Chemotherapeutic elimination of genetic components
by Roger Vincent Miller

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Plant Pathology
Montana State University

Abstract:
Nonchromosomal genetic components (NGCs), in a broad sense, are found in many biological systems. In plant pathology, these components are important as vectors of pathogenicity and interspecific competitions. Chemotherapy, a widely used approach has had little applicability for controlling these genetic elements. In this study, a series of rapid biological systems was developed for detection of chemotherapeutants active against such genetic components.

Five bacterial systems, each characterized by a visible change when a genetic alteration occurred in the cells, were chosen and tested against 67 known or potentially active agents. The systems detected 75 percent of the agents tested. At least three assays were required to detect all 75 percent of the agents. Fifty percent of eighteen agents shown to have activity against barley stripe mosaic virus (BSMV) were detected by the bacterial systems. One of the assays was also used for screening barley cultivars for possible virus resistance. A cultivar having activity in the bacterial system was shown to have resistance to mechanical transmission of barley stripe mosaic virus.

BSMV strain MI-3 of was developed as a rapid eukaryotic prescreen for viricides. Eighteen compounds alone or in combination with butylated hydroxyanisol (BHA) repressed symptom development. Five combinations of agents were multiplicitively and isobolographically analyzed for synergy. These combinations failed to indicate synergy in four of the five cases. The use of an immunological technique (ELISA) indicated an almost complete absence of viral antigen in asymptomatic plants derived from treated seed. Six treatments were tested with this technique.

Activity of viricides or plasmid "curing" agents is often difficult to demonstrate due to chemotherapeutic efficacies of less than 100 percent. A mathematical model based on stochastic branching processes was developed. This formula was compared to spontaneous losses of a resistance plasmid, RK2, from E. coli. Predictions obtained from the formula correlated highly with the latter part of the loss curve. Expanded or redefined parameters of this basic model might provide a method for ascertaining viricidal activity.

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MONTANA STATE UNIVERSITY
Bozeman, Montana

May 1983

APPROVAL

of a thesis submitted by

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This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

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ABSTRACT

Nonchromosomal genetic components (NGC's), in a broad sense, are found in many biological systems. In plant pathology, these components are important as vectors of pathogenicity and interspecific competitions. Chemotherapy, a widely used approach has had little applicability for controlling these genetic elements. In this study, a series of rapid biological systems was developed for detection of chemotherapeutants active against such genetic components.

Five bacterial systems, each characterized by a visible change when a genetic alteration occurred in the cells, were chosen and tested against 67 known or potentially active agents. The systems detected 75 percent of the agents tested. At least three assays were required to detect all 75 percent of the agents. Fifty percent of eighteen agents shown to have activity against barley stripe mosaic virus (BSMV) were detected by the bacterial systems. One of the assays was also used for screening barley cultivars for possible virus resistance. A cultivar having activity in the bacterial system was shown to have resistance to mechanical transmission of barley stripe mosaic virus.

BSMV strain MI-3 of was developed as a rapid eukaryotic prescreen for viricides. Eighteen compounds alone or in combination with butylated hydroxyanisol (BHA) repressed symptom development. Five combinations of agents were multiplicitively and isobolographically analyzed for synergy. These combinations failed to indicate synergy in four of the five cases. The use of an immunological technique (ELISA) indicated an almost complete absence of viral antigen in asymptomatic plants derived from treated seed. Six treatments were tested with this technique.

Activity of viricides or plasmid "curing" agents is often difficult to demonstrate due to chemotherapeutic efficacies of less than 100 percent. A mathematical model based on stochastic branching processes was developed. This formula was compared to spontaneous losses of a resistance plasmid, RK2, from E. coli. Predictions obtained from the formula correlated highly with the latter part of the loss curve. Expanded or redefined parameters of this basic model might provide a method for ascertaining viricidal activity.

INTRODUCTION

The chemotherapeutic elimination of genetic components has many applications in plant pathology. Included in these applications would be the elimination of the genetic basis for pathogenicity in bacteria, fungi and viruses, and the development of prescreens for detecting advantageous characteristics such as virus resistant cultivars and new chemotherapeutic agents. Effective target chemotherapeutic studies could lead to an understanding of pathogenicity, host specificity and disease resistance. However, due to the intimacy of specific genetic components to their host organisms, effective chemotherapy has eluded most research efforts. Chromosomal genetic components are usually inaccessable to chemotherapeutic elimination, although highly mutatable regions and prophage and episomal insertion points are known to react to chemotherapeutic agents (144). In addition, some genetic functions of chromosomal origin have nonchromosomal regulatory systems (144,180,181) allowing the nonchromosomal system to be the chemotherapeutic target.

The most common genetic entities subject to chemotherapy are the nonchromosomal genetic components or

NGC's. After the discovery of bacterial plasmids by Lederberg in the 1950's (180,181), NGC's were found to exist in a wide variety of both prokaryotic and eukaryotic cells. NGC's include a diverse group of nonautonomously replicating entities including plasmids, episomes, replicons, insertion sequences, transposons, closed circular DNA, mitochondria (260), chloroplasts (260), bacteriophage, viruses and viroids (144,180,181,206,207,226). NGC's have been found in virtually all genera of prokaryotes (39,144,180) including the Cyanobacteria (136), Streptomyces (101,179), and Myxobacteria (123). In eukaryotic cells, plasmid-like NGC's have been found in yeast (82,91,92,235,249,254); the ascomycete Podospora anserina (238); green monkey kidney cells (53); and cytoplasmic male-sterility of grains (2,3,4,200,201) and sugarbeets (197). From the prevalence and diversity of NGC's in both eukaryotic and prokaryotic cells, it is becoming more evident that NGC's span every conceivable transitory type from viroids (226) to satellite viruses (121,122,174), to RNA and DNA viruses (156,206,207), to plasmids (144). The diversity of NGC's and the ability of these components to be interspecifically and intergenerically transferred and expressed (144,180,181,206) has obscured

the classifications of these genetic components.

The importance of NGC's in disease etiologies of prokaryotic origin are rapidly becoming evident. Loss of virulence during *in vitro* culturing, originally attributed to spontaneous mutation, altered physiology or environment, is often associated with loss of altered plasmids (39,134). Other plasmid mediated phenotypic characteristics include fertility factors, antibiotic resistance, heavy metal resistance, phage sensitivity or resistance, bacteriocin sensitivity or resistance, catabolic pathways, restriction enzyme synthesis and symbiotic nitrogen fixation (32,33,39,51,134,143,144,180, 181,206,220,250). Plasmid or bacteriophage mediated virulence of animal pathogenic prokaryotes has been well established and found to usually involve either antigenic determinants or toxin production (64,78,79,196; Pappenheimer, A. M., Jr., 1978. Diptheria: molecular biology of an infectious process. Trend. Biochem. Sci. (TIBS). October, 1978: N220-N222). Plasmid mediated avirulence rather than virulence has been shown in *Vibrio cholerae* (232). Although not shown to mediate pathogenicity, plasmids have been found in enteroinvasive *Escherichia coli* (99) and *Legionella pneumophila* (163).

Plasmids have been found in all genera of plant

pathogenic prokaryotes (37,38,39,46,47,48,105,113,134, 172,194,236,258,270). Definitive association of plasmids with virulence has been shown with Agrobacterium tumefaciens (48,108), Agrobacterium rhizogenes (172,270), and one strain (but not all strains) of Pseudomonas syringae pv. savastanoi (36,37,38). Production of Agrocin 84 by Agrobacterium radiobacter and sensitivity to the Agrocin by A. tumefaciens are both mediated by plasmids (125). Bacterial invasion and establishment of species of the non-pathogenic Rhizobium have been shown to be mediated by large (70 to 400 megadaltons) plasmids (118,152,182,183,278). Although not conclusively demonstrated, plasmid mediated virulence has been implicated in Pseudomonas syringae (D. C. Sands, G. Warren, D. F. Myers and A. L. Scharen. 1978. Plasmids as virulence markers in Pseudomonas syringae, p. 39-45. In: Proc. Internat. Conf. Plant Path. Bact., 4th, Angers, France), in Pseudomonas solanacearum (C. O. Ofuya and K. S. Wood. 1981. Cell wall degrading polysaccharides and extrachromosomal DNA in wilts caused by Psuedomonas solanacearum, p. 270-279. In: Proc. Internat. Conf. Plant Path. Bact., 5th, Cali, Columbia), and in Erwinia stewardii (D. L. Coplin, R. D. Frederick, M. H. Tindal and S. L. McCammon. 1981. Plasmids in virulent and

avirulent strains of Erwinia stewardii, p. 379-388. In: Proc. Internat. Conf. Plant Path. Bact., 5th, Cali, Columbia). Gantotti et al. (72) found two of three strains of Pseudomonas phaseolicola possessed plasmid mediated production of phaseotoxin. Chemotherapeutic elimination of plasmids may be useful as a unique approach for control of these and subsequent pathogens shown to have plasmid mediated functions required for virulence.

Chemotherapeutic elimination of prokaryotic plasmids has applicability for control of pathogens and as prescreens for potential antiviral and/or antitumor compounds. Induced plasmid loss, often termed "curing", has been utilized primarily for ascertaining plasmid mediated functions. Cultivation of bacteria at elevated temperatures (106,108,158,251,265) with thymine starvation (36,180,192) or with various chemical treatments results in the loss of plasmids (32,180,210). Surface active agents such as sodium dodecylsulfate induce plasmid loss in certain systems (36,106,180,193,217). Other chemical agents indicating antiplasmid chemotherapeutic efficacy include the acridine dyes (36,109,144,180,210,263), ethidium bromide (20,36,180,210), mitomycin C (107), rifampicin (210),

crystal violet (36) and other tricyclic compounds (12,169), amikacin (214), 5-iodo-2'-deoxyuridine (135), coumermycin A_1 (49), guanidine (26), and oxolinic acid (247). Most of these agents interact directly with DNA such as intercalcation by acridine or cross-linking by mitomycin C. Oxolinic acid and coumermycin A_1 inhibit DNA gyrase (247,248) rather than interacting with the DNA directly. Richmond (210) points out that none of the agents have universal activity with respect to different plasmids. For example, ethidium bromide and acridine are effective against F factors but ineffective against many of the R factors. Col factors are known to have 15 to 30 copies per cell (144) and thus tend to be resistant to chemotherapy. The use of anti-plasmid agents for chemotherapy is therefore limited to cases where the target plasmid has been characterized as to chemotherapeutic sensitivity (210). Lang and Baker (135), in one of the few in vivo tests, showed loss of virulence in cultures of Agrobacterium tumefaciens in the presence of 5-iodo-2'-deoxyuridine on carrot discs.

A problem arises in showing induced loss of bacterial plasmids or viruses. These NGC's are spontaneously lost at various rates under conditions which are selective for NGC free host cells. Under

putative non-selective conditions, loss rates range between 10^{-2} to 10^{-6} per cell generation, depending upon the NGC involved and prevailing environmental conditions. High background spontaneous loss rates make it difficult to demonstrate induced loss of NGC's. NGC's with low spontaneous rates are often insensitive to chemotherapeutants. Models proposed by various investigators (105,113,119,142,202) fail to adequately describe plasmid behavior for demonstration of induced elimination. These models are primarily theoretical and not proposed for use in chemotherapeutic studies. A general mathematical formula for prediction of behavior of both plasmids and viruses would be advantageous for demonstration of chemotherapeutic efficacy.

The chemotherapeutic elimination of plant viruses has progressed slowly due to the biochemical similarity of the pathogen with its host (255). Work conducted in the 1950's and 1960's centered around use of 8-azaguanine and 2-thiouracil (15,65,66,67,68,129,145,154,157). These early studies and subsequent studies have employed primarily cut leaf disc or half-leaf disc *in vitro* tests (157,231,255). Field trials of these *in vitro* promising compounds were generally ineffective. However, a variety of compounds has shown antiviral activity in these *in*

vitro assays. Plant hormones including cytokinin (5, 128, 133, 166, 167,209,255), indoleacetic acid (255) and gibberillic acid (255) have been shown to inhibit viral replication of tobacco mosaic virus (TMV), tobacco ringspot virus, potato virus X, papaya leaf reduction virus and cauliflower mosaic virus. Antibiotics showing antiviral activity in plant systems include actinomycin D (225,255), nofomycin (87,224,255), chloramphenicol (156,255), daunomycin (255), cytovirin (88,228,229,255), blasticidin S (108,156), trichothecin (156) and cycloheximide (156,232). Herbicides such as 2,4-D (147,156,230,255) have been shown to reduce virus replication rates as have fungicides (69,70,156,255,256). Thermal treatment, UV treatment, X-rays, heavy metals and cations have all been shown to affect viral replication of plant viruses (156). The greatest percentage of the tests were conducted *in vitro*, but Hansen (98) showed complete repression of apple chlorotic spot virus symptoms in *Chenopodium quinoa* when treated with 250 ug/ml virazole, applied as a soil drench. Latency or permanent loss of the virus was not established. An alternative to treatment of mature plants or use of cut leaf assays would be to treat seeds for either seed-borne viruses or as a prophylactic treatment.

Barley stripe mosaic virus (BSMV) is a plant virus having some characteristics which make it a candidate for antiviral chemotherapy testing. Streissle (242) indicated that acute viral infections are usually insensitive to chemotherapy due to rapid onset and development of the disease. Subacute infections are probably more sensitive. As BSMV in the dormant seed represents a subacute infection, it may prove more sensitive to chemotherapy than acute infections in mature plants. The virus is a single stranded positive sense RNA virus classified as Hordeivirus (155). The virion is rod-shaped (155). Natural hosts are Hordeum vulgare and Triticum aestivum (9). Experimental hosts include Avena sativum, Zea mays, Spinacea olevacea, Nicotiana tabacum cv. Samsun, Beta vulgaris and various species of Chenopodium (9). The MI-3 strain of the BSMV is a tripartite virus (T. W. Carroll, 1980. Department of Plant Pathology, Montana State University, personal communication). The virus is seed-borne, allowing an in vivo inhibition chemotherapeutic approach, as opposed to leaf disc or cut leaf assays (156,255). Of over 200 known seed-borne viruses (24,151), BSMV has one of the highest transmission rates, reported up to 100 percent (151). The virus is favored by an elevated temperature

of 21 to 27°C (111). Mayhew and Carroll (159) showed an association of the virus with microtubules, suggesting a possible chemotherapeutic target. Strains of the virus contain polyadenylate sequences on the 3' nucleotide end (1), and a 7-methylguanosine 5' cap (24). The virus produces characteristic chlorotic streaking (24), which allows rapid evaluation of disease symptoms. Due to the relatively well characterized nature of the virus, seed-borne transmission, growth of infected plants and characteristic symptoms, the virus appears suitable for antiviral compound testing. The virus can present problems to plant breeders. This is particularly true with the recurrent selection male-sterile-facilitated populations. The virus can be spread via pollen, causing high infection rates in these non-selfing populations and preventing release of the barley lines. The BSMV assay, therefore, presents two applications: first, as an assay for detection of antiviral compounds for use in plant, animal and bacterial chemotherapy; and secondly, for development of chemotherapeutic approaches for elimination of BSMV from high value experimental populations.

Imperative to effective chemotherapeutic elimination of genetic components is the detection and development of

new agents or analogs. As a universally effective agent against genetic components has not been found (210,221), a diverse battery of prescreens is needed to detect potentially active compounds. The use of bacterial prescreens has the advantage of high sensitivity, rapidity and inexpensiveness (271). A bacterial prescreen for antitumor agents that has been developed involves induction of the bacteriophage lambda (59,60,75,102,140,198). This system has been used extensively to screen for natural products and was shown to detect compounds such as azaserine, mitomycin C, and streptonigrin. However, not all equally efficacious agents such as actinomycin D, 6-diazo-5-oxo-L-norleucine and puromycin were detected by these assays. Other lambda based systems developed for detecting anticancer agents have coupled enzyme systems to promoters for visual detection (58,74,271). Fleck, according to White (271), utilized a lambda based assay which can resolve agents which either induce or repress the phage. Garner (73) and Moreau et al. (173) used lambda systems to screen for carcinogenicity. Agents which lambda systems failed to detect were found to induce antiphage activity of SP10 in Bacillus subtilis (271). Baccichetti et al. (10) showed detection of tilorone analogs by T_1, T_2 and

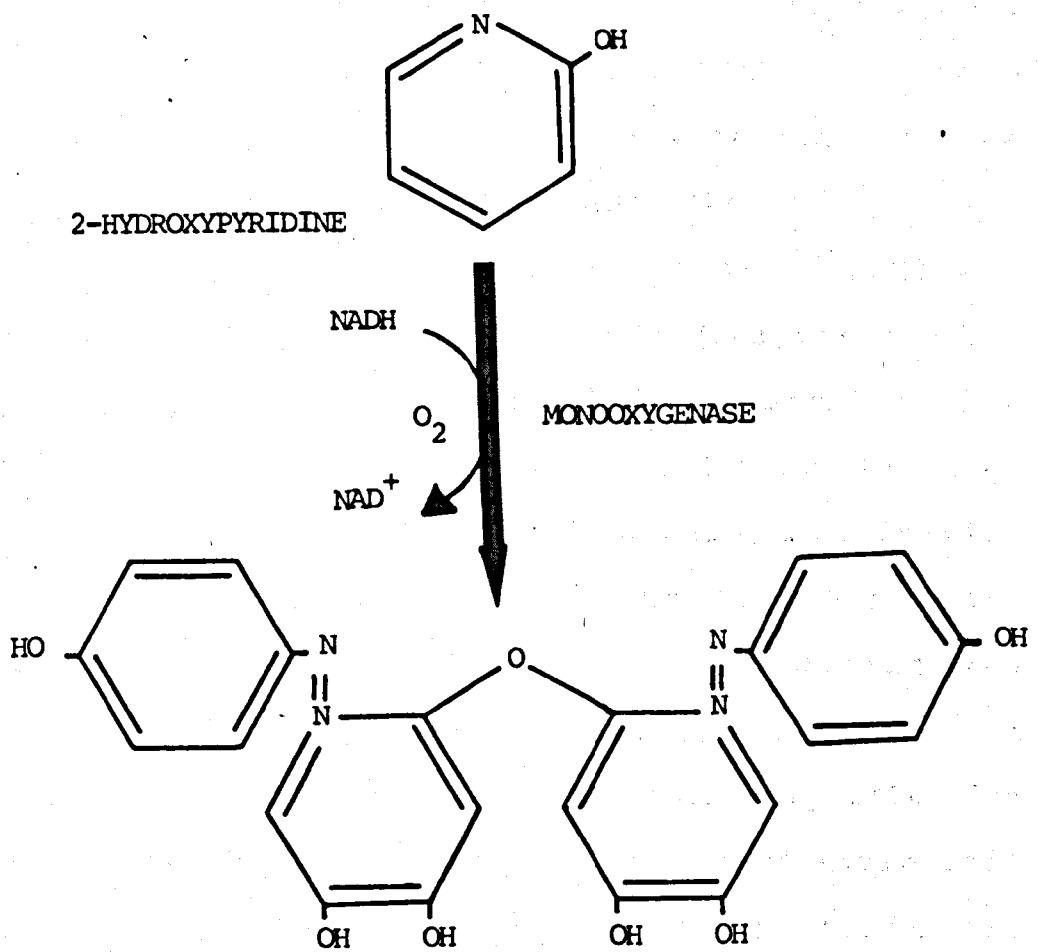
PhiX-174 bacteriophage. Hanka et al. (97) used bacteriocidal activity in Streptococcus faecalis to detect ARA-C. The Ames test (160) has been used extensively to detect potential carcinogenic compounds. Another assay developed for carcinogen testing utilized R factors in Salmonella typhimurium (161). Bacterial respiration and antimetabolite tests developed by UpJohn Laboratories were used to detect antitumor compounds (271). Eukaryotic prescreens which have been developed include respiration and antimetabolite yeast systems, animal tissue culture, organ culture and embryonated eggs (186,237,271). Ebringer (56,57) developed a prescreen with Euglena gracilis which detected inhibitors of DNA or protein synthesis including naladixic acid, novobiocin, porfiromycin and lincomycin via loss of plastids. Bernard and Riow (17) described an assay for intercalators detected by induced kinetoplast loss from the uniflagellate Trypanosoma cruzi. Limitations of these prescreens include: inability to detect indirect responses such as immune responses; inability to detect pro-drugs, requiring host mediated activation; inability to predict therapeutic index of the chemotherapeutant; and inability to define the biochemical target for selective toxicity.

The purpose of this research was to develop a series of biological assays for the detection of chemotherapeutants exhibiting efficacy for elimination or genetic components. A series of bacterial assays for this expressed purpose was developed and tested against known or potentially active chemotherapeutants. An *in vivo* plant viral system, seedborne BSMV, was developed as an additional prescreen along with development schemes for chemotherapeutic elimination of the virus. Results obtained from the bacterial assays were compared for ability to detect antitumor agents and agents active against BSMV. Utilization of a bacterial assay for detection of virus-resistant cultivars was also tested. Parameters for optimal sensitivity of the bacterial prescreens and BSMV assay were studied.

MATERIALS AND METHODS

Bacteria and Bacterial Nonchromosomal Genetic Components:

The gram-positive coryneform soil saprophyte, Arthrobacter crystallopoietes (Ensign and Rittenberg) was obtained from Dr. P. E. Kolenbrander (National Institutes of Health, Bethesda, MD). This bacterial prescreen for anti-NGC compounds was originally developed by Sands et al. (D. C. Sands, A. L. Scharen, G. Warren and U. Matern. 1979. A plate assay for plasmid curing agents, Abstract 578. In the Ninth International Congress of Plant Protection and the 71st Ann. Meet., Amer. Phytopath. Soc., Washington, D. C.). When grown in the presence of 2-hydroxypyridine, the bacterium produces a purple chromophore (Figure 1) which appears metallic green with oblique lighting. The chromophore production was originally thought to be plasmid mediated (131,268), which served as the basis for the anti-NGC assay. The bacterium was maintained on nutrient agar (Difco) (Appendix). Pigment formation assays were conducted on PYHT agar (131,132) (Appendix) at 30°C. The initial 72 hours of cultivation were conducted in a dark incubator to facilitate rapid growth. Subsequent cultivation was conducted in a incubator with 16 hours fluorescent



4,5,4',5'-tetrahydroxy-3,3'-diazadiphenoquinone-(2,2')

Figure 1. Arthrobacter crystallopoietes chromatophore formation from 2-hydroxypyridine. According to Kolanbrander and Weinberger (132).

lighting per day. It was found that green pigment production required light activation. Plasmid isolations from A. crystallopoietes were conducted on cells grown on MVGC medium (268) (Appendix).

Escherichia coli C600 with the plasmid pRK2 was obtained from Dr. Steven Lam in our department. This system was used to elucidate NGE loss rates and for comparison of the data to a theoretical model based on stochastic branching theory. This strain, a derivative of E. coli K12 Yale strain Y10, has the genotypic characteristics shown in Table 1. The strain was maintained on LB_a (Appendix) plates and cultivated at 37°C. A derivative of the strain, obtained by selection of cells expressing the spontaneous loss of antibiotic resistance was also maintained. This strain was maintained on LB agar (Appendix) at 37°C. Plasmid RK2 confers resistance to ampicillin, tetracycline, and kanamycin. The plasmid, an R factor, was originally isolated from Klebsiella aerogenes (synonomous with K. pneumoniae) (115) and is similar to or the same as pRP1 and pRP4 (253). The plasmid contains 56.4 kilobase pairs with a molecular weight of 40-48 megadaltons (115,252,253), 60 percent G+C content (115) and comprises approximately 3 percent of the cellular DNA in E. coli

Table 1. Some genotypic characteristics of Escherichia coli strains used in this study.

E. coli strain	Genotypic Characteristics[a]	References
C600	thr-1, leu-6, thi-1, $supE_{44}$, lacY1, tonA21,	11
GY4015	thr-1, leu-6, thi-1, $supE_{44}$, lacY1, tonA21, ampA601, -	R. Devoret[b],
GY5022	ProA, his, trp, lac, envA, ampA1, strA, papa	R. Devoret[b],
GY5027	proA, his, trp, lac, envA, ampA1, strA, uvr B34, papa	R. Devoret[b],
MM294	endo I-, thi, r_k^-, m_k^+	F. Fuller[c]
RR1	proA, leu-6, thi-1, lacY1, rpsL20, hsdR, hsdM, ara-14, galK2, xyl-5, mtlA, supE44	42

[a] Phenotypic traits according to Bachman (11):
ampA: ampicillin resistance
ara: Arabinose utilization
endoI: endonuclease (non-methylating) activity
envA: cell envelop and anomolous cell division
galK: galactose utilization
his: histidine biosynthesis
hsd: host specificity (phage)
lac: lactose utilization
leu: leucine biosynthesis
m^+_k: methylation (endonuclease) activity
mtlA: mannitol utilization
pro: proline biosynthesis
r_m: restriction endonuclease
rpsL: ribosomal protein; same as strA
StrA:streptomycin resistance
thi: thiamine biosynthesis
thr: threonine biosynthesis
tonA: T_1, T_5, colicin M receptor
trp: tryptophan biosynthesis
uvrB: uv repair endonuclease

[b] R. Devoret, 1982. Personal communication. Laboratoire d'Enzymololgie, CNRS, Gif-sur-Yvette, France.

[c] F. Fuller, 1982. A family of cloning vectors containing the lac uv5 promoter. Paper in preparation.

(110). Based on an average of 300 megadaltons of DNA in the E. coli chromosome (239), pRK_2 would have approximately 2 copies per cell.

The following E. coli strains were provided by Dr. R. Devoret (Laboratoire d'Enzymologie, Gif-sur-Yvette, France) for use in a lambda induction test. The genotypic characterization of all these strains is given in Table 1. GY4015 is a derivative of C600 (see previous strain characterization). It was used as a lytic indicator of phage activity released by induction of lamda from lysogenized strains. The bacterium was maintained on LB (Appendix) and cultivated at 37°C. The other two strains, GY5022 and GY5027, are resistant to streptomycin and lysogenized with phage lambda. GY5022 is a derivative of D22 cured of K12 and lysogenized with papa. GY5027 is a recombinant of D22 cured of K12 and GY2526 (a derivative of HfrH). The recombinant was lyogenized again with papa, but as opposed to GY5022 lacking UV repair endonuclease. Long term maintenance of the lysogenic strains, GY5022 and GY5027, were an LaCa medium with streptomycin-killed GY4015 (Appendix). Prior to induction tests, the lysogenic strains were grown up for 12 hours in LBE medium. GY5022 and GY5027 were cultivated at 37°C. Induction tests were conducted

on GTamp (Appendix).

Escherichia coli MM294 with pOP203-13 was obtained from Dr. Jesse Jaynes in our department. The genotypic characteristics of this bacterium are shown in Table 1. The bacterium was maintained on LB_{tet} (Appendix) plates to retain plasmid containing cells. Chemotherapeutically induced loss of the plasmid was conducted in buffered LB medium. Loss of the plasmid was ascertained by concurrent loss of derepressed B-galactosidase activity as shown on X-gal medium (Appendix). The plasmid, pOP203-13, is a derivative of pBR322 (ColE derivative) and contains the lac UV3 promoter and repressor binding sites resulting in constitutive derepression of the genomic lac operon (Fuller, F. 1982. A family of cloning vectors containing the UV5 promoter. Manuscript in preparation). Thus plasmidless cells can be detected by lack of blue pigment formation on X-gal plates (Appendix), making this system ideal as a prescreen for detecting anti-NGC compounds.

Escherichia coli RR1 with pBR329 was obtained from Dr. Jesse Jaynes in our department. The organism was utilized as a bacteria prescreen for anti-NGC compound by induced loss of plasmid mediated chloramphenicol resistance. The genotypic characteristics of this

bacterium are shown in Table 1. The bacterium was maintained on LB_{chl} (Appendix) plates to facilitate retention of the plasmid. The plasmid, pBR329, confers resistance to ampicillin, tetracycline and chloramphenicol (42). The plasmid was constructed by insertion of a portion of pBR328 (carrying chloramphenicol resistance) into pBR327. The plasmid is a ColE derivative with approximately 30 copies per cell. Chemotherapeutic induced loss of pBR329 was ascertained on LB_{cv} plates (Appendix) according to the method of Proctor and Rownd (204). Chloramphenicol resistance was indicated on the plates by the uptake of the rosalin dye.

Barley Cultivars and Viral Nonchromosomal Genetic Components:

Seed stocks of the barley cultivar Vantage (CI 7324) usually containing 60 to 70 percent seed-borne barley stripe mosaic virus (BSMV) was used to test chemotherapeutic efficacy of the compounds against this plant viral NGC. Vantage seed infected with the MI-3, BSMV strain, isolated and characterized by Dr. T. W. Carroll of our department, was utilized throughout the study. MI-3 is characterized with tripartite RNA species and high virulence. Other barley cultivars utilized in mechanical transmission studies included Black Hulless

(CI 666), Modjo (CI 3212) and Mobet (PI 467884) provided by Dr. T. W. Carroll of our department; CI 7155, CI 9530 and Alpine (CI 9578) provided by Mr. R. Eslick in the Department of Plant and Soil Science at Montana State University; and var. Aeofsi and var. violacean provided by Dr. U. Matern, Institut fur Biologie II der Universitat, Lefhrstuhl fur Biochemie der Pflazen, Federal Republic of Germany. All barley seeds were grown in 5 cm square pots with eight seeds per pot or in 35 x 50 x 10 cm flats with 150-350 seeds per flat.

Chemotherapeutic Agents:

A diversity of agents, totaling 67 compounds, was tested for chemotherapeutic efficacy against the bacterial plasmid prescreens, bacteriophage prescreen and against barley stripe mosaic virus. The chemotherapeutic agents and abbreviations used throughout this study are listed in Table 2 along with the supplier. The mode of action of these agents and references to the modes of action are also given in Table 2. Specific inhibition sites are given in Table 3. The agents had previously been shown to have antiviral or anticancer activity or were analogs to known antiviral compounds.

Table 2. Agents tested for chemotherapeutic efficacy.

Compound[a]	Abbrev.	Mode of Action	Specific Site of Action[b]	References
N-Acetyl-D-galactosamine (S)	AGL	Lectin binding antagonist		227
Acridine orange (A)	AOR	Integrated into ssDNA or ssRNA; intercalates into dsDNA; inhibits DNA polymerase and DNA dependent RNA polymerase activity	11	71,269,274
Adenine-9-B-D-arabinofuranoside (S)	ARA	Incorporated into DNA inhibiting DNA polymerase and DNA dependent RNA polymerase. Also inhibits pyrimidine synthesis, adenylate cyclase and polynucleotide phosphorylase		14,55,58
Amygdalin (Laetrile) (N)	AMY	Unknown		Merck[c]
6-Azauridine (S,P)	AZU	Pyrimidine antimetablite	14	18
Berberine sulfate (S)	BER	Plasmid curing agent		63
Bouvardin (N)	BOU	Inhibits protein synthesis		243
8-Bromoinosine (P)	BIN	Inosine analog		Merck[c]
Bruceantin (N)	BRU	Inhibits initiation phase of protein synthesis		28
Butylated hydroxyanisol (S)	BHA	Antioxidant, perturbs cell membrane and viral membrane functions		123
4-t-Butylcatechol (S)	BCT	Antioxidant, perturbs cell membrane and viral membrane functions		Merck[c]

Table 2. (Continued)

Compound[a]	Abbrev.	Mode of Action	Specific Site of Action[b]	References
Caffeine (S)	CAF	Inhibits phosphodiesterase activity causing rise of AMP intercalates into dsDNA, binds to adenosine in ssDNA, induces membrane associated calcium release.		127,276
Camptothecin (N)	CAM	Promotes degradation of both RNA and DNA; may intercalate into dsDNA		16,43,149, 259
L-Canavanine sulfate (S)	CAN	Arginine analog, inhibits functional protein synthesis		192
Chloramphenicol (S)	CHL	Binds to 50s subunit of bacterial 70s ribosomes, inhibits protein synthesis		195
Colchicine (S)	COL	Inhibits mitosis, covalently binds to tubulin inhibiting polymerization into microtubules		117,171
Cordycepin (S)	COR	Inhibits purine and pyrimidine synthesis, ribonucleotide reductases, DNA polymerase and DNA dependent RNA polymerase incorporated into RNA	1,2,8,13	16,71,126,246
Coumermycin A_1 (G)	COA	Prevents ATP coupling, inhibits DNA gyrase activity		62,76,77, 216,247
Cycloheximide (S)	CYC	Binds to 60s subunit of 80s eukaryotic ribosomes, inhibits protein synthesis		239,264

Table 2. (Continued)

Compound[a]	Abbrev.	Mode of Action	Specific Site of Action[b]	References
Cytosine-B-d arabinofuranoside (P)	CAR	Direct incorporation into DNA; inhibits DNA polymerase and DNA dependent RNA polymerase	13	55,171,264
2-Deoxy-D-glucose (S)	DOG	Lectin antagonist, incorporated into some viral glycoproteins		14,40,41,176
6-Diazo-5-oxo-L-norleucine (S)	DON	Glutamine analog, inhibits purine synthesis	2,4,10,16	18,71,126,171
N^6,O^2-Dibutyl adenosine 3',5'-cyclic monophosphate (S)	AMP	Cellular regulation, phosphorylation of tubulin prior to polymerization of microtubules		266
Ellipticine (N)	ELL	Intercalates into dsDNA, inhibits DNA polymerase and DNA dependent RNA polymerase, activity causes ssDNA strand breaks		130,191,261
Ethidium bromide (A)	EBR	Intercalates into dsDNA, inhibits DNA polymerase and DNA dependent RNA polymerase activity		71,86
Ethyl methanesulfonate (S)	EMS	Mutagen methylation of guanine, produces purine analogs		177
5-Fluoroorotic acid (S)	FOA	Nucleic acid precursor; pyrimidine analog	13	

Table 2. (Continued)

Compound[a]	Abbrev.	Mode of Action	Specific Site of Action[b]	References
p-Fluoro-DL-phenylalanine (S)	FPA	Inhibits protein synthesis, incorporates into proteins producing functionally impaired proteins; may inhibit electrostatic binding and penetration by viruses		16,186
5-Fluorouracil (S)	5FU	Incorporated into DNA and RNA; inhibits pyrimidine synthesis	34,35,41	18,150, 171,218
Guanosine (S)	GUS	Feedback inhibitor of purine synthesis	6,7,23,33	139
4-Hydroxypyrazolo-[3,4-d]-pyrimidine (A)	HPP	Hypoxanthine analog; inhibits xanthine oxidase; inhibits purine degradation		18,171
8-Hydroxyquinoline (A)	8HQ	Inhibits amino acid charging of tRNA, RNA synthesis and protein synthesis; induces ppGpp synthesis; affects cellular regulation		126,184
5-S-Isobutyl-5'-deoxyadenosine (S)	IDA	Inhibits methylation of RNA, especially 5' cap		16
Kinetin (S)	KIN	Increases cell membrane calcium levels, promotes plant cell division and chloroplast development		219
Lapechol (N)	LAP	Inhibits oxidative phosphorylation		185,245
Levamisol (S)	LEV	Interacts with ribosomes inhibiting mRNA		16

Table 2. (Continued)

Compound[a]	Abbrev.	Mode of Action	Specific Site of Action[b]	References
6-Mercaptopurine riboside (S)	MPR	Inhibits purine synthesis	17,18,23	18,170,171.211
2-Mercaptopyrimidine (S)	MCP	Pyrimidine analog		
Methotrexate (S)	MTX	Folic acid antagonist, inhibits folic acid reductase	9,15,45	18
N^3-methyl-5-iodouridine (S)	MIU	Uracil analog		
-Methyl-D-mannoside (S)	MMS	Lectin antagonist, incorporated into some viral glycoproteins		41
Mitomycin C (S)	MTC	Reduced form covalently cross-links dsDNA, bifunctional alkylating agent which alkylates primarily guanosine		71,82,114, 124,148,261
Naladixic acid (S)	NAL	Binds to DNA/gyrase complex; inhibits DNA gyrase activity		62,76,190, 248
Nitidine chloride (N)	NIT	Plant alkaloid		244,245
Novobiocin (S)	NOV	Inhibits ATP coupling DNA gyrase activity		62,76,216,247
Oxolinic acid (W)	OXA	Binds to DNA/gyrase complex; inhibits DNA gyrase activity		76,77,247, 248
a-Peltatin (N)	PEL	Binds to tubulin inhibiting polymerization into microtubules; inhibits nucleoside		117

Table 2. (Continued)

Compound[a]	Abbrev.	Mode of Action	Specific Site of Action[b]	References
Phosphonoformic acid (S)	PFA	Folic acid antagonist, inhibits dTTP and purine synthesis and DNA polymerase	7,9,15,45	6,30,43,50
Quinacrine (S)	QAC	General enzyme inhibitor, uncouples oxidative phosphorylation		18,171
Quinacrine mustard (S)	QAM	Monofunctional alkylating agent which alkylates guanosine primarily; intercalates		8,137,138
Quinine sulfate (S)	QNS	Intercalates into dsDNA, inhibits DNA polymerase and DNA dependent RNA polymerase		63,95
Taxol (N)	TAX	Alkylating agent, promotes microtubule formation		27,223,244
Theophylline (S)	THP	Inhibits phosphodiesterase activity causing rise in cAMP levels, intercalates into ssDNA, induces membrane associated calcium release		127
2-Thiouracil (S)	THU	Uracil analog		
Thymidine (S)	THY	Allosteric inducer of dGTP and dATP production, feedback inhibitor of purine and pyrimidine synthesis	1,13	139
Tilorone R10024DA (S)	T24	Intercalates into dsDNA; induces interferon; inhibits DNA polymerase and DNA dependent RNA polymerase;		29,243, 261,274

Table 2. (Continued)

Compound[a]	Abbrev.	Mode of Action	Specific Site of Action[b]	References
Tilorone R10233DA (S)	T33	See T24		
Tilorone R10556DA (S)	T56	See T24		
Tilorone R10874DA (A)	T74	See T24		
Tilorone R11002DA (A)	T02	See T24		
Tilorone R11043DA (S)	T43	See T24		
Tilorone R11513DA (S)	T13	See T24		
Tilorone R11567DA (S)	T67	See T24		
Tilorone R11877DA (S)	T77	See T24		
5-Trifluoromethyluracil (P)	TFU	Uracil analog, covalently binds to amino acids		171
Trigonelline (S)	TRG	Nicotinic acid analog, affects cellular metabolism		22

Table 2. (Continued)

Compound[a]	Abbrev.	Mode of Action	Specific Site of Action[b]	References
Vinblastine sulfate (S)	VBS	Binds to tubulin inhibiting polymerization into microtubules; inhibits amino acid transport, protein synthesis, RNA and DNA polymerases, and mRNA synthesis		18,44,171
Virazole (Ribovarin) (I)	VRZ	Inibits nucleic acid synthesis especially guanosine and 5' capping of mRNA	9	16,55,96,211

[a]Supplier indicated parenthetically as follows:

S Sigma Chemical Company, St. Louis, M.O.

A Aldrich Chemical Company, Milwaukee, W.I.

N National Cancer Institute, Bethesda, M.D.

P P-L Biochemical, Inc., Wilwaukee, W.I.

G Godfrey Science and Design, Inc., Syracuse, N.Y.

W Warner-Lambert Company, Pharmeceutical Research Division, Ann Arbor, M.I.

I ICN Corporation, Irvine, C.A.

[b]Specific sites of action are given in Table 3.

[c]The Merck Index, an encyclopedia of chemical and drugs, nineth edition, Merck and Co., Inc., Rahway, N.J.

Solubilization and Application of Chemotherapeutic Agents:

All chemotherapeutic agents were solubilized in dimethyl sulfoxide (DMSO) and diluted in distilled water to give a one percent (v/v) concentration of DMSO and the appropriate concentration of test agent. In bacterial prescreens where greater than 100 ug/ml concentrations were utilized, compounds in Table 4 required additional solvation or were partially insoluble and tested as partial suspensions. Agents were added aseptically to DMSO which had been sterilized by autoclaving. Sterile water was then added.

Bacterial disc assays were conducted using sterile 12.7 mm analytical filter paper discs (Schleicher and Schuell, Inc., Keene, NH). The discs were found to absorb 100 ul of aqueous solution. Generally, chemotherapeutic agents were tested at concentrations of 1000 ug/ml, 100 ug/ml and 10 ug/ml on the filter paper discs. Levels used of ethidium bromide, mitomycin C and novobiocin were based on previous bactericidal data. Each plate contained only one chemotherapeutic disc.

BSMV assays were conducted by allowing infected seed to imbibe aqueous solutions (with 1 percent v/v DMSO) of the chemotherapeutic agents for 24 hr. Preliminary data

Table 3. Specific sites of action of chemotherapeutic agents on purine and pyrimidine biosynthesis[a].

Number of specific site to action[b]	Enzyme(s) inhibited	Inhibits	End Product
1	Ribophosphate pyrophosphokinase	Pur, Pyr	5-phospho- -D-ribose 1-phosphoric acid
2	Amidophosphoribosyl transferase	Pur	5-phospho- -D-ribosylamine
3[c]	phosphoribosylglycinamide formyltransferase	Pur	5'-phosphoribosyl-n-formylglycinamide
4[d]	phosphoribosyl formylglycinamide synthetase	Pur	5'-phosphoribosyl-n-formylglycinamididine
5[c]	phosphoribosylamino imidazole-carboxamide transferase	Pur	5-phosphoribosyl-4-carboxyamide-5-formamidoimidazole
6	Adenylosuccinate synthetase	Pur	Adenylsuccinic acid
7	Adenylsuccinate lyase	Pur	Adenylic acid
8	Ribonucleoside diphosphate reductase	Pur,Pyr	Deoxyribonucleosides
9	Inosine monophosphate dehydrogenase	Pur	Xanthosine 5'-phosphate

Table 3. (Continued)

Number of specific site to action[b]	Enzyme(s) inhibited	Inhibits	End Product
10	Nucleoside monophosphate kinase	Pur,Pyr	Nucleoside diphosphates
11	Aspartate transcarbamoylase	Pyr	N-carbamoyl-aspartic acid
12	Orotate phosphoribosyl transferase	Pyr	Orotidine 5'-phosphate
13	Orotidine 5'-phosphate decarboxylase	Pyr	Uridine monosphosphate
14	Cytidine triphosphate synthetase	Pyr(CTP)	Cytidine triphosphate
15[d]	Thioredoxin reductase	Pur,Pyr	Thioredoxin
16[c]	Thymidylate synthetase	Pyr	Deoxythymidylic acid

[a] From A. L. Lehninger, (139).

[b] Specific site of action number referred to in Table 2.

[c] Folate antagonists do not directly interact with these enzymes; instead inhibit folate mediated methylation.

[d] Required for ribonucleotide diphosphate reductase activity (8).

Table 4. Solubilization of DMSO insoluble chemotherapeutic agents.

Chemotherapeutic Agent	Cosolvent
Bouvardin	10% 1N NaOH
Bruceantin	10% 1N NaoH
Ellipticine	Insoluble -suspension
Guanosine	10% 1N NaOH
Kinetin	10% 1N NaOH
Lapachol	10% 1N NaOH
Methotrexate	10% 1N HCl
Nitidine chloride	NaOH
Taxol	Insoluble - suspension
Tilorone R10233 DA	Insoluble - suspension
Tilorone R10556 DA	Insoluble - suspension

with 20 seeds indicated seeds imbibed 42.97±4.17 percent of the dry seed weight in the allotted 24 hr. Concentrations of chemotherapeutic agents were applied based on dry seed weight. Thus 100 ug/g seed required approximately 250 ug/ml of solution.

Arthrobacter Prescreen:

The Arthrobacter prescreen was conducted using the method of Sands et al. (D. C. Sands, A. L. Scharen, G. Warren and U. Matern. 1979. A plate assay for plasmid curing agents, Abstract 578. In the Ninth International Congress of Plant Protection and the 71st Ann. Meet., Amer. Phytopath. Soc., Washington, D. C.). Pigment producing cells of Arthrobacter crystallopoietes obtained from PYHT agar plates, were suspended in 5 ml sterile 0.05 M potassium phosphate buffer, pH 7.2. Optical density of the bacterial suspension was obtained on a Model 800-3 Klett-Summerson photoelectric colorimeter (Klett Manufacturing Company, NY) with a green filter. Serial dilutions of the bacterial suspension, based on a conversion rate of 3 Klett units equivalent to 10^6 colony forming units (CFU's), were made into sterile phosphate buffer. PYHT agar plates were seeded with 100 to 200 ul of the bacterial suspension yielding 500 to 1000 CFUs/plate. The bacteria were spread evenly across the

plate using a triangular bent glass rod. A disc containing a chemotherapeutic agent, DMSO, 1 N HCL or 1 N NaOH was placed in the center of the agar plate. Plates were cultivated as described above and evaluated for loss of pigment production proximal to the disc. Non-pigmented colonies were cultured in media free of the chemotherapeutic agent to determine permanence of the non-pigmented state.

Plasmid mediation of pigmentation of the bacterium was tested. Pigmented colonies requiring photoactivation and non-pigmented cells (spontaneously arising in photoactivated pigmented colonies) were subjected to plasmid isolation. Plasmids were isolated by an agarose gel electrophoresis modification of the method described by Gross et al.(90). Bacterial cells were grown in MVGC broth shake cultures for 7 days at ambient temperatures. Cells were collected by centrifugation at 10,000 g for 10 minutes. The cells were washed with TE buffer (0.05 M Tris, 0.02 M EDTA, pH 8.0) and centrifuged at 10,000 g for 10 minutes. The cells were resuspended in TE buffer at a rate of 2 ml/Klett unit on a liter basis. Proteinase VI (Sigma Chemical Company) at a rate of 500 ug/ml, predigested for 30 minutes in TE buffer, and 50 ug/ml grade III lysozyme (Sigma Chemical Company) were

added to the cell suspension. Sarkosyl NL-30 (ICN Pharmaceuticals) to a final volume of 1 percent (v/v) was added to the cell suspension. The suspension was gently shaken to aid mixing and incubated at 37°C for 1 hr. At the conclusion of the hour, 1 M lithium chloride (3 percent v/v) and 2 volumes distilled phenol were added to the lysed cells. The alkali denaturization step was eliminated due to alkali instability of the plasmid. The solution was gently inverted and centrifuged at 6000 g for 5 minutes. The phenol layer was discarded and 2 volumes of cold 95 percent ethanol (-10° C) was added to the solution. The solution was gently mixed and placed in the freezer for 12 hr. The solution was then centrifuged at 6000 g for 5 minutes and the supernatant layer discarded. The pellet was air dried and resuspended in as small an amount of distilled water as possible in which the pellet was completely dissolved. A 10 ul aliquot of the sample was then electrophoresed on a vertical 0.7 percent agarose gel. The agarose (Sigma Type IV) was dissolved in TBE buffer [89 mM Tris, 89 mM boric acid, 2.5 mm disodium EDTA, pH 8.3, according to Davis et al. (52) by heating. The electrophoresis was conducted at 150 volts for 3 hr. After electrophoresis, the gel was stained by immersion into a 0.6 ug/ml

solution of ethidium bromide (Aldrich Chemical Company) for 5 to 10 minutes. The DNA bands were viewed and photographed using a UV transilluminator and a Polaroid Land camera with an orange filter and loaded with 665 positive/negative film (52).

Lambda Prescreens:

The lambda prescreens were conducted according to the method of Moreau et al (173) for Inductest I. Indicator bacteria (GY4015), cultivated in LB broth, was diluted to a density of $1x10^8$ to $5x10^8$ cells per ml into soft overlay agar (Appendix). Lysogenic bacteria, either GY5022 or GY5027 grown on LB broth, were added at a density of $1x10^3$ cell per ml soft agar. The bacterial suspension in soft agar was poured over GTamp agar plates (Appendix), at a rate of 2.5 ml per plate. Discs containing chemotherapeutic agents were placed on the center of the plates. The plates were refrigerated at 4^oC overnight to facilitate diffusion of the chemotherapeutic agents. The plate was incubated at 37^oC for 24 hours and evaluated for prophage induction. Positive induction was indicated by plaque formation in the indicator lawn proximal to the chemotherapeutic disc. Comparisons of chemotherapeutic induction and severity were made between the UV repair deficient (GY5027)

bacterial strain and the UV nondefective repair (GY5022) bacterial strain.

B-galactosidase (pOP203-13) Prescreen:

The B-galactosidase prescreen was conducted using E. coli MM294 (pOP203-13) (Table 1). Bacteria grown on LB_{tet} agar were plated out on X-gal agar. A colony expressing B-galactosidase activity was suspended in sterile 0.05 M potassium phosphate buffer. Cell density was determined with a Klett colorimeter with a conversion factor of 10 Klett units for 10^6 cells per milliliter. X-gal agar plates were seeded from a dilution of the bacterial suspension for a final density of 800 to 1000 CFUs per plate. Seeding was accomplished via a triangular bent glass rod. Filter paper discs containing the chemotherapeutic agents were placed in the centers of the seeded agar plates. The plates were refrigerated at 4^oC overnight to facilitate diffusion of the chemotherapeutic agents. The plates were then incubated at 37^oC for 48 hours. At the conclusion of the incubation period, observations were made on the bactericidal zones and B-galactosidase production (blue pigment colonies). Altered physiological responses of the B-galactoside-production were induced overproduction (darker blue colonies) or repression (white colonies)

proximal to the chemotherapeutic disc. Colonies lacking expression (possible plasmid loss) or expressing overproduction of B-galactosidase were streaked onto fresh X-gal agar plates to ascertain irreversibility of the altered plasmid's physiological state without the presence of the chemotherapeutant. All 67 chemotherapeutic agents were tested with this assay.

Chloramphenicol Resistance (pBR329) Prescreen:

The pBR329 prescreen was conducted using E. coli RR1 (pBR322) (Table 1). Bacteria were maintained on LB_{chl} for selection of plasmid-containing colonies. Bacteria were removed from the plate, suspended in sterile 0.05 M potassium phosphate buffer, pH 7.2. Cell density was ascertained by optical density of the suspension, determined on a Klett Colorimeter with a conversion of 10 Klett units for 10^6 cells per ml. An appropriate dilution was made into phosphate buffer to give rise to 500 to 1000 CFU's per plate upon seeding onto LB_{cv} medium. LB_{cv} medium (Appendix) was utilized according to the method of Proctor and Rownd (204) to allow visual distinction between cells expressing chloramphenicol resistance and cells not expressing chloramphenicol resistance. Chemotherapeutic discs of the 67 test compounds were placed in the centers of seeded plates.

The plates were refrigerated for 12 hours to facilitate diffusion. The plates were then incubated at 37°C and colony pigmentation (lack of pigment indicative of chloramphenicol sensitivity) assessed after 72 hours. Colonies lacking pigmentation were selected and plated onto LB_{chl} to confirm permanence of loss of chloramphenicol resistance.

Barley Stripe Mosaic Virus Assay:

Vantage barley (CI 7324) maintained in maximally infected field populations, expressing approximately 65% seed infection, was utilized for the BSMV assay. In one test, BSMV infestation levels were only 31 percent due to error in seed lots. Seeds were allowed to imbibe the chemotherapeutant for 24 hours as described above and planted into 10 cm^2 pots. All chemotherapeutic agents were applied based on ug/g dry seed weight, designated henceforth in the BSMV tests as ug/g. After the preliminary testing, the experimental design throughout the remainder of BSMV experiments utilized 30 replications per treatment, and generally 60 replications were conducted on control plants treated with only 1 percent DMSO. All experiments were conducted using a completely randomized design. Randomization was instigated using a random number generation program on a