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Isolation of the Virulence Gene(s) in the New, Live, Oral Vaccine Candidate *Salmonella typhimurium* SR-11 Fad

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ISOLATION OF THE VIRULENCE GENE(S) IN THE NEW, LIVE, ORAL

VACCINE CANDIDATE *Salmonella typhimurium* SR-11 Fad⁻

BY

JAMES H. ALLEN

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE

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A U.S.A. B.A.S.I.C. MASTER OF SCIENCE THESIS

OF

JAMES H. ALLEN

APPROVED:

Thesis Committee

Major Professor

Paul J. Cohen

Shari Koller

David R. Nelson

Thomas J. Rockett

DEAN OF THE GRADUATE SCHOOL

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Abstract

A DNA fragment containing the defective virulence gene(s) in the live oral vaccine candidate *Salmonella typhimurium* SR-11 Fad⁻ was isolated. Genomic DNA fragments from the vaccine candidate, constructed by Tn10d::*cam* transposon mutagenesis, were probed with the 1.4 Kbp chloramphenicol resistance gene contained in Tn10d::*cam* and analyzed by Southern hybridization. Two of the fragments, an 8.0 Kbp *Hind* III fragment and a 4.5 Kbp *Pst* I fragment, were isolated, cloned into *E. coli* HB101, and mapped. The *Pst* I fragment, containing approximately 2.9 Kbp of flanking genomic DNA, was subcloned into the sequencing vector pBluescript II SK + (Stratagene, La Jolla, CA) and mapped.

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Preface

This research is presented here in the standard format for thesis.

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Introduction

Poultry production is reduced significantly by *Salmonella* bacteria. The U.S. poultry industry hatches approximately 7.5 billion eggs annually in incubation facilities, and nearly \$77 million is lost to the poultry industry each year due to *Salmonella* outbreaks in poultry farms (1). The health care costs associated with human salmonellosis caused by eating contaminated eggs and poultry is estimated at \$4 billion annually (1). Vaccination of chickens against *Salmonella* would both reduce losses to the poultry industry and the poultry-associated human salmonellosis.

Typhoid fever, an infectious disease caused by the bacterium *Salmonella typhi*, remains a serious public health problem in developing countries causing an estimated 600,000 deaths annually (2). Two *Salmonella typhi* vaccines, the live oral *S. typhi* Ty21a and a vaccine based on the Vi polysaccharide capsule of *S. typhi*, currently are licensed in several countries (3). Each of these vaccines, while effective, still suffers from drawbacks such as fevers after administration, incomplete protection, and a loss of protection with time (3). Therefore biotechnology is being used to develop new attenuated *S. typhi* live oral vaccines (3).

Live oral vaccines, based on deletions into genes necessary for *Salmonella* pathogenesis, have shown great promise (4, 5). For example, the live oral *S. typhimurium* Δcya (adenylate cyclase) Δcrp (cAMP receptor protein) vaccine is based on deletions in a *Salmonella* global regulatory system and is effective in animals and humans (4, 5). Another *S. typhimurium* live oral vaccine, avirulent and immunogenic in mice and cattle, is based on *aro* (aromatic compounds) deletions; this strain is auxotrophic for *p*-

aminobenzoic acid and 2,3-dihydroxybenzoate which are unavailable in mammalian cells (6, 7). In addition, *Salmonella* strains containing deletions in genes of the *phoP-phoQ* regulon (regulation of virulence) are effective mouse and human vaccines (8, 9).

The use of attenuated *Salmonella* strains as live vaccines is a safe and effective means of inducing significant humoral and secretory antibody responses in animal species: humans, cattle, sheep, rabbits, fowl, and mice (10). Live *Salmonella* invade the Peyer's patches, where they present their numerous antigens directly to the T and B lymphocytes of the mucosal-associated lymphoid tissues (10). This elicits the mucosal immune system to produce antigen-specific immune responses (10). Because of the significant humoral and secretory antibody responses, live attenuated *Salmonella* strains show great promise for carriers of antigenic determinants from other pathogenic microorganisms (10).

The Vaccine Candidate: *Salmonella typhimurium* SR-11 Fad⁻. The live oral vaccine candidate, *Salmonella typhimurium* SR-11 Fad⁻, was constructed by Tn10d::*cam* transposon mutagenesis of the SR-11 parent strain (11). The mutant strain was selected for its inability to catabolize oleate and citrate as sole carbon sources (11). It was phenotypically designated Fad⁻ (Fatty acid) for its inability to catabolize fatty acids as a sole carbon source (11). The SR-11 Fad⁻ strain also was unable to utilize acetate and isocitrate as carbon sources, but could utilize glucose, glycerol, pyruvate, and succinate as sole carbon sources (11).

Salmonella typhimurium SR-11 Fad⁻, administered perorally, was completely avirulent in BALB/c mice at a dose as high as 10⁹ colony forming units (cfu) (11). In

contrast, the SR-11 parent strain proved lethal at doses of greater than 10^4 cfu (11). The vaccine candidate also was found to be protective in BALB/c mice (11) as well as avirulent and protective in chickens (Dr. Paul S. Cohen: personal communication). A single oral dose of 10^7 SR-11 Fad⁻ cells protected BALB/c mice against a lethal dose of 10^9 cells of the virulent SR-11 *Salmonella typhimurium* parent strain (11).

In order for a vaccine to be useful, it must be safe. Therefore it should not contain antibiotic resistance genes. Since the SR-11 Fad⁻ vaccine candidate was constructed by Tn10d::cam transposon mutagenesis (11), it contains a chloramphenicol resistance gene. This chloramphenicol cassette is interrupting a virulence gene(s) and is blocking the expression of that virulence gene(s). Further development of this vaccine candidate required the isolation of this interrupted virulence gene(s). The virulence gene(s) and the chloramphenicol resistance cassette can then be permanently deleted, yielding a safer live oral vaccine candidate.

Materials and Methods

Agarose gel electrophoresis. Agarose gels were made with concentrations between 0.50% and 0.90% of electrophoresis grade agarose (Fisher Scientific, Fair Lawn, NJ) in TAE buffer (40 mM Tris-acetate; 1.0 mM EDTA, 5 Prime→3 Prime, Inc., Boulder, CO) and ethidium bromide (final concentration: 0.2-0.4 µg/ml; Sigma Chemical Co., St. Louis, MO) (12). Several DNA standard markers were used: phage lambda cut with *Hind* III (Gibco BRL, Gaithersburg, MD) and 1 Kbp DNA ladders (Gibco BRL, Gaithersburg, MD and Promega Corporation, Madison, WI).

Bacterial strains. See Table 1.

Calf intestinal alkaline phosphatase. Calf intestinal alkaline phosphatase (CIAP) (Promega Corporation, Madison, WI) was employed for the hydrolysis of 5'-phosphate groups from vector DNA, following incubation with a restriction enzyme. Three successive CIAP reactions, following the manufacturer's protocol, were performed on vectors prior to ligation to an insert.

DIG-labeled probe. A DIG-labeled (digoxigenin) probe was made from the 1.4 Kbp chloramphenicol cassette using A DIG High Prime DNA Labeling & Detection Starter Kit II (Boehringer-Mannheim, Germany): 750 ng of template DNA (chloramphenicol cassette) was denatured by boiling for 10 minutes and quickly cooled in an ice/ethanol bath; the now single-stranded template DNA was added to a labeling reaction mixture of deoxynucleoside triphosphates (dATP, dGTP, dCTP, dTTP and DIG-dUTP) and the Klenow fragment of DNA polymerase III; labeling of the template occurred, in this DNA synthesis reaction, by the random incorporation of DIG-dUTP (a

steroid hapten covalently bound to deoxycytidine triphosphate) over a 25 hour period at 37°C. The labeling reaction was stopped by heat inactivation at 65°C for 10 minutes.

Endonuclease digests. The restriction endonucleases, *Bam* HI, *Eco* RI, *Hind* III, *Pst* I, *Pvu* II, and *Xho* I were purchased from Gibco BRL (Gaithersburg, MD) and/or Promega Corporation (Madison, WI). The manufacturer's protocols for restriction endonuclease digestions were modified for volumes of 20-50 μ l using nuclease-free, deionized, distilled water (Sigma Chemical Co., St. Louis, MO), and DNA was digested overnight with an excess of enzyme ($>5U/\mu$ g DNA).

Incubation of bacterial cultures and plates. All liquid bacterial cultures, except where noted, were grown overnight at 37°C with aeration in the appropriate antibiotic. All plates inoculated with bacteria, except where noted, were incubated at 37°C for 12-18 hours.

Isolation of DNA. (Note: volume of reagents used and/or centrifugation speed and time varied according to the initial volume of the bacterial cultures and/or bacterial stain.) Genomic DNA was isolated by a modified protocol from *Current Protocols in Molecular Biology* (13). Bacterial cells from an overnight culture were pelleted by centrifugation, resuspended in TE Buffer, gently lysed with sodium dodecyl sulfate (SDS) (BioRad Laboratories, Richmond, CA), and incubated at 37°C for one hour in proteinase K (Sigma Chemical Co., St. Louis, MO) to degrade the proteins. Hexadecyltrimethylammonium bromide (10% CTAB in 0.7 M NaCl, Sigma Chemical Co., St. Louis, MO) and NaCl was added to the lysate and incubated at 65°C for 15 minutes: the CTAB solution complexes with denatured protein and polysaccharides; the

salt neutralizes macromolecules. An emulsion was formed by the addition of an equal volume of chloroform/isoamyl (24:1) (Sigma Chemical Co., St. Louis, MO) to the lysate. The aqueous phase, containing the DNA, was extracted after centrifugation leaving behind an organic phase of cell wall debris, denatured protein, and polysaccharides complexed to CTAB. A second extraction with phenol/chloroform/isoamyl (25:24:1) (Sigma Chemical Co., St. Louis, MO), removed residual CTAB precipitate. The DNA was precipitated in 100% isopropanol (Sigma Chemical Co., St. Louis, MO) and pelleted by centrifugation. The DNA pellet was rinsed in 70% ethanol to remove excess salt, lyophilized, and resuspended in TE buffer. Incubation (37°C for greater than 1 hour) of the genomic DNA in RNase A (Sigma Chemical Co., St. Louis, MO) degraded most of the contaminating RNA. The DNA underwent a final phenol/chloroform/isoamyl extraction to remove the RNase A and any residual proteins. After precipitation in 100% ethanol, the genomic DNA was ready for manipulation.

Genomic DNA also was isolated by using the G NOME[®] genomic DNA isolation kit (BIO 101, Vista, CA). The protocol from the manufacturer was modified by increasing the initial volume of overnight liquid bacterial cell culture to 10 ml per miniprep. The kit used a “Cell Suspension Solution,” “RNase Mixx,” “Cell Lysis/Denaturing Solution,” “Protease Mixx,” and “Salt-Out Mixture” of unspecified composition and replaced the chloroform steps of the standard protocol (13) with incubation at 4°C followed by centrifugation. A final precipitation of the genomic DNA in 100% ethanol completed the manufacturer’s protocol. Genomic DNA isolated by the

kit contained less RNA and protein contaminants than genomic DNA isolated by the standard protocol (13).

Quantities of plasmid DNA greater than 10 μg were isolated by modified alkaline lysis (14). A minimum of 100 ml of bacterial cells from an overnight liquid culture were pelleted by centrifugation and resuspended in GTE Buffer (50 mM glucose; 10 mM EDTA [ethylenediaminetetraacetic acid]; 25 mM Tris-HCl [Tris[hydroxymethyl]aminomethane hydrochloride], Fisher Scientific, Fair Lawn, NJ). The resuspended cells were added to a lysis solution, containing 0.2 N NaOH (Sigma Chemical Co., St. Louis, MO) and 1.0% SDS (wt/vol), and incubated on ice for 10 minutes. An appropriate volume of potassium acetate solution (5 M KOAc, pH 4.8) was mixed into the cell lysate and centrifuged. The supernatant, containing the plasmid DNA, was extracted with phenol/chloroform/isoamyl alcohol and precipitated in 100% isopropanol at -20°C for 30 minutes. The plasmid DNA was pelleted by centrifugation, rinsed in 70% ethanol to remove excess salt, lyophilized, and resuspended in TE buffer. Incubation (37°C for greater than 1 hour) of the plasmid DNA in RNase A degraded most of the contaminating RNA. The plasmid DNA underwent a final phenol/chloroform/isoamyl extraction to remove the RNase A and any residual proteins. After a final concentration by precipitation in 100% ethanol, the plasmid DNA was ready for manipulation.

Quantities of plasmid DNA less than 10 μg were isolated by WizardTM Plus Minipreps DNA Purification Systems (Promega Corporation, Madison, WI). The protocol from the manufacturer was modified by increasing the initial volume of

overnight liquid bacterial cell culture to 25 ml per miniprep. The bacterial cells were pelleted by centrifugation and resuspended in Cell Resuspension Solution (50 mM Tris, pH 7.5; 10 mM EDTA; 100 µg/ml RNase A). Cell Lysis Solution (0.2 N NaOH; 1.0% SDS) was added to the resuspended cells, mixed by inversion of the microcentrifuge tubes, and incubated at room temperature for 5 minutes. Neutralization Solution (1.32 M KOAc) was mixed into the cell lysate and centrifuged. The supernatant, containing the plasmid DNA, was pipetted into a 3 ml syringe (Becton Dickenson and Co., Franklin Lakes, NJ) attached to a Wizard™ Minicolumn. Wizard™ DNA Purification Resin was added to the lysate and plunged through the minicolumn. The minicolumn was rinsed with Column Wash Solution (80 mM KOAc; 8.3 mM Tris-HCl, pH 7.5; 40 µM EDTA; 55% ethanol), removed from the syringe and dried by centrifugation. Plasmid DNA was eluted off the minicolumn by the addition of TE buffer and centrifugation. The plasmid DNA was then ready for manipulation.

DNA was purified, when necessary, by extraction with phenol/chloroform/isoamyl alcohol (15). Phase Lock Gel™ (5 Prime→ 3 Prime, Inc., Boulder, CO) microcentrifuge tubes were used during the extraction process according to the manufacturer's protocol: an equal volume of DNA sample (genomic or plasmid) and phenol/chloroform/isoamyl alcohol were added to a Phase Lock Gel™ microcentrifuge tube, mixed, and centrifuged (14,000 x g) for two minutes. A thick density barrier was formed between the upper aqueous phase, containing the DNA, and the protein-laden interface and the lower organic phase. The upper aqueous phase was then simply poured off into sterile microcentrifuge tubes. A second extraction of the trapped organic phase

with TE buffer and phenol/chloroform/isoamyl alcohol removed any remaining DNA. The DNA samples were pooled, concentrated by ethanol precipitation, lyophilized, and resuspended in an appropriate volume of TE buffer.

DNA was concentrated, when necessary by ethanol precipitation (15). GenElute™ (Supelco, Bellefonte, PA) LPA (linear polyacrylamide DNA carrier) was used in all ethanol precipitations of plasmid DNA. The protocol from the manufacturer was modified by increasing the sodium acetate (Sigma Chemical Co., St. Louis, MO) concentration to 0.3 M to facilitate a more complete precipitation of the DNA in 100% ethanol (Quantum Chemical CO, Tuscola, IL). Precipitated DNA was pelleted by centrifugation (12,000 x g), lyophilized, and resuspended in TE buffer (10 mM Tris-Cl; 1.0 mM EDTA, 5 Prime→3 Prime, Inc., Boulder, CO). DNA was frozen at -20°C for short-term storage and -78°C for long-term storage.

Concentrations of DNA were estimated by ethidium bromide dot quantitation (16). Sample DNA, in a series of dilutions in TE Buffer and ethidium bromide (1.0 µg/ml final concentration), was spotted onto Parafilm® M (American Can Company, Greenwich, CT) and viewed on a ultra-violet transilluminator. A series of standards, lambda DNA cut with *Hind* III (Gibco BRL, Gaithersburg, MD), was spotted above the sample DNA. Comparison of the fluorescence of the sample to these standards provided an estimate of the DNA concentration in the sample (16).

DNA was also recovered from agarose gels. Bands of electrophoresed genomic or plasmid DNA were cut from agarose gels and added to GenElute™ Minus EtBr Spin Columns (Supelco, Bellefonte, PA) prewashed in TE Buffer. The columns were inserted

onto microcentrifuge tubes and centrifuged (12,000 x g) for 10 minutes. The columns retained the agarose and ethidium bromide while the DNA was eluted by the TE buffer. The DNA was then concentrated by ethanol precipitation.

Ligase reactions. T4 DNA ligase (Promega Corporation, Madison, WI) reactions were performed according to the manufacturers protocol for greater than 12 hours at 16°C. T4 DNA ligase catalyzed the formation of a 3' to 5' phosphodiester bonds between the 3'-hydroxyl group of one restriction fragment and the 5'-phosphate group of another restriction fragment while their sticky ends are transiently base-paired (17).

Media and antibiotics. Luria broth (Lennox formulation: 5g NaCl /L, Sigma Chemical Co., St. Louis, MO; 10 g tryptone /L, Difco Laboratories, Detroit, MI; 5g yeast extract /L, Difco Laboratories, Detroit, MI; pH 7.5) and Luria agar plates (Lennox formulation for Luria broth plus 15 g Bacto agar /L, Difco Laboratories, Detroit, MI; pH 7.5) were used exclusively. Antibiotics were purchased from Sigma Chemical Co. (St. Louis, MO) and added to the media at a final concentration as follows: 100 µg/ml ampicillin; 30 µg/ml chloramphenicol; 50 µg/ml nalidixic acid; 100 µg/ml streptomycin sulfate; 10 µg/ml tetracycline hydrochloride. MacConkey agar (Difco Laboratories, Detroit, MI) and XLD agar (Difco Laboratories, Detroit, MI) were used to characterize initial strains and all transformants.

Southern blotting. DNA, digested with restriction enzymes, was electrophoresed in a 0.85% agarose gel and photographed on an ultra-violet transilluminator (18). The DNA was partially depurinated by gently shaking the agarose gel in 0.25 M HCl bath for 30 minutes (19). The partial depurination cleaved DNA strands into fragments 1 to 2 Kb

in length improving their mobility in the gel (19). The gel was rinsed with distilled water and bathed in 0.4 M NaOH on an orbital shaker for 20 minutes. The DNA within the gel was denatured at this step yielding single-stranded DNA (19). The single-stranded DNA was then transferred overnight to a Magna Charge Nylon (Micron Separations, Inc., Westboro, MA) membrane (positively-charged) by upward capillary action using 0.4 M NaOH as the transfer buffer (19). The negatively-charged DNA was immobilized on the positively-charged nylon membrane during the transfer. The membrane was rinsed in 2x SSC (0.3 M NaCl; 30 mM sodium citrate; pH 7.0; 5 Prime→3 Prime, Inc., Boulder, CO) twice to remove excess salt and agarose fragments (19).

A DIG High Prime DNA Labeling & Detection Starter Kit II (Boehringer-Mannheim, Germany) was used to further hybridize and develop the blot. The DIG-labeled 1.4 Kbp chloramphenicol cassette probe, denatured by boiling for 5 minutes and quickly cooled in an ice/ethanol bath, was diluted in prewarmed standard hybridization buffer (5x SSC; 0.1 % [wt/vol] N-lauroylsarcosine; 0.02% [wt/vol] SDS; 1% [vol/vol] Blocking reagent) to a final concentration of 15 ng/ml. The probe was hybridized to the membrane for 12.5 hours at 68°C with gentle agitation. At this point, the single-stranded DNA probe in solution hybridized to homologous single-stranded DNA bound to the membrane. The membrane was then rinsed in post-hybridization solutions: twice with 2x SSC, 0.1% SDS at room temperature and twice with 0.1% (vol/vol) SSC, 0.1% (wt/vol) SDS at 68°C with gentle agitation. The post hybridization washes (stringency washes) removed any non-specifically bound probe from the membrane.

To detect the probe, the membrane was rinsed for 5 minutes in washing buffer (0.1 M maleic acid, Sigma Chemical Co., St. Louis, MO; 0.15 M NaCl; 0.3% [vol/vol] Tween 20[®], Bio-Rad Laboratories, Hercules, CA; pH 7.5). The membrane was then incubated for 30 minutes in blocking solution (0.1 M maleic acid ; 0.15 M NaCl; 10% [vol/vol] Blocking reagent). The membrane was then incubated for 30 minutes in anti-DIG-AP conjugate antibodies (anti-digoxigenin Fab fragments conjugated to alkaline phosphatase) diluted to 75 mU/ml in blocking solution. The anti-DIG-AP conjugate antibodies bound the DIG-labeled 1.4 Kbp chloramphenicol cassette probe via the hapten digoxigenin coupled to dUTP. Non-specific binding of the antibodies to the membrane was decreased by the blocking solution. The membrane was then washed twice for 15 minutes in washing buffer and equilibrated for 5 minutes in detection buffer (0.1 M Tris-HCl; 0.1 M NaCl; 50 mM MgCl₂; pH 9.5). The substrate CSPD[®] was added to the membrane in a development folder and incubated at room temperature for 5 minutes. Enzymatic dephosphorylation of the substrate CSPD[®] by alkaline phosphatase (anti-DIG-AP conjugate antibodies bound to the DIG-labeled probe) emitted light at a maximum wavelength of 477 nm. The light emissions were recorded on Hyperfilm-ECL High Performance Chemiluminescence Film (Amersham Life Science, Buckinghamshire, England) with exposure times ranging from 30 seconds to 5 minutes.

Transformations. Competent recipient cells are required for high-efficiency transformation by electroporation (20). *Escherichia coli* HB101 competent recipient cells were prepared for all transformations according to modified protocol from *Current Protocols in Molecular Biology* (20). An overnight culture of *Escherichia coli* HB101

cells were grown in Luria broth containing 100 µg/ml streptomycin sulfate; the fresh overnight culture was diluted (1:100) into Luria broth lacking the antibiotic and grown to an optical density (absorbance @ 600 nm) of 0.5 to 1.0. The cells were chilled on ice and pelleted by centrifugation (4000 x g) for 15 minutes in a cold rotor. The supernatant was poured off and the cells were resuspended in the same volume of cold, sterile, distilled, deionized water (ddiH₂O). The bacterial cells were pelleted by centrifugation, washed again in sterile ddiH₂O, and washed once in 20 ml of 10% glycerol (vol/vol) (Sigma Chemical Co., St. Louis, MO). The washed cells were resuspended in 10% glycerol (vol/vol) to a final concentration of approximately 10¹⁰ cells/ml. These competent recipient cells were used immediately and/or frozen at -78°C for up to 6 months.

Electroporation with high voltage was utilized to introduce plasmid DNA into the competent recipient cells (20). Each transformation contained 40 µl of chilled competent recipient cells and 1-2 µl of heat-inactivated ligase reaction. These were electroporated in 0.2 cm electrode gap cuvettes (Bio-Rad Laboratories, Hercules, CA) using a Bio-Rad Pulse Controller and Bio-Rad Gene Pulser (Bio-Rad Laboratories, Hercules, CA) at 2.5 kV, 25µF, and 200Ω. Time constants ranged between 4.4 msec to 4.8 msec. The electroporated cells were then incubated, shaking, for 1 hour at 37°C in Luria broth. During this time, CIAP-treated plasmids (when used) were repaired as they replicated within the cell. After incubation, 200 µl aliquots of the transformation mixture were plated onto Luria agar containing the appropriate antibiotic.

Vectors. The vector, pBR322 (Figure 1), was isolated from *Escherichia coli* HB101 (pBR322). The vector, pBluescript II SK (+), was purchased from Stratagene (La

Location of the chloramphenicol resistance cassette. A first and obvious candidate, *Salmonella typhimurium* SR-11 Pad^r, had been constructed previously by Tn¹⁰/*cat* transposon mutagenesis of the SR-11 parent strain (11). The location of the inserted chloramphenicol cassette within the chromosome of SR-11 Pad^r was unknown. As a first step in determining the location of the chloramphenicol cassette inserted into SR-11 Pad^r, it was necessary to isolate the cassette for future use as a probe. It was known that the 1.4 Kbp chloramphenicol resistance gene is flanked by Bam HI endonuclease restriction sites (21). Therefore, genomic DNA from SR-11 Pad^r was isolated and digested with Bam HI. The vector pBR322, which contains a unique Bam HI site (Figure 1), was also digested with the endonuclease Bam HI. The cut vector was then dephosphorylated with calf intestinal alkaline phosphatase (CIAP) to prevent self-ligation. A ligation reaction (Figure 2) using a 3:1 insert to vector molar ratio was performed.

The ligation reaction was electroporated into *E. coli* HB101 competent cells. Transformants were selected on Luria agar plates containing chloramphenicol. One of the chloramphenicol-resistant transformants was isolated and found to be resistant to ampicillin. The clone was also determined to be sensitive to tetracycline which suggested an interruption of the tetracycline resistance gene in the vector pBR322 by the chloramphenicol cassette (Figures 2, 3). The transformant was designated: *E. coli* HB101 (pBIA1). The plasmid pBIA1 was digested with Bam HI and electrophoresed on a 0.7% agarose gel. Two bands were observed: a band at approximately 4.3 Kbp which

Results

Isolation of the chloramphenicol resistance cassette. A live oral vaccine candidate, *Salmonella typhimurium* SR-11 Fad⁻, had been constructed previously by Tn10d::cam transposon mutagenesis of the SR-11 parent strain (11). The location of the inserted chloramphenicol cassette within the chromosome of SR-11 Fad⁻ was unknown. As a first step in determining the location of the chloramphenicol cassette inserted into SR-11 Fad⁻, it was necessary to isolate the cassette for future use as a probe. It was known that the 1.4 Kbp chloramphenicol resistance gene is flanked by *Bam* HI endonuclease restriction sites (21). Therefore, genomic DNA from SR-11 Fad⁻ was isolated and digested with *Bam* HI. The vector pBR322, which contains a unique *Bam* HI site (Figure 1), was also digested with the endonuclease *Bam* HI. The cut vector was then dephosphorylated with calf intestinal alkaline phosphatase (CIAP) to prevent self-ligation. A ligase reaction (Figure 2) using a 3:1 insert to vector molar ratio was performed.

The ligation reaction was electroporated into *E. coli* HB101 competent cells. Transformants were selected on Luria agar plates containing chloramphenicol. One of the chloramphenicol-resistant transformants was isolated and found to be resistant to ampicillin. The clone was also determined to be sensitive to tetracycline which suggested an interruption of the tetracycline resistance gene in the vector pBR322 by the chloramphenicol cassette (Figures 2, 3). The transformant was designated: *E. coli* HB101 (pJHA1). The plasmid pJHA1 was digested with *Bam* HI and electrophoresed on a 0.9% agarose gel. Two bands were observed: a band at approximately 4.3 Kbp which

represents the vector pBR322 and a 1.4 Kbp band corresponding to the chloramphenicol resistance gene (Figure 4: lane 4). The plasmid pJHA1 was also mapped with *Eco* RI since the chloramphenicol cassette and the vector pBR322 were known to contain a unique *Eco* RI restriction sites (21) (Figure 1). Bands at approximately 4.6 Kbp and 1.2 Kbp (Figure 4: lane 3) confirmed the predicted map of pJHA1 (Figure 5).

Southern blot of *Salmonella typhimurium* SR-11 Fad⁻. The goal of the southern hybridization was to determine the sizes of fragments of SR-11 Fad⁻ genomic DNA containing an intact chloramphenicol cassette. When hybridized to a labeled chloramphenicol cassette probe, different sized fragments will correspond to different restriction endonucleases employed to digest the SR-11 Fad⁻ genomic DNA. In order to reach this goal, the chloramphenicol cassette must be digested with various restriction endonucleases to determine which enzymes leave the chloramphenicol cassette intact. Since the plasmid pJHA1 now contained the 1.4 Kbp chloramphenicol cassette (Figure 3), it was mapped with the restriction endonucleases *Eco* RI, *Hind* III, *Pst* I, *Pvu* II, and *Xho* I. Restriction enzymes which left the chloramphenicol cassette intact appeared on an electrophoresed agarose gel as a single band: the circular plasmid being cut and linearized at a unique restriction site in pBR322 (Figure 1), the vector used in constructing pJHA1. The restriction endonucleases *Hind* III and *Pst* I digests of pJHA1 each revealed a single band indicating an intact chloramphenicol cassette (Figure 6: lanes 3, 5).

Genomic DNA from *Salmonella typhimurium* SR-11 Fad⁻ was digested separately with *Hind* III and *Pst* I, electrophoresed on 0.85% agarose gel, and blotted to a positively-charged nylon membrane. The membrane was hybridized with the 1.4 Kbp

chloramphenicol cassette DIG- labeled chemiluminescent probe and developed (see Materials and Methods). The blot revealed a *Hind* III fragment of approximately 8.0 Kbp (Figure 7: lane 6) and a *Pst* I fragment of approximately 4.2 Kbp (Figure 7: lane 7). The *Hind* III fragment should contain approximately 6.6 Kbp of *Salmonella typhimurium* SR-11 Fad⁻ genomic DNA flanking the chloramphenicol cassette. Likewise, the *Pst* I fragment should contain approximately 2.8 Kbp of *Salmonella typhimurium* SR-11 Fad⁻ genomic DNA flanking the chloramphenicol cassette. Equally important, the southern blot revealed that the virulent parent strain of *Salmonella typhimurium* SR-11 did not contain the chloramphenicol cassette (Figure 7: lanes 1, 5), as did the SR-11 Fad⁻ vaccine candidate strain.

Cloning of the *Hind* III and *Pst* I fragments of *Salmonella typhimurium* SR-11 Fad⁻ genomic DNA. Genomic DNA from the SR-11 Fad⁻ strain was isolated and digested with *Hind* III or *Pst* I . The vector pBR322 also was digested with *Hind* III or *Pst* I (Figure 1). Ligase reactions using a 3:1 insert to vector molar ratio were performed (see Materials and Methods). The ligation reactions were electroporated into *E. coli* HB101 competent cells. Transformants were selected on Luria agar plates containing chloramphenicol.

Plasmid DNA from two of the *Hind* III chloramphenicol resistant transformants was isolated, and the clones were characterized. Both clones were determined to be ampicillin-resistant and tetracycline-resistant. Also, both clones contained plasmids. These were designated *E. coli* HB101 (pJHA3) and *E. coli* HB101 (pJHA4) since they may or may not have the same *Hind* III insert orientation.

Subsequent mapping with the restriction endonuclease *Eco* RI revealed *E. coli* HB101 (pJHA3) as a spontaneous chloramphenicol mutation in the chromosome of *E. coli* HB101. The clone also contained the insertless, self-ligated vector pBR322. This insertless vector conferred the observed resistance to ampicillin and tetracycline.

Better results were observed with *E. coli* HB101 (pJHA4): mapping with *Eco* RI revealed three bands greater than 4.0 Kbp. One band represented the pBR322 vector with its unique *Eco* RI site (Figures 1). The *Eco* RI site in the 1.4 Kbp chloramphenicol resistance gene is 600 bp into the cassette (21). This suggested that fragments of SR-11 *Fad*^r genomic DNA at least 3.2 Kbp in size flanked the chloramphenicol cassette. However, the flanking genomic DNA was much larger than the ideal 1 Kbp (financial consideration), and pJHA4 was not sequenced.

Plasmid DNA from two of the *Pst* I chloramphenicol resistant transformants also were isolated, and the clones were characterized. Both clones were determined to be ampicillin-sensitive and tetracycline-resistant. This suggested an interruption of the ampicillin resistance gene in the vector pBR322 by the chloramphenicol cassette (Figure 1). These clones were designated *E. coli* HB101 (pJHA5) and *E. coli* HB101 (pJHA6) since they may or may not have the same *Pst* I insert orientation.

Subsequent mapping of the plasmid pJHA6 with *Bam* HI, *Eco* RI, and *Pst* I revealed it to be approximately 9.4 Kbp in size. The *Eco* RI digest of pJHA6 revealed two bands: approximately 6.9 Kbp and 2.5 Kbp (Figure 8: lane 2). The *Pst* I digest of pJHA6 revealed one tall band between approximately 4.2 Kbp and 4.5 Kbp (Figure 9: lane 3) representing the vector (pBR322 is 4.361 Kbp in size) and the *Pst* I insert; a

second band at approximately 600 bp was also observed, but due to its low intensity, is not visible in the duplicate photograph (Figure 9: lane 3). The *Bam* HI digest of pJHA6 showed three bands: approximately 5.7 Kbp, 2.3 Kbp, and a 1.4 Kbp band corresponding to the chloramphenicol cassette (Figure not shown). Five bands were observed with the *Bam* HI-*Pst* I double digest of pJHA6 (Figure 9: lane 5): approximately 3.2 Kbp, 1.9 Kbp, 1.4 Kbp, 1.1 Kbp and 0.6 Kbp (the 0.6 Kbp band is not visible in the duplicate photograph due to its low intensity). The bands at 1.1 Kbp appear to represent two bands: one on top of the other. Five bands also were observed with the *Eco* RI-*Pst* I double digest of pJHA6: approximately 3.6 Kbp, 2.7 Kbp, 1.7 Kbp, 0.8 Kbp and 0.6 Kbp (Figure 8: lane 4). Initially, the 600 bp fragment (a very low intensity band in 0.5% and 0.7% agarose gels) were puzzling because they were not observed when the plasmid pJHA6 was double digested with *Bam* HI and *Pst* I (Figure 9: lane 5). The answer was in the construction of pJHA6: the 5.1 Kbp *Pst* I insert was not isolated from a gel. Instead, SR-11 Fad⁻ genomic DNA was digested with *Pst* I and this digested DNA was used in the ligation reaction. The *Pst* I digest of SR-11 Fad⁻ genomic DNA either was incomplete or the small 600 bp fragment was simply ligated to the end of the fragment containing the chloramphenicol cassette (Figure 10).

Cloning the 4.5 Kbp *Pst* I fragment using a sequencing vector. Thirty-six micrograms of plasmid DNA from *E. coli* HB101 (pJHA6) was isolated, digested with *Pst* I, and electrophoresed on a 0.7% agarose gel. The 4.5 Kbp band was cut from the gel, purified, and ethanol-precipitated (see Materials and Methods). This removed the unwanted 600 bp artifact fragment (Figure 10). The 4.5 Kbp fragment (insert) was

ligated into the multiple cloning site of the cloning vector, pBluescript II SK + (Stratagene, La Jolla, CA) (Figure 11). The ligation reactions were electroporated into *E. coli* HB101 competent cells. Transformants were selected on Luria agar plates containing ampicillin and chloramphenicol. Three of the thousands of transformants were characterized and plasmid DNA from each was isolated from two of the clones. After preliminary mapping with *Bam* HI, *Eco* RI, and *Pst* I, one of the clones was designated *E. coli* HB101 (pJHA7).

Plasmid DNA from *E. coli* HB101 (pJHA7) was again isolated and digested with *Bam* HI, *Eco* RI, and *Pst* I. All the digests confirmed the size of the plasmid: approximately 7.4 Kbp (Figure 12). The *Bam* HI-*Eco* RI double digest of pJHA7 (Figure 12: lane 9) was identical the *Bam* HI-*Eco* RI-*Pst* I triple digest of pJHA7 (Figure 12: lane 10) since they are only several base pairs apart in the multiple cloning site of the sequencing vector pBluescript II SK + (Stratagene, La Jolla, CA) (Figure 11). Both digests of pJHA7 revealed five bands: approximately 2.9 Kbp, 2.0 Kbp, 1.1 Kbp, 0.8 Kbp and 0.6 Kbp (Figure 12: lanes 9, 10). These double and triple digests confirmed the remaining single and double digests of pJHA7 (Figure 12, lanes 3, 4, 5, 7, 8). The virulence gene(s) interrupted by the chloramphenicol cassette has been isolated. The chloramphenicol cassette is flanked by approximately 1.0 Kbp and approximately 1.9 Kbp fragments of *Salmonella typhimurium* SR-11 *Fad*⁻ genomic DNA (Figure 13).

Since primers for the T3 and T7 promoter sites (Figures 11, 13) in this sequencing vector have already been made, this step will facilitate the sequencing of the unknown gene(s). The plasmid, pJHA7, has been sent out for sequencing.

Discussion

Salmonella typhimurium SR-11 Fad⁻, the vaccine candidate, was phenotypically designated Fad⁻ (Fatty acid) for its inability to metabolize fatty acids as a sole carbon source (11). The SR-11 Fad⁻ strain also is unable to utilize acetate, citrate, and isocitrate as carbon sources, but can utilize glucose, glycerol, pyruvate, and succinate as sole carbon sources (11). Since SR-11 Fad⁻ can utilize succinate and pyruvate but not citrate and isocitrate, there appear to be at least two blocks in the tricarboxylic acid cycle (TCA): one prior to succinate and one in the glyoxylate bypass (11). Both blocks result in a reduction in the synthesis or activity of enzymes within the TCA cycle (11). These data suggest that the mutation is regulatory in nature.

S. typhimurium SR-11 Fad⁻ is a worthy live oral vaccine candidate since it is both avirulent and immunogenic in BALB/c mice (11) and chickens (Dr. Paul S. Cohen: personal communication). Since SR-11 Fad⁻ grows on several sugars subject to catabolite repression, it is not the same live oral vaccine candidate as those based on Δcya (adenylate cyclase) and Δcrp (cyclic 3', 5'-AMP receptor protein) mutations (4, 16). Furthermore, since SR-11 Fad⁻ grows on glucose supplemented minimal media without the addition of aromatic compounds, it is not same as live oral vaccine candidates based on $\Delta aroC$, $\Delta aroD$ mutations (11).

In order for a vaccine to be useful, it must be safe. Therefore it should not contain antibiotic resistance genes. Since the SR-11 Fad⁻ vaccine candidate was constructed by Tn10d::*cam* transposon mutagenesis (11), it contains a chloramphenicol resistance gene. This chloramphenicol cassette appears to interrupt the expression of a regulatory gene

necessary for the expression of virulence. The goal of this research was to isolate the gene(s) interrupted by the chloramphenicol cassette. This virulence gene(s) and the chloramphenicol resistance cassette could then be permanently deleted yielding a safer live oral vaccine candidate.

The first step toward that goal was to isolate the chloramphenicol cassette and use it as a probe in Southern hybridization analysis of genomic DNA. This step revealed the size of the restriction enzyme-digested DNA fragments containing the chloramphenicol cassette. The goal was reached when the 4.5 Kbp *Pst* I-digested fragment was cloned using the sequencing vector pBluescript II SK + (Stratagene, La Jolla, CA) (Figures 11, 13). The resulting plasmid, pJHA7, contains 1.0 Kbp and 1.9 Kbp fragments of SR-11 *Fad*⁻ genomic DNA flanking the chloramphenicol cassette (Figure 13). The flanking DNA is the virulence gene(s).

The virulence gene(s) in pJHA7 can now be sequenced (22) taking advantage of primers for the T3 and T7 promoters and the known sequences of the 70 bp inverted repeats flanking the chloramphenicol cassette (Figure 13). If the results of the sequencing reveal that the virulence gene(s) lies within a large operon, it may exhibit a polar effect on downstream genes in the operon (23). If this is the case, then the larger 8.0 Kbp *Hind* III fragment in the plasmid pJHA4 can be sub-cloned into the sequencing vector and the entire operon can be sequenced.

Either fragment of SR-11 *Fad*⁻ genomic DNA (1.0 Kbp or 1.9 Kbp) in the plasmid pJHA7 (Figure 13) can now be made into a probe. With this probe, other genera and species of intracellular bacteria such as *Salmonella choleraesuis* (swine pathogen),

Salmonella dublin (bovine pathogen), *Salmonella gallinarum* (poultry pathogen), *Escherichia coli*, and *Yersinia* can be screened for virulence gene(s) by Southern hybridization (19). If they contain the virulence gene(s), then other chloramphenicol resistant vaccine candidates (easily selected) can be made by homologous recombination of the 4.5 Kbp *Pst* I fragment utilizing an allelic exchange vector (24, 25).

Cárdenas and Clements (10) noted that, "The use of live attenuated *Salmonella* strains as delivery vectors of heterologous antigens to the secretory immune system constitutes a promising approach for the development of new vaccines against a number of diseases." It is now recognized that live oral *Salmonella* vaccines stimulate significant humoral and secretory antibody responses *en route* (10). Thus, the SR-11 Fad⁻ vaccine candidate shows great promise as a delivery vehicle of heterologous antigens.

Tables

Table 1. Bacterial strains used in this study.

Strain	Relevant Genotype	Relevant Phenotype
<i>Salmonella typhimurium</i> SR-11	<i>gyrA1816</i>	nalidixic acid resistant
<i>Salmonella typhimurium</i> SR-11 Fad ⁻	<i>gyrA1816</i> <i>Tn10d::cam</i>	nalidixic acid resistant chloramphenicol resistant
<i>Escherichia coli</i> HB101	<i>rpsL20</i>	streptomycin resistant

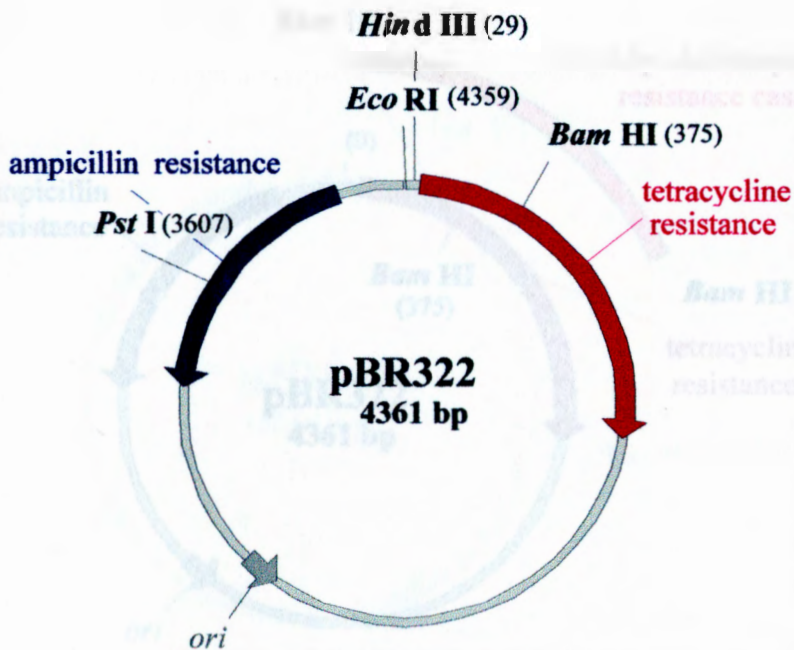


Figure 1. The unique *Bam* HI, *Eco* RI, *Hind* III, and *Pst* I restriction enzyme sites in the cloning vector pBR322. The blue section represents the ampicillin resistance gene (*bla*) and the purple section represents the tetracycline resistance gene (*tet*). The arrows designate the direction of transcription. The gray arrow depicts the origin and direction of replication. The numbers in parentheses denote base pairs of DNA.

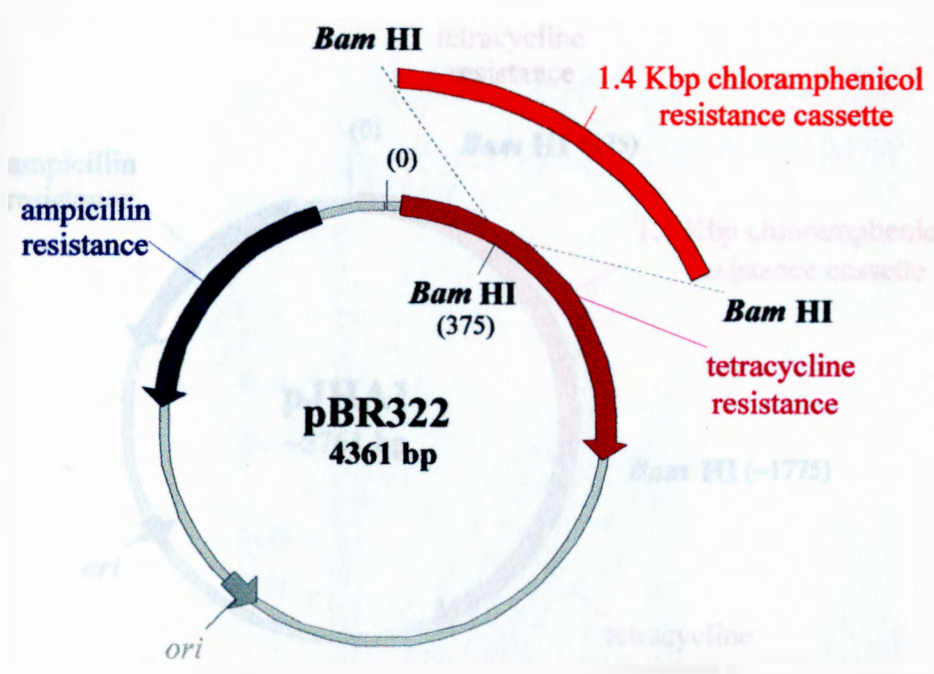


Figure 2. The construction of pJHA1. The *Bam* HI-digested chloramphenicol cassette (red) was ligated into the unique *Bam* HI restriction enzyme site in the cloning vector pBR322. The blue section represents the ampicillin resistance gene (*bla*) and the purple section represents the tetracycline resistance gene (*tet*). The arrows designate the direction of transcription. The gray arrow depicts the origin and direction of replication. The numbers in parentheses denote base pairs of DNA.

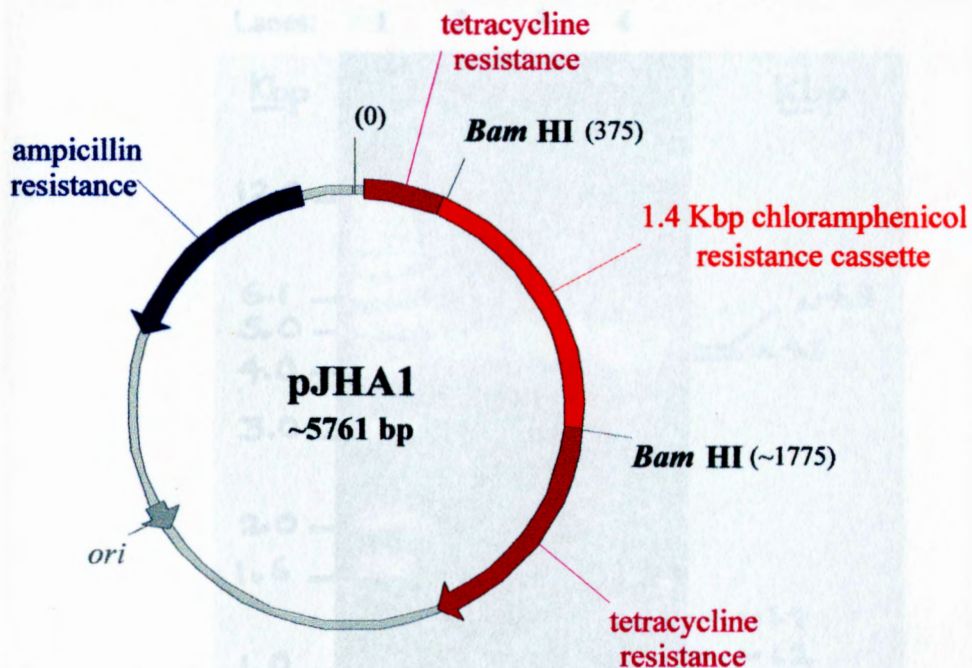


Figure 3. The interruption of the tetracycline resistance gene (purple) by the 1.4 Kbp chloramphenicol resistance cassette (red) in the plasmid pJHA1. The ampicillin resistance gene (blue) remains intact. The arrows designate the direction of transcription. The gray arrow depicts the origin and direction of replication. The numbers in parentheses, which denote base pairs of DNA, are approximate.

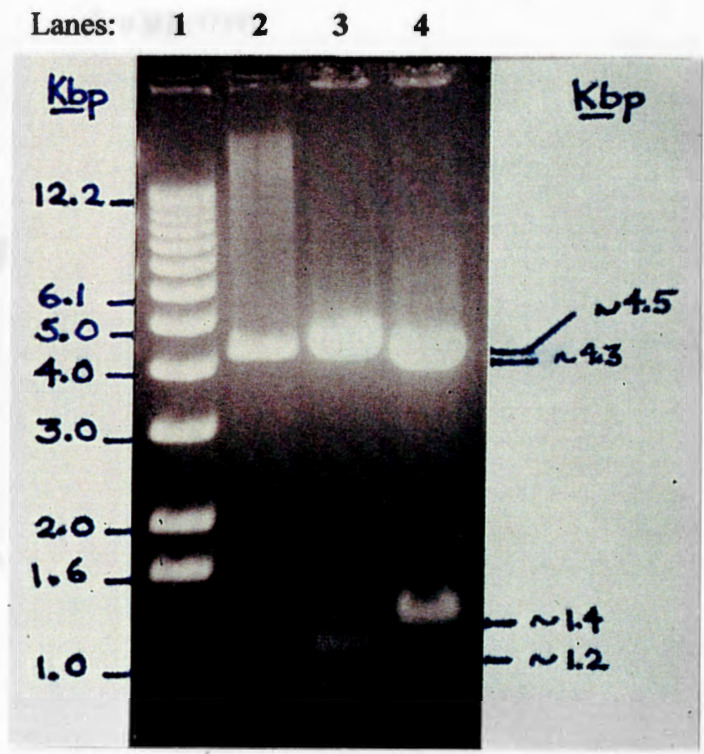


Figure 4. Agarose (0.9%) gel analysis of the plasmid pJHA1. Lane 1: 1 Kbp ladder; lane 2: *Eco* RI-digested pBR322; lane 3: *Eco* RI-digested pJHA1; lane 4: *Bam* HI-digested pJHA1.

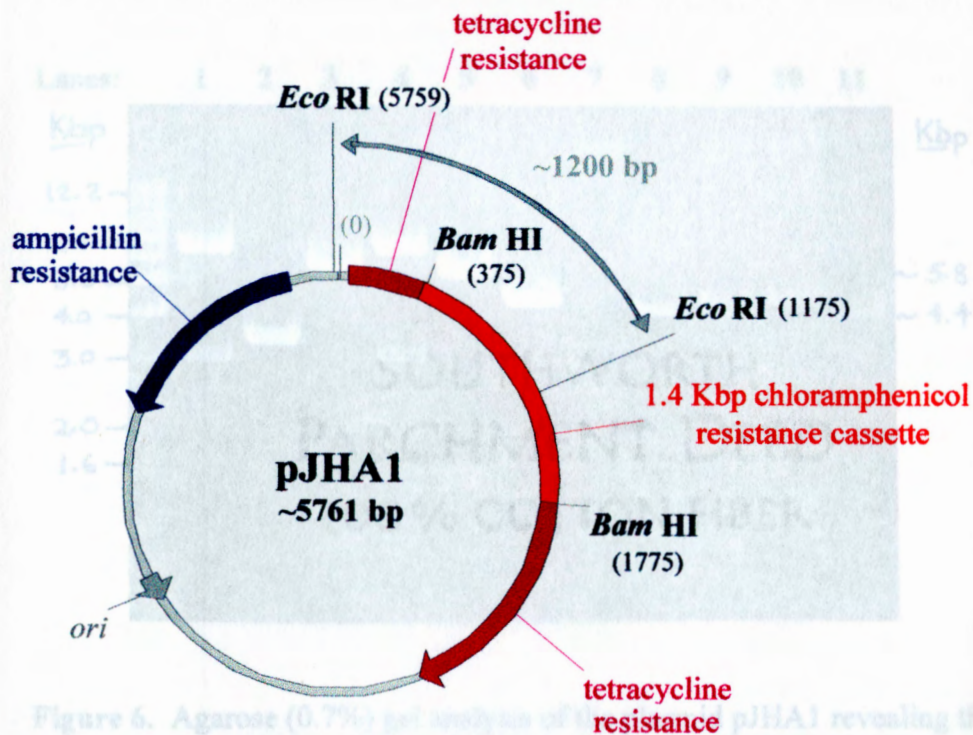


Figure 6. Agarose (0.7%) gel analysis of pJHA1 revealing the

Figure 5. The plasmid pJHA1 mapped with the restriction endonucleases *Bam* HI and *Eco* RI. The tetracycline resistance gene (purple) is interrupted by the chloramphenicol resistance cassette (red). The ampicillin resistance gene (blue) remains intact. The arrows in these genes designate the direction of transcription. The gray arrow (*ori*) depicts the origin and direction of replication. The numbers in parentheses, which denote base pairs of DNA, are approximate.

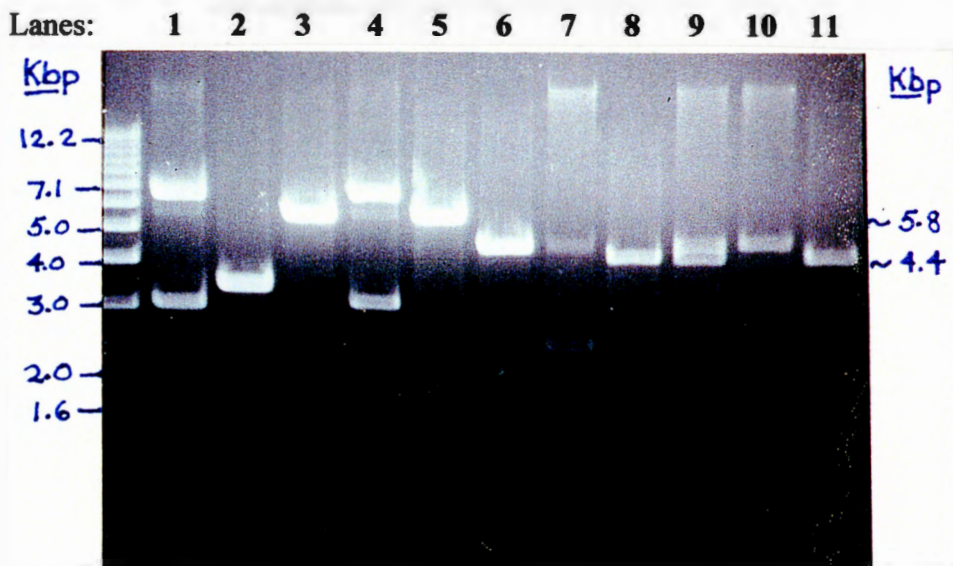


Figure 6. Agarose (0.7%) gel analysis of the plasmid pJHA1 revealing the unique *Hind* III and *Pst* I restriction enzyme sites (lanes 3, 5). Lanes 7-11 are control digests to determine and/or confirm unique restriction endonuclease sites in the vector pBR322. Unique sites (Lanes 3, 5, 8, 9, 11) are represented by single a band of linearized DNA. Lane 1: undigested pJHA1; lane 2: *Pvu* II-digested pJHA1; lane 3: *Hind* III-digested pJHA1; lane 4: *Xho* I-digested pJHA1 (either pJHA1 does not contain an *Xho* I restriction site or this was an ineffective digest); lane 5: *Pst* I-digested pJHA1; lane 6: *Eco* RI-digested pJHA1 (a band at approximately 1.2 Kbp was also observed, but due to its low intensity, it is not visible in this duplicate photograph); lane 7: undigested pBR322; lane 8: *Pvu* II-digested pBR322; lane 9: *Hind* III-digested pBR322; lane 10: *Xho* I-digested pBR322 (pBR322 does not contain an *Xho* I restriction site); lane 11: *Pst* I-digested pBR322.

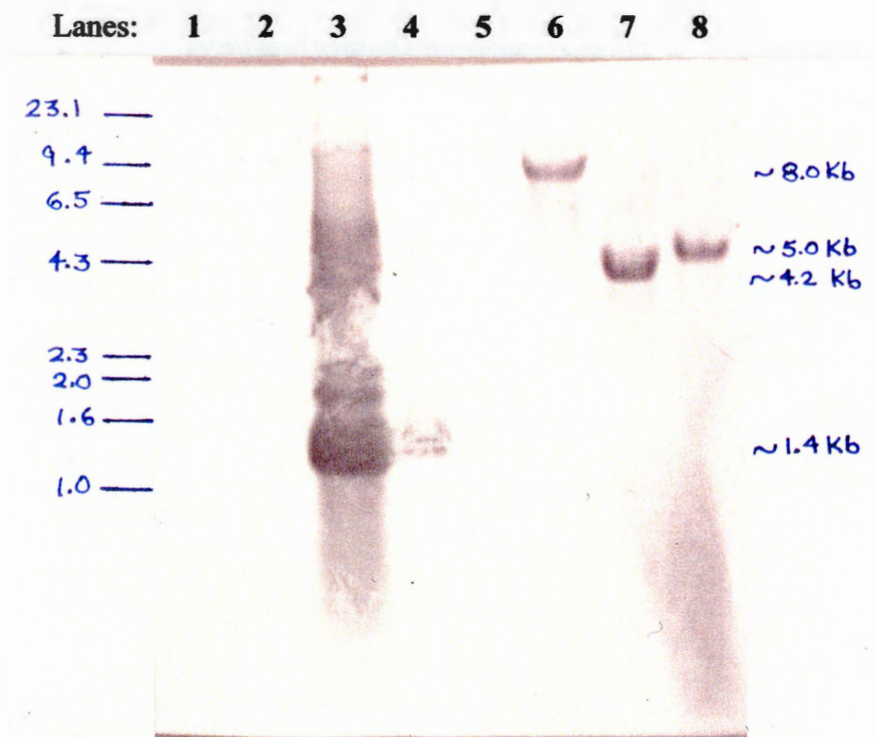


Figure 7. Southern hybridization analysis of *Salmonella typhimurium* SR-11 genomic DNA using the 1.4 Kbp chloramphenicol cassette as a probe. Lane 1: undigested SR-11 wild-type DNA; lane 2: undigested SR-11 Fad^- DNA; lane 3: *Bam* HI-digested pJHA1; lane 4: *Bam* HI-digested SR-11 Fad^- DNA; lane 5: *Bam* HI-digested SR-11 wild-type DNA; lane 6: *Hind* III-digested SR-11 Fad^- DNA; lane 7: *Pst* I-digested SR-11 Fad^- DNA; lane 8: *Eco* RI-digested SR-11 Fad^- DNA.

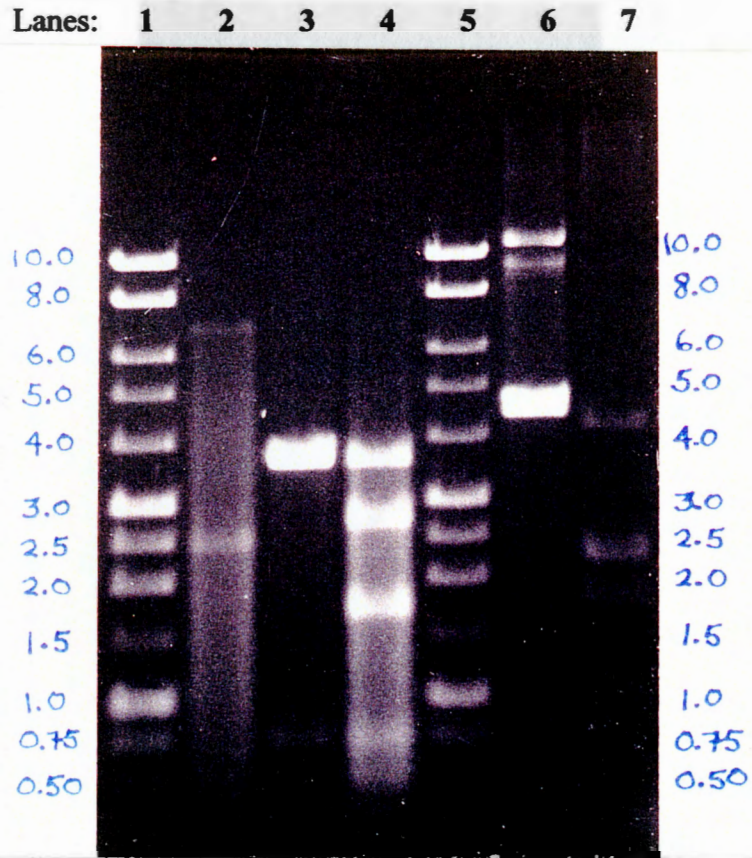


Figure 8. Agarose (0.5%) gel analysis of the plasmid pJHA6. Lane 1: 1 Kbp ladder; lane 2: *Eco* RI-digested pJHA6; lane 3: *Eco* RI, *Pst* I-digested pBR322; lane 4: *Eco* RI, *Pst* I-digested pJHA6; lane 5: 1 Kbp ladder; lane 6: undigested pJHA6; lane 7: undigested pBR322.

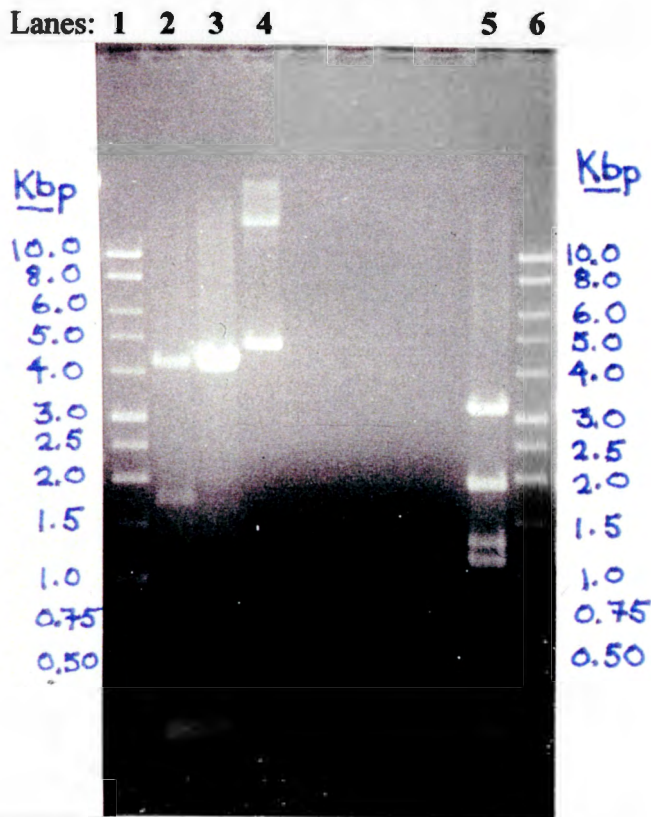


Figure 9. Agarose (0.7%) gel analysis of the plasmid pJHA6. Lane 1: 1 Kbp ladder; lane 2: *Pst* I-digested pBR322 (the band at approximately 1.7 Kbp represents an irreversibly-denatured form of the vector pBR322); lane 3: *Pst* I-digested pJHA6 (a band at approximately 600 bp was also observed, but due to its low intensity, it is not visible in this duplicate photograph); lane 4: undigested pJHA6; lane 5: *Bam* HI, *Pst* I-digested pJHA6 (a band at approximately 600 bp was also observed, but due to its low intensity, it is not visible in this duplicate photograph); lane 6: 1 Kbp ladder.

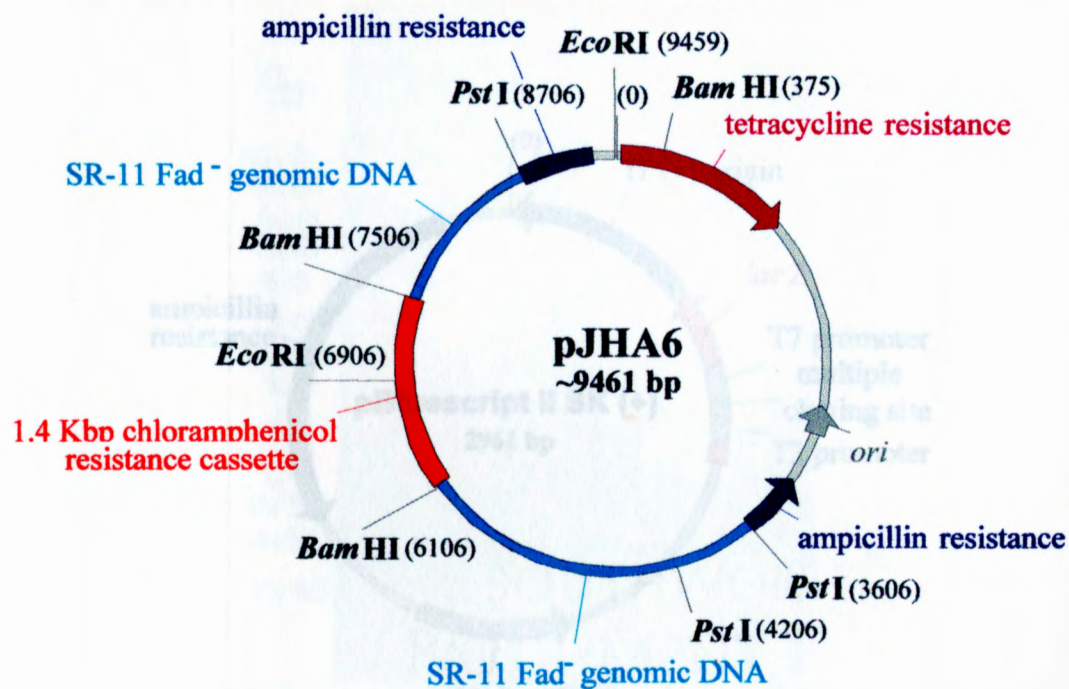


Figure 10. The plasmid pJHA6 mapped with the restriction enzymes *Bam* HI, *Eco* RI, and *Pst* I. The ampicillin resistance gene (blue) is interrupted by the 4.5 Kbp *Pst* I insert and a 600 bp *Pst* I artifact. The tetracycline resistance gene (purple) remains intact. The arrows designate the direction of transcription. The gray arrow depicts the origin and direction of replication. The numbers in parentheses, which denote base pairs of DNA, are approximate.

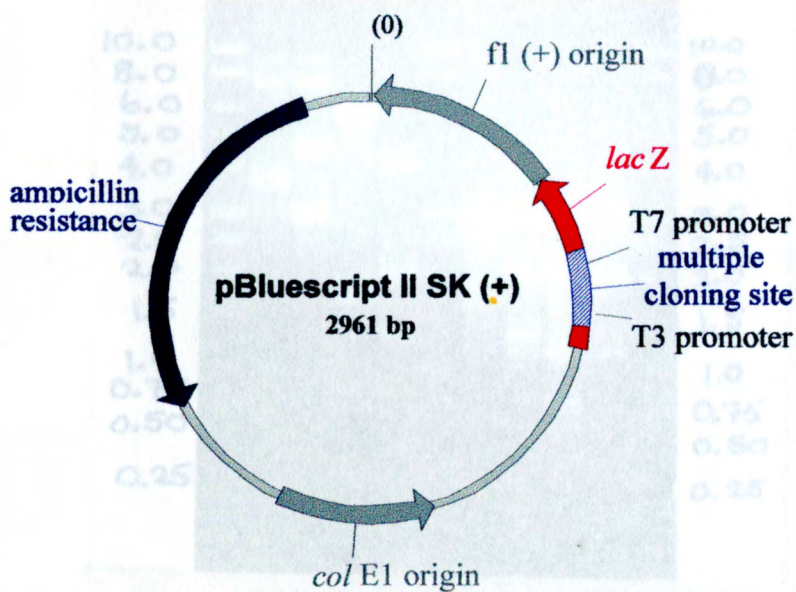


Figure 11. Map of the sequencing vector pBluescript II SK + (Stratagene, La Jolla, CA). The multiple cloning site contains the *Bam* HI, *Eco* RI, and *Pst* I restriction endonuclease sites. The blue arrow represents the ampicillin resistance gene (*bla*) and its direction of transcription. Two origins of replication (gray arrows) are included in this sequencing vector. The *lac Z* gene is represented by the fuchsia arrow.

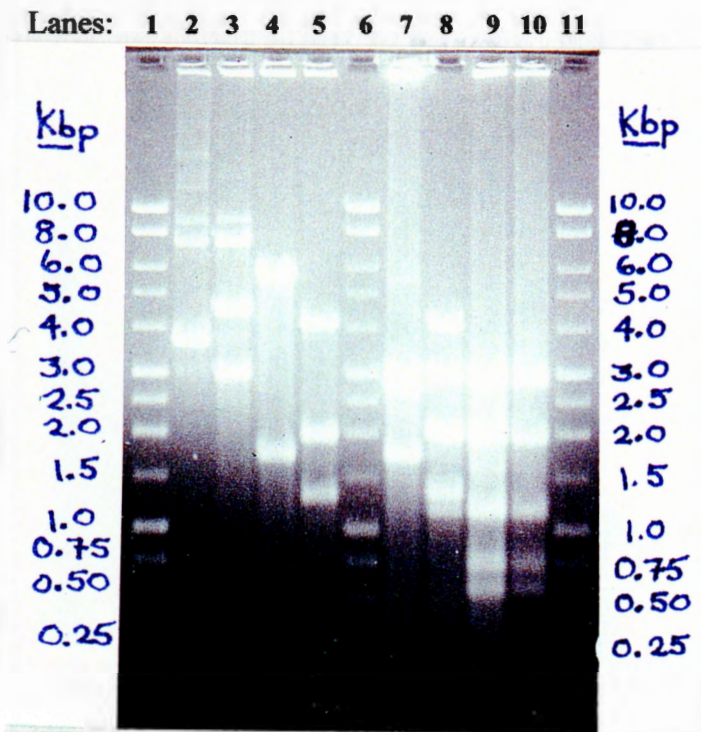


Figure 12. Agarose (0.7%) gel analysis of the plasmid pJHA7. Lane 1: 1 Kbp ladder; lane 2: undigested pJHA7; lane 3: *Pst* I-digested pJHA7 (the upper two bands reveal that this digest was incomplete); lane 4: *Eco* RI-digested pJHA7; lane 5: *Bam* HI-digested pJHA7; lane 6: 1 Kbp ladder; lane 7: *Eco* RI, *Pst* I- digested pJHA7; lane 8: *Bam* HI, *Pst* I-digested pJHA7 (the upper band at approximately 4.1 Kbp reveals that this digest was incomplete); lane 9: *Bam* HI, *Eco* RI-digested pJHA7; lane 10: *Bam* HI, *Eco* RI, *Pst* I-digested pJHA7; lane 11: 1 Kbp ladder.

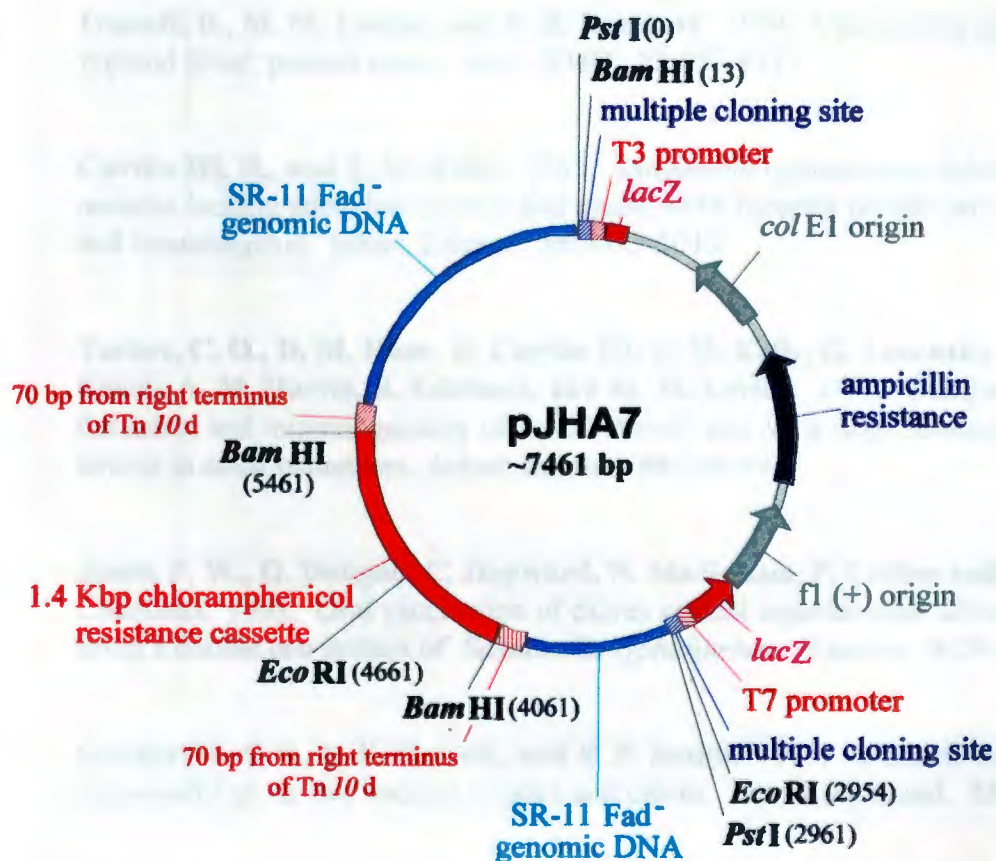


Figure 13. The plasmid pJHA7 mapped with the restriction enzymes *Bam* HI, *Eco* RI, and *Pst* I. The ampicillin resistance gene (blue arrow) remains intact. The sequences of the T3 and T7 promoters (orange-brown diagonals) and the 70 bp inverted repeats (orange-brown diagonals) flanking the chloramphenicol cassette (red) are known. The numbers in parentheses, which denote base pairs of DNA, are approximate.

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