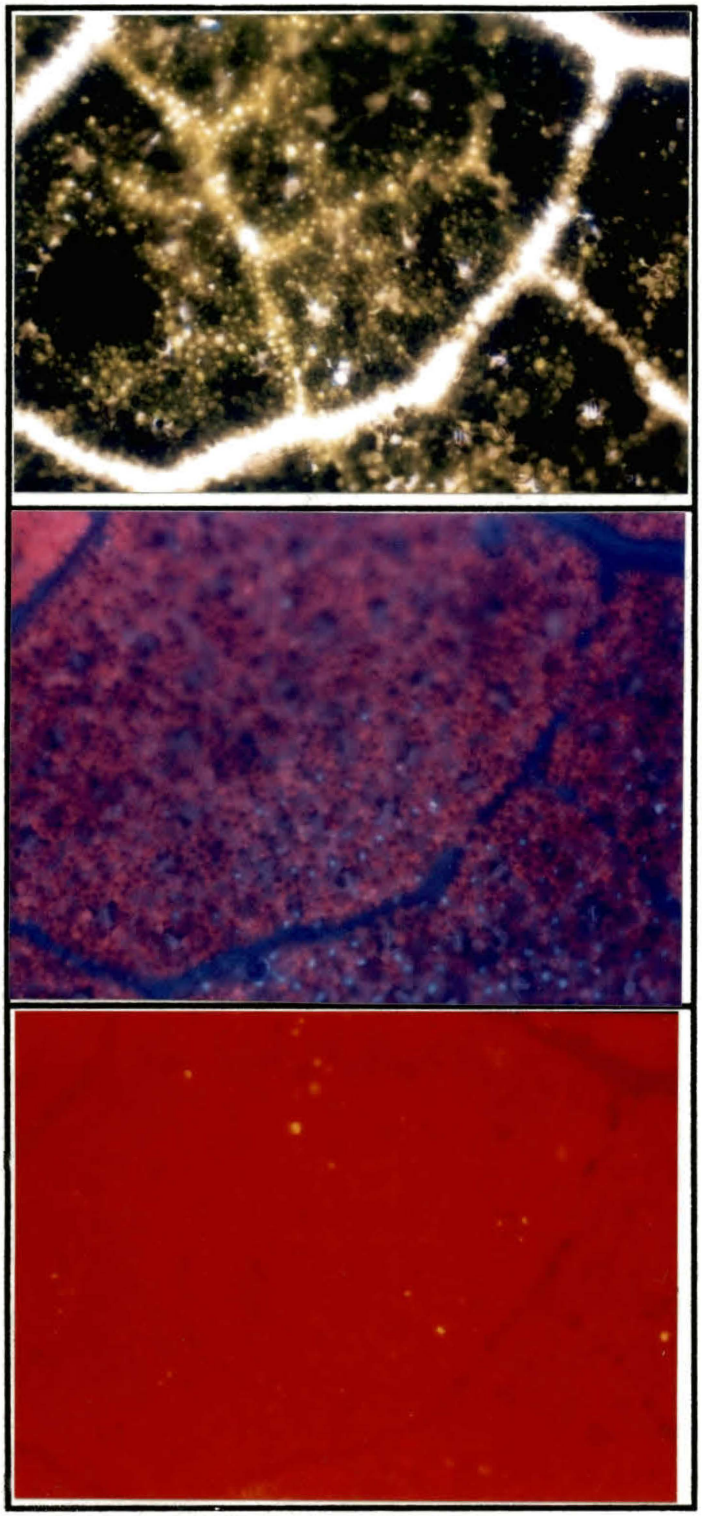
APPENDIX B

UNUSUAL SYMPTOMOLOGIES TWO WEEKS POST-INFECTION

Suspected Tn*3*-HoHo1 mutants of pUFA809 conjugated into *Xcm* strain H, isolated from AcB5 51B leaves as being either avirurent or virulent, were inoculated onto AcB5 51B leaves at low inoculum concentrations. At 2 weeks post-infection, samples of the leaves were viewed in a Nikon Optiphot microscope equipped with episcopic-fluorescence capability using a mercury lamp (12 V, 100 W) and a B2 filter cassette for red fluorescence (IF460-485 excitation filter, DM510 dichroic mirror, and 520W emission filter) or a UV filter cassette for blue fluorescence (UV330-380 excitation filter, DM400 dichroic mirror, and 420K emission filter).

Figure 40 contains micrographs from a sample of a leaf inoculated with transconjugant 66.15 which was purified from a suspected avirulent lesion of an Ac B5 51B leaf from the *Xcm* H(pUFA809::Tn*3*-HoHo1) transconjugant screening (Chapter IV). Figures 41 and 42 contain micrographs of samples from an AcB5 51B leaf inoculated with 133.17, a *Xcm* H(pUFA809::Tn*3*-HoHo1) transconjugant that was virulent on Ac B5 51B.

In Figure 40, blue fluorescent cells (panel b) are seen in a region separate from the yellow fluorescent cells (panel c). Perhaps the blue fluorescence is due to scopaletin. In Figure 41 panels b and c, red epidermal cells are seen (not unusual). There is, however, a diffuse yellow fluorescence spreading beyond the large water-soaked center and which contains localized regions of brighter yellow flurescence (Figure 41 panel c). No brightly blue fluorescent cells are seen (Figure 41 panel b). In Figure 42 panels b and c both red

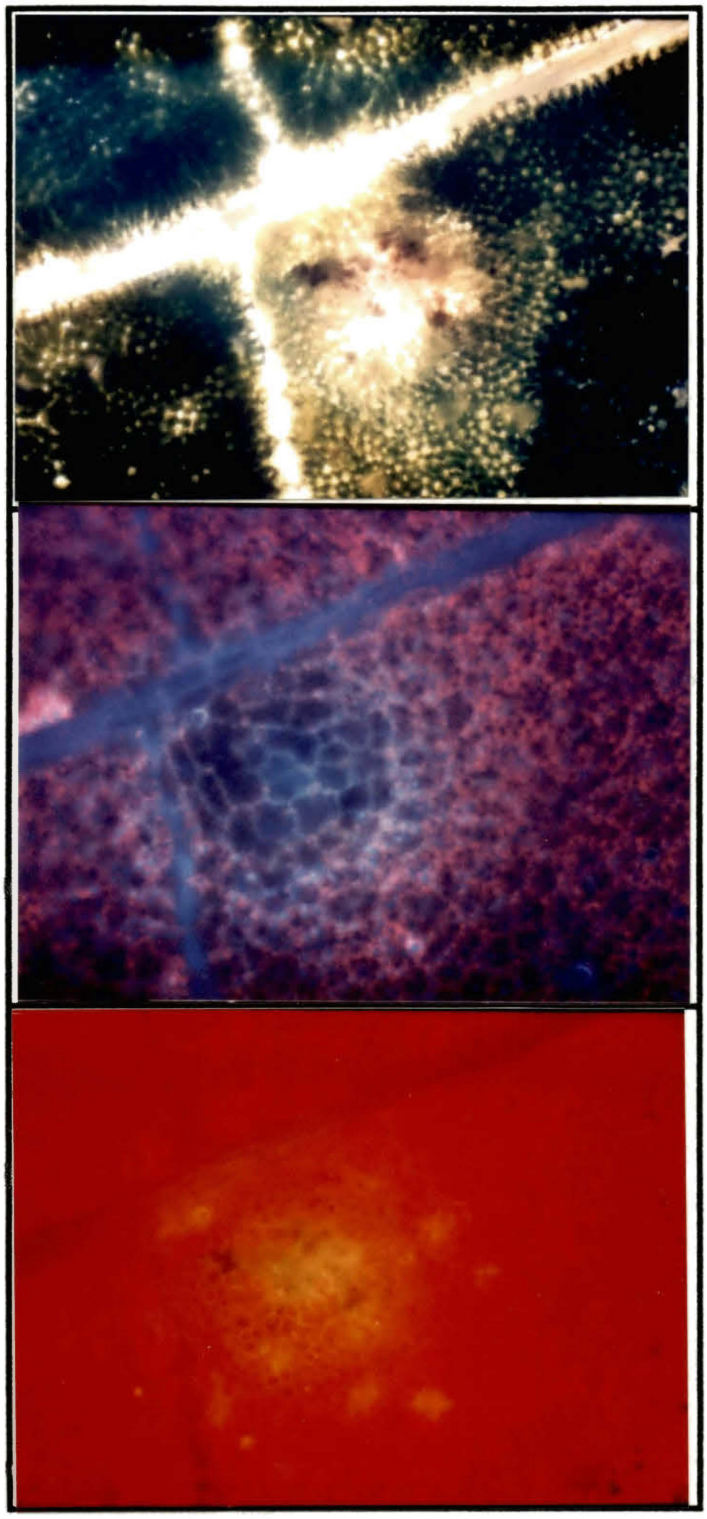


a) transmitted light

b) blue fluorescence (emission)

c) red fluorescence (emission)

Figure 40. Ac B_5 51B Inoculated with the Avirulent 66.15 *Xcm* H(pUFA809::Tn*3*-HoHo1) Transconjugant.



a) transmitted light

b) blue fluorescence (emission)

c) red fluorescence (emission)

Figure 41. Ac B_5 51B Inoculated with the Virulent 133.17 *Xcm* H(pUFA809::Tn*3*-HoHo1) Transconjugant, Water-soaked Lesion A.

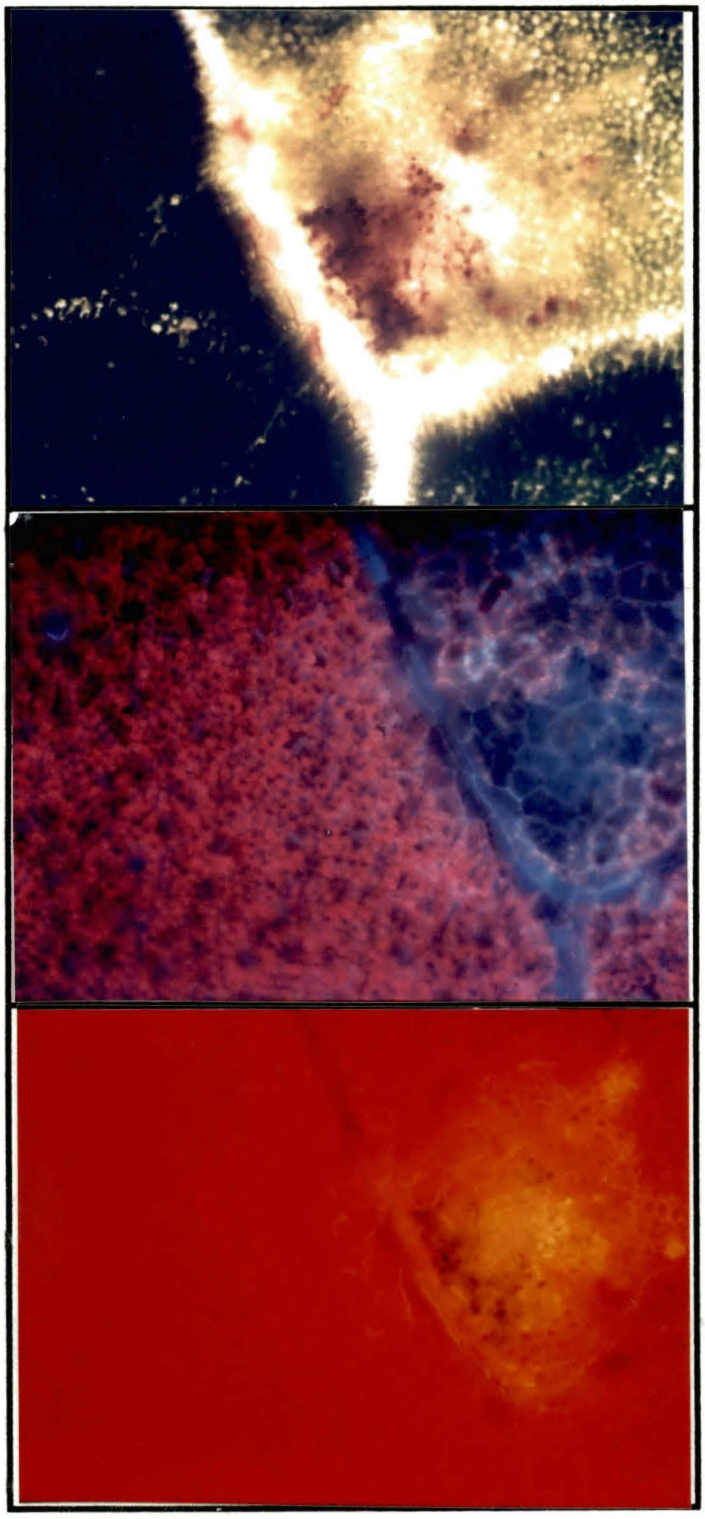


Figure 42. Ac B_5 51B Inoculated with the Virulent 133.17 *Xcm* H(pUFA809::Tn*3*-HoHo1) Transconjugant, Water-soaked Lesion B.

epidermal cells and the diffuse yellow fluorescence noted in Figure 41 panel c are seen. A region of the leaf across the vein from the water-soaked lesion (Figure 42 panel b) is considerably more pink than a similar region of Figure 41 panel b.

VITA

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