**GALL FORMATION BY *ERWINIA*
SPECIES ON DOUGLAS-FIR**

BY

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ABSTRACT

Bacterial galls on Douglas-fir (*Pseudotsuga menzeisii* [Mirb.] Franco), collected from the southern tip of Vancouver Island, the Greater Vancouver area and the Hope region of British Columbia, were generally globose in shape with rough, irregular surfaces and measured between 0.5 and 2.0 cm in diameter. The galls were generally located on the tips of branches or twigs of 10- to 20-year old Douglas-fir trees.

The bacterial gall disease appeared to affect few Douglas-fir trees in the collection areas and bacterial galls were not found on any other coniferous species. Furthermore, there have been no reports of serious damage to natural forests in British Columbia due to bacterial gall disease. Young, greenhouse-grown Douglas-fir seedlings occasionally died if the tip of the main stem was artificially inoculated. Often new growing tips would be produced affecting the growth form of the seedlings.

Two types of gall-forming *Erwinia* spp. were isolated from Douglas-fir galls. Typical isolates, tentatively identified by fatty acid analysis as *Erwinia salicis*, produced galls which were rough and irregular in shape composed of multiple outgrowths marked by a single or cross-shaped fissure. The atypical isolate, tentatively identified by fatty acid analysis as *Erwinia herbicola* subsp. *herbicola*, produced galls which were smooth and generally round in shape with the surface cracking as the gall expanded.

Colonies of the typical isolates grown on casein-peptone-glucose media were characteristically round, slightly domed with

somewhat concentric ridging observed near the margins of the colonies. Three to 4 day old colonies of the atypical isolates grown on casein-peptone-glucose media were characteristically round and concave while older colonies produced an extracellular slime and were more irregular in shape. In Luria Broth, the typical isolates grew at temperatures of up to 32°C while the atypical isolate grew at temperatures of up 34°C. The typical isolate was resistant to a wider range of antibiotics than the atypical isolate.

Polyclonal antisera were produced against glutaraldehyde-fixed whole cells of both the typical T-2789 and atypical A-0181 gall-forming *Erwinia* isolates. The purified antisera were isolate specific as tested by immunodiffusion and an indirect ELISA against several different phytopathogenic bacteria including *Pseudomonas syringae* pv. *syringae, Erwinia herbicola* subsp. *herbicola, Agrobacterium tumefaciens, Rhizobium leguminosarum* and *Erwinia carotovora* subsp. *carotovora.*

Plasmid profiles of the typical *Erwinia* isolates contained one band while the atypical isolate characteristically contained 4 to 5 bands which appeared to be different forms of at least one plasmid. Restriction digests of the typical isolates suggested a size of approximately 50 kb while complex digestion profiles were obtained for the atypical isolates because of the difficulty in isolating individual plasmid types. From visual estimates against HindIII-digested lambda DNA, a size of between 10 and 20 kb was suggested for the fastest moving plasmid band of the atypical isolate. No homology was observed between the different plasmid types characteristic of the two isolates. The

role of the plasmid DNA of the atypical isolate in pathogenesis was not determined because curing of the plasmid(s) was not successful using high temperature treatments plus chemical curing agents.

Heat treatment experiments, in which the pathogen was selectively killed at various times after inoculation, demonstrated that the bacteria are required to be present for gall induction and continued development of the gall for both of the gall-forming *Erwinia* isolate types.

Pathogenicity of the isolated bacteria was tested on 14 conifer species, other than Douglas-fir, including *Abies*, *Chamaecyparis*, *Pinus* and *Thuja* spp. The typical isolates were weakly pathogenic on *Abies*, *Larix* and *Picea* spp. The atypical isolate was weakly pathogenic on *Abies*, *Chamaecyparis*, *Larix*, *Picea* and *Pinus* spp. Due to the limited damage caused on the conifers tested and to their infrequent occurrence, these gall-forming pathogens do not appear to be of economic importance to the forestry industry.

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GENERAL INTRODUCTION

The forest industry in B.C., and Canada as a whole, is now reaching a critical period in terms of maintaining its competitive position internationally. The forests of Canada are the nation's most valuable natural asset with approximately one million families depending directly or indirectly on the forest industry for their livelihood (Reed, 1989). World consumption of industrial roundwood is growing at an average of 2.5% annually and for Canada to keep its share of the market, it must increase the growth and yield of its forests while maintaining the high quality and low cost of the raw material.

Genetic improvement of conifers must be carried out, in conjunction with reforestation, to provide high-quality, faster-growing trees. A study of the productivity of some Douglas-fir (*Pseudotsuga menziesii* [Mirb.] Franco) and loblolly pine (*Pinus taeda* L.) plantations showed that silvicultural and genetic manipulation has increased their productivity 70 and 300%, respectively, in comparison to natural growth forests (Reed, 1989). Research into genetic improvement of plants in general has centered on the use of the Ti plasmid of *Agrobacterium tumefaciens* (Smith and Townsend, Conn.), the causal agent of crown gall, as a cloning vector (Barton and Chilton, 1983; Fraley *et al.*, 1983). The studies to date have mostly involved dicotyledonous plants as the recipients although some monocots and gymnosperms are within the wide host range of *Agrobacterium* (De Cleene and De Ley, 1976). Recently the transformation of several conifer species such as white spruce (*Picea glauca*

(Moench) Voss), Englemann spruce (*Picea englemannii* Parry ex Engelm.) and Sitka spruce (*Picea sitchensis* (Bong.) Carr) as well as loblolly pine by *Agrobacterium* has been shown to occur (Ledig, 1985; Sederoff *et al.*, 1986; Dandekar *et al.*, 1987; Ellis *et al.*, 1989).

Douglas-fir, native to the coast and interior of B.C., has also been shown to be susceptible to *A. tumefaciens* (De Cleene and De Ley, 1976). However, *A. tumefaciens*, is not commonly found as a natural pathogen of Douglas-fir. Therefore, it would be of interest to investigate other gall-forming bacterial pathogens of Douglas-fir with regard to their mechanism of gall formation and their potential for harboring a plasmid with capabilities similar to the *Agrobacterium* Ti plasmid.

Galls on conifers can be caused by either fungi, insects, bacteria, mistletoes or environment-related stresses. One of the gall-forming fungal pathogens is *Endocronartium harknessii* (J.P. Moore) Y. Hirat, the causal agent of Western gall rust on such hosts as lodgepole and ponderosa pines (Sinclair *et al.*, 1987). Forms of *Cronartium quercuum* (Berk.) Miy. ex Shirai cause Pine-oak gall rust on different pine species such as loblolly, ponderosa and Scots pine. Conifer galls can also be caused by insect pests such as the Cooley Spruce gall adelgid (Sinclair *et al.*, 1987). A review of the literature on gall diseases on conifers in general, reveals very few reports of bacterial incitants. The first published report of a bacterial gall disease on conifers was in 1888 by Vuillemin (cited by Hansen and Smith, 1937). The author isolated bacteria from galls located on the twigs, branches and upper stem of *Pinus*

halepensis Mill. (Aleppo pine). A similar organism was isolated from galls on *Pinus cembra* L. (Swiss stone pine) in 1911 by von Tubeuf. The first published report of a bacterial gall disease on Douglas-fir was in 1933 by Hansen and Smith in California (Hansen and Smith, 1933).

Agrobacterium tumefaciens and *A. pseudotsugae* (Hansen and Smith 1937) Savulescu 1947 are the only two bacterial phytopathogens described as causing galls on conifers, more specifically, Douglas-fir (De Cleene and De Ley, 1976; Hansen and Smith, 1937). The work by Hansen and Smith on *A. pseudotsugae* has not been confirmed and the isolated pathogen is not even listed in the most recent version of Bergey's Manual of Determinative Bacteriology (Krieg and Holt, 1984). In addition to the small number of pathogens causing gall formation on conifers, the frequency of occurrence of these phytopathogens is very restricted (R.S. Hunt, *unpublished*).

Galls attributed to bacteria have been reported on Douglas-fir from southern Arizona to B.C. and Alberta (Sinclair *et al.*, 1987). There have been over 52 reports of galls of suspected bacterial origin in B.C. since 1946, although extensive damage to the hosts was not observed (R.S. Hunt, *unpublished*). Samples of a gall disease on Douglas-fir have been collected in the southwestern corner of British Columbia. The disease was suspected to be of a bacterial origin due to the different symptoms in relation to those associated with the well-documented fungal galls. A strain of bacteria was isolated from these galls in the mid 1980's (Muehlchen, 1985). Several Douglas-fir gall specimens were collected or received since that

time.

One of the first steps to be taken when working with a relatively unknown phytopathogen, such as the Douglas-fir gall-forming bacterium, is to choose a method for the detection and/or identification of the organism in mixed culture or in plant material. There are many methods of identification or detection of phytopathogenic agents such as selective media (Meneley and Stanghellini, 1976), serological assays (Miller and Martin, 1988), plasmid profiles (Morales and Sequeira, 1985), DNA:DNA hybridization using either chromosome (Thompson *et al.*, 1989) or plasmid probes (Gilbertson *et al.*, 1989) and genome and/or plasmid fingerprinting (Cooksey and Graham, 1989). The method used depends upon facilities and funds available, number of samples to be processed, degree or level of specificity desired and time available.

The serological detection and identification of phytopathogenic bacteria is widely used as a tool in the research laboratory. Serology takes advantage of the recognition of antigenic determinants on the pathogen that are often characteristic of the species. The first report of the use of a serological test to identify a plant pathogen was in 1918 by Jensen working with *Agrobacterium tumefaciens* (cited by De Boer, 1987). Infectious agents in diseased tissue, even when in low concentration or in a latent phase, can be detected with serological assays (Miller and Martin, 1988).

For the Douglas-fir gall-forming bacterium to be considered as a gene vector, the pathogen's mechanism of gall formation must involve a transfer of genes to the host plant genome, a

transformation event, such as that which occurs in the *A. tumefaciens* system. The *A. tumefaciens* genes that are integrated into the host plant genome encode for enzymes involved in the production of phytohormones (Watson *et al.*, 1975; Weiler and Spanier, 1981). Once the transfer of the bacterial genes to the plant cells occurs, the presence of the pathogen is no longer required for the continued development of the galls (Chilton *et al.*, 1977). The bacterial genes are transcribed and translated as host DNA and effect the growth of gall tissue.

Transformation of host plant cells does not occur in the *Pseudomonas syringae* pv. *savastanoi* ([Smith 1908] Young, Dye & Wilkie 1978) system. Rather, *P. syringae* pv. *savastanoi*, the causal agent of olive and oleander knot, produces tumors on its host plants through the production of phytohormones by the bacterium itself. The phytohormones cause hypertrophy, hyperplasia and vascular differentiation of the host cells surrounding the invading bacterium (Smidt and Kosuge, 1978). Therefore, the presence of the bacterium is required for initiation as well as continued development of the tumor.

Although gall formation by the two phytopathogens arises in the host plants by different mechanisms, the basis of gall formation is similar in both systems. The production of phytohormones, namely auxins and cytokinins, is required for the initiation and development of the plant tumors (Weiler and Spanier, 1981; Comai *et al.*, 1982). Auxins, such as indole acetic acid (IAA), regulate cell expansion and elongation while cytokinins, such as trans-zeatin, regulate cell division (Davis

et al., 1985). Overproduction of these phytohormones in combination leads to the unregulated growth and division of host plant cells and hence to the production of a tumor or gall. The actual site of production of the phytohormones distinguishes between the *A. tumefaciens* and *P. syringae* pv. *savastanoi* systems.

Whether or not the mechanism of gall formation by the Douglas-fir gall-forming bacterium involves a transfer of genes from a plasmid to the host plant cells, as occurs in the crown gall system, the role of any plasmid DNA in pathogenesis remains an interesting question. Plasmids are autonomously replicating extrachromosomal pieces of DNA. Plasmids contain, on average, about 2% of the bacterial chromosome but can constitute up to a third of the genome (Coplin, 1989). Many phytopathogenic bacteria, including species in the genera, *Agrobacterium*, *Clavibacter*, *Erwinia*, *Pseudomonas*, and *Xanthomonas*, are known to carry one or several plasmids.

Plasmids encode for many different phenotypic characteristics which are often nonessential yet advantageous to the bacterium, including antibiotic resistance, metal degradation, nitrogen fixation, conjugation, the production of bacteriocins and restriction enzymes (Trevors, 1985). Most of the plasmids identified in plant pathogenic bacteria are still labelled as cryptic or have no known function (Coplin, 1989). However, in both the *A. tumefaciens* and *P. syringae* pv. *savastanoi* systems, genes involved in the production of phytohormones, and hence pathogenicity, have been located on plasmids (Liu and Kado, 1979; Comai *et al.*, 1982). Furthermore,

genes involved in host specificity have been located on plasmids of phytopathogenic bacteria (Coplin, 1989).

The focus of this study on the Douglas-fir gall-forming bacteria was not their role as phytopathogens, but rather whether or not the bacteria carried plasmids that could serve as potential gene vectors for the genetic engineering of conifers. The shift in research interest of bacterial gall formers is due in part to the results of extensive studies of the phytopathogenic gall formers, *A. tumefaciens* and *P. syringae* pv. *savastanoi*. More specifically, the successful use of the Ti plasmid of *A. tumefaciens* as a vector for introducing resistance genes into economically important plants has become an important research tool (Barton and Chilton, 1983; Chilton, 1983). Therefore the isolation of a relatively unknown bacterial gall former prompted investigation into its potential economic value as an alternate vector to the Ti plasmid of *Agrobacterium tumefaciens*.

When considering the scant amount of information on bacterial gall formers on Douglas-fir and the focus of this study, the specific objectives were the following:

1) to describe the galls produced, isolate the causal agent and produce antisera against the gall-forming pathogens;

2) to determine if the mechanism of gall formation involves the transformation of host plant cells;

3) to determine if there is a correlation between plasmid presence and pathogenicity.

CHAPTER 1

ISOLATION, DESCRIPTION AND

SEROLOGICAL DETECTION OF THE DOUGLAS-FIR

GALL-FORMING BACTERIA

INTRODUCTION

The Douglas-fir gall disease, reported by Hansen and Smith (1937), in some instances suppressed growth and caused dieback of the host tree. The affected Douglas-fir trees were located in areas where the health of the host was already compromised such as in damp areas by streams or ponds. The smooth, globose galls formed on the twigs, branches and upper main stems of young Douglas-fir trees. The galls measured between one millimeter to several centimeters in diameter. A characteristic cross-shaped marking was present across the face of the galls. Galls occurring on the main stem of young Douglas-fir seedlings can cause deformation and even death of the seedling. The bacterium was not pathogenic on any of the conifers tested, including *Pinus halepensis* Mill., *P. lambertiana* Dougl., *Pinus radiata* Don. and *Tsuga heterophylla* (Raf.) Sarg. (Hansen and Smith, 1937), except for *Pseudotsuga macrocarpa* (Vasey) Mayr (Smith, 1940). The bacterium was determined to be highly specific to *Pseudotsuga* spp. *Adelges cooleyi* (Gillette), the Cooley spruce gall adelgid, was suggested as an insect vector of the bacterium.

The structure of the Douglas-fir gall was likened to that

of galls produced by *Pseudomonas syringae* pv. *savastanoi*, in which the galls are composed of groups of rapidly dividing cells. The Hansen and Smith bacterium was located in relatively large intercellular spaces in the centers of these groups of cells. The isolated gall bacterium was determined to be a Gram-negative, non-motile, facultative aerobe (more appropriately termed facultative anaerobe using current terminology). It was named by Hansen and Smith as *Bacterium pseudotsugae* (1937) and later renamed to *Agrobacterium pseudotsugae* (Salvulescu, 1947). The work done on *A. pseudotsugae* has never been confirmed.

The first step in the study of any 'new' disease is the description of the disease symptoms on the host plant. The identification and description of the pathogen through biochemical tests, which is a separate study in itself, was not considered a priority in this study because the major aim was to investigate the mechanism of gall formation. Classification of the bacterial isolates using a fatty acid analysis system (Hewlett Packard Microbial Identification System) was regarded as suitable for the context of this research. Fatty acid analysis is touted as being very reliable for identification purposes, due to the genetic stability of the fatty acids (Anonymous, 1985; Stead, 1988). In this system, comparisons of high resolution gas chromatographic analyses of the cell wall fatty acids were made with a computer library of profiles from various known bacterial species and the outcomes were recorded as similarity indices (Miller, 1984; Stead, 1988). A tentative identification by the fatty acid analysis system in addition to visual observations allowed for comparison to other gall-forming

phytopathogens.

Some of the recognized phytopathogenic bacteria that cause galls on their host plants include *Agrobacterium tumefaciens*, *Pseudomonas syringae* pv. *savastanoi* and *Erwinia herbicola* f.sp. *gypsophilae* (Brown) Miller, Quinn and Graham 1981. *A. tumefaciens* is the causal agent of crown gall which affects many dicots, monocots and gymnosperms. Galls form on roots and stems especially at the crown area or base of the stem (Sinclair *et al.*, 1987). *Pseudomonas syringae* pv. *savastanoi* is the pathogen that is responsible for olive and oleander knot as well as bacterial knot on ash. The major symptoms include galling or knot formation as well as dieback of twigs and branches (Sinclair *et al.*, 1987).

Finally, crown and root galls on *Gypsophila paniculata*, resembling those caused by *A. tumefaciens*, were first described in the 1930's. The isolated pathogen was originally named *Bacterium gypsophilae* but has since been reclassified as *Erwinia herbicola* f.sp. *gypsophilae* (Miller *et al.*, 1981; Cooksey, 1985). *E. herbicola* is also reported to cause galling symptoms on roses and carnations (Maas Geesteranus and Barendsen, 1966). Galls are usually formed at the cut end of rooted cuttings or at graft unions when below the soil line. Little work however has been done on this gall-forming strain of *E. herbicola* in relation to the mechanism of gall formation. However, a recent study done on the mechanism of gall development by *E. herbicola* f.sp. *gypsophilae*, suggests a mechanism similar to *P. syringae* pv. *savastanoi* (Clark *et al.*, 1989). Tentative identification of the Douglas-fir gall-forming bacteria can aid, by comparisons

with other gall-forming bacteria, in the understanding of the
disease cycle and in solving the question of the mechanism of
gall formation.

The production of polyclonal antisera against bacterial
pathogens has been used for their detection and identification.
Both polyclonal antisera and monoclonal antibodies have been
produced against such bacteria as *Erwinia carotovora*,
Pseudomonas syringae, *Corynebacterium sepedonicum* and
Xanthomonas spp. (De Boer, 1982; Allan and Kelman, 1977;
Civerolo and Fan, 1982; Benedict *et al.*, 1989). Polyclonal or
monoclonal antibodies can be produced against the bacterial
antigens depending upon the facilities available and the
specificity required (De Boer, 1987). Serological assays
involving polyclonal antisera are fairly specific for most
detection purposes.

As a heterologous mixture of antibodies, polyclonal
antisera often is criticized as not being very specific towards
the immunogen. This problem can be alleviated partially by
careful purification and preparation of immunogen. Isolation of
the immunoglobulin G (IgG) fraction also can increase the
specificity of the antiserum (Miller and Martin, 1988). The
specificity of the antiserum must be tested before routine use
in qualitative and quantitative assays.

The measurement of specificity of antisera depends upon the
serological method employed and the number of organisms tested
(De Boer, 1982). Ouchterlony double diffusion or
immunodiffusion was chosen as one of the techniques to test the
specificity because it allows the visualization of relationships

between antigens (De Boer *et al.*, 1979). The second technique chosen was an indirect ELISA as a modification of this technique was to be used in the experiments as outlined in Chapter 2.

Many different serological assays have been used to detect bacteria including latex agglutination, Ouchterlony double diffusion or immunodiffusion, immunofluorescence and immunoelectron microscopy (De Boer, 1982; Briansky *et al.*, 1982; da Roche *et al.*, 1986; Benedict *et al.*, 1989). The enzyme-linked immunosorbent assay (ELISA) has become a widely used assay for both qualitative and quantitative detection of phytopathogens including viruses, mycoplasma-like organisms, fungi and bacteria. ELISA was first introduced as a serological assay in the area of plant pathology in 1976 (Clark and Adams, 1977) for the detection of plant viruses.

There are a seemingly infinite number of variations of the basic ELISA procedure. Variations arise in the type of solid-support used (Lazarovits *et al.*, 1987), the times of incubation, the number and sources of antibodies used in a single assay and the presence or absence of the coating antibody step. An enrichment step occasionally is used as the initial step of the assay to increase the bacterial cell concentration in the test sample. This modification is an attempt to increase the level of detection (Béguin *et al.*, 1984). Such a modification was used for the detection of live bacteria in heat-treated, inoculated branches as outlined in Chapter 2.

The objectives of these experiments were to firstly describe the Douglas-fir galls collected from various locations in B.C. The second objective was to describe the bacterial

incitants of these galls and to obtain a tentative
identification through the use of fatty acid analysis. A third
objective was the production of polyclonal antisera against the
gall-forming bacteria to facilitate detection and identification
of inoculated bacteria in the host tissues.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions:

The gall-forming bacterial isolates all originated from Douglas-fir galls collected from four locations in southwestern British Columbia. Two sites were on the southern tip of Vancouver Island at the edges of Lorna Lake and Cordova Bay near Victoria. One site was in the Dunbar area of greater Vancouver and the final site was in the Hope region (Table 1.1). Except for the Hope site, in which Douglas-fir was the dominant species, these areas were marginal growth areas for Douglas-fir, either in mixed forests or in urban areas.

The bacteria were grown on 0.01% casein hydrolysate, 0.5% peptone (Difco), 0.5% glucose (BDH) (CPG media; Kelman, 1954) pH 7.0 plates for quick identification of the characteristic growth of the isolates. Nutrient agar (Difco) + 1% glucose (NA/G media) pH 7.0 plates were used occasionally if a shorter growth period was required. A temperature of 21-23°C was used for growth of the cultures. Broth cultures were grown either in Luria broth (LB) (1.0% Tryptone [Difco], 0.5% Yeast Extract [Difco], 0.8% NaCl) pH 7.0 (Maniatis *et al.*, 1982) or Nutrient Broth (NB) (Difco) pH 7.0 at room temperature or in 21°C growth chambers on rotary shakers (150 rpm). Antibiotic resistances were tested through growth of the isolate(s) on NA/G media containing filter-sterilized antibiotics.

Bacterial isolates, A-0181, T-2739, T-2763 and T-2789, were sent away to be identified by Microbial ID, Inc., Newark,

Table 1.1. Gall-forming *Erwinia* isolates and bacterial species used to test the specificity of polyclonal antisera

Bacterial species	Isolated by	Geographic location	Growth media[a]
Family Enterobacteriaceae			
Erwinia isolates			LB
(gall-forming)			CPG
Typical isolates			
T-2721	Muehlchen, UBC	Cordova Bay	
T-2722	Muehlchen, UBC	Cordova Bay	
T-2739	Muehlchen, UBC	Lorna Lake	
T-2763	DeYoung, UBC	Vancouver	
T-2774	DeYoung, UBC	Vancouver	
T-2789	DeYoung, UBC	Vancouver	
Atypical isolate			
A-0181	DeYoung, UBC	Hope	
Erwinia carotovora			LB
subsp. *carotovora*			NA/G
(Jones 1901) Bergey *et al.*, 1923 strain E379	Copeman, UBC	Creston	
Erwinia herbicola			LB
subsp. *herbicola*			NA/G
(Lohnis 1911) Dye 1964 strain 2273	NCPPB #2273		
Family Rhizobiaceae			
Agrobacterium pseudotsugae			LB
(Hansen and Smith, 1937)			CPG
Salvulescu 1947 strain 180	NCPPB #180		
Agrobacterium tumefaciens (Smith and Townsend, 1907) Conn 1942			YDP
			NKS
strain B-1	Copeman, UBC	Vancouver	
strain CH3	Dion, Laval	Quebec	
Rhizobium leguminosarum			YMB
(Frank 1879) Frank 1889	Holl, UBC	Vancouver	YMA
Family Pseudomonadaceae			
Pseudomonas syringae			LB
van Hall 1902 cherry strain	Copeman, UBC	Vancouver	NA/G

[a]The media used included: Luria broth (LB), casein hydrolysate-peptone-glucose (CPG) (Kelman, 1954), Nutrient agar (Difco) supplemented with 1% glucose (NA/G), yeast-dextrose-peptone (YDP) (Moore, 1977), NKS (Brisbane and Kerr, 1983) and yeast-mannitol broth (YMB) (Vincent, 1970).

Delaware, USA, using the Hewlett Packard 5898A Microbial

Identification System (Miller, 1984).

Authentic cultures used in the antisera specificity tests

are listed in Table 1.1. All chemicals used were from Sigma

Chemical Company unless otherwise noted.

Plant materials:

Douglas-fir trees used for pathogenicity tests were

obtained as 1-year-old seedlings from year-end grading at the

University of British Columbia Faculty of Forestry Nursery. The

various tree species used in the host range study were obtained

from Dr. R.S. Hunt at the Pacific Forest Research Center in

Victoria, B.C. Seedlings were planted out in large styrofoam

cups (600 ml) in a standard soil mix with 2400 cm^3 peat and 200

cm^3 Osmocote, a slow release fertilizer (14N-6.0P-11.6K), per

0.16 m^3 of sterilized soil. Fertilizer, 20-20-20, was applied

once every two weeks at a rate of 0.06 g/L.

Direct Isolations/Inoculations:

Direct isolations were carried out through maceration of

gall tissue in a small amount of LB broth in sterile dimple

plates. No surface sterilization was necessary although surface

tissue was removed with sterile tools if there was a sufficient

amount of tissue remaining to sample. After 15-20 min, loopfuls

of the resulting suspension were streaked onto CPG plates.

Colonies appeared after 4 to 5 days.

Artificially-induced galls were produced through stab

inoculations using straight pins. Branches were inoculated with

5 to 7-day-old bacterial colonies from CPG plates, 3-5

centimeters (cm) below the branch tip. The branch diameters

measured between 2-5 millimeters (mm) at the point of inoculation. The pins were often unavoidably pushed through the branch and hence through the stele of the branch.

Polyclonal Antisera Production:

Late log phase cultures grown in NB were harvested by centrifugation (2100 X g (maximum) for 15 min), washed three times with phosphate buffered saline (PBS) pH 7.3 and adjusted in PBS to an optical density of 1.0 unit at 660 nm. The whole cells were fixed with 2.0% glutaraldehyde by dialysis for 2 h and washed with several changes of PBS (pH 7.3) at $4^{\circ}C$ over a 20 h period (Allan and Kelman, 1977). The glutaraldehyde-fixed whole cell preparations were then mixed 1:1 with Freund's complete adjuvant (Difco). Two milliliter (ml) aliquots were injected intramuscularly into the hind legs of New Zealand white rabbits. A schedule of 5 weekly injections was followed by test bleedings 10 days after the last injection. Booster shots were administered once a month.

Bleedings were done either by nicking the marginal vein or by sedating the rabbit with Innovar Vet and inserting a catheter into the auricle artery (10-20 ml per bleeding). Whole blood was allowed to clot by placing at $37^{\circ}C$ for 1 h and then at $4^{\circ}C$ overnight. The samples were then centrifuged at 2100 X g (maximum) for 10 min at $4^{\circ}C$. The supernatants were poured off and stored in aliquots at $-20^{\circ}C$ or at $4^{\circ}C$ with 0.02% sodium azide as a preservative.

IgG Purification:

The IgG fraction of the antiserum was purified by a modified Clark and Adams method (1977). One ml of whole antiserum was diluted with 9 ml of distilled water and mixed with 10 ml of saturated ammonium sulfate at room temperature for 30 min. The precipitate was centrifuged out at 2100 X g (maximum) at 10°C. The pellet was then resuspended in 2 ml of half-strength PBS. The resulting sample was then run through a DEAE-cellulose column pre-equilibrated with half-strength PBS and monitored at 254 nm with an ISCO Model UA-4 Absorbance Monitor. The first major peak was collected. Protein concentration was measured on a Hewlett Packard spectrophotometer at 254 nm assuming an extinction coefficient of 1.4.

Antisera Sensitivity:

Testing of the specificity the whole antisera and the IgG fractions were carried out using an indirect ELISA (Voller *et al.*, 1979). This assay involved the incubation of *Erwinia* cells directly in the microplate wells (200 μl/well). Microtiter plates (Titertek) were previously sterilized for 15 min under a UV light (General Electric G25T8 25 W germicidal lamp). The antigens were used a concentration of 5×10^8 cells/ml in PBS pH 7.3 ($A_{660} = 0.14$ for A-0181 and $A_{660} = 0.28$ for T-2789). After a 1 h incubation step at room temperature, the wells were emptied with two, 10-sec rinses of tap water from a homemade plate washer. A 45-min blocking step using 200 μl/well 'Blotto' (0.1% Carnation Instant Skim Milk Powder diluted in PBS pH 7.3) (Ellis, 1988) followed at room temperature. Plates were dumped

(not rinsed) and the antiserum diluted in PBS plus 0.05% Tween
20 and 0.1% Blotto (200 μl/well) was incubated for 1 h at room
temperature. An antiserum dilution series (one hundred-fold
dilutions between 10^{-1} and 10^{-12}) was replicated at least three
times. Two, 10-sec washes removed unbound immunoglobulins.
Goat anti-rabbit alkaline phosphatase enzyme conjugate diluted 1
in 1000 with PBS/Tween-20/Blotto, 200 μl/well, was incubated at
room temperature for 1 h. Two, 10-sec washes were followed by
addition of the substrate, p-nitrophenyl phosphate, diluted 0.6
mg/ml in substrate buffer (10% diethanolamine; pH 9.8).
Absorbance readings at 405 nm were taken on a Titertek Multiskan
plate reader, either 30 or 45 min, after substrate addition.

Specificity: Immunodiffusion:

Antiserum specificity was tested using Ouchterlony double
diffusion (3 mm diameter wells were spaced 4 to 5 mm apart) (De
Boer *et al.*, 1979). Bacterial cells, taken from 1 to 2-week old
cultures, were suspended in sterile distilled water to form very
turbid suspensions. Whole antisera or IgG fractions were added
undiluted to the middle wells and the reactions were allowed to
proceed for 16-24 h at room temperature.

Several different phytopathogenic bacteria were also tested
to determine the specificity of the antisera: *Erwinia
carotovora* subsp. *carotovora*, *E. herbicola* subsp. *herbicola*
strain 2273, *Agrobacterium pseudotsugae* strain 180, *A.
tumefaciens* strain B-1, *Rhizobium leguminosarum* (formerly
trifolii), *Pseudomonas syringae* (cherry strain), along with the
atypical isolate A-0181 and two typical isolates, T-2789 and
T-2722.

Specificity: Indirect ELISA:

An indirect ELISA as outlined above for the determination of the antisera sensitivity was also used to determine the specificity of the antisera. The phytopathogenic bacteria tested by immunodiffusion were also used in the indirect ELISAs except for *Agrobacterium pseudotsugae*.

Tests involving the whole T-2789 antiserum were carried out with a single concentration of cells, 5-7 X 10^8 cells/ml, and 10-fold dilutions of antiserum ranging from 10^{-2} to 10^{-6} (4 replications per dilution). Experiments with the purified IgG fractions were carried out with varying concentrations of cells and a single concentration of antiserum. Concentrations of 5 X 10^8, 5 X 10^7, 5 X 10^6, 5 X 10^5, 5 X 10^4 cells per ml were used for the indirect ELISAs with 4 replications per cell concentration per bacterial strain. The antiserum dilutions used were 10^{-4} for T-2789 and 10^{-3} for A-0181.

Host Range:

A host range study was carried out involving the following tree species: *Abies amabilis* Dougl. ex Forbes, *A. grandis* (Dougl. ex D. Don) Lindl., *A. lasiocarpa* (Hook.) Nutt., *Chamaecyparis nootkatensis* (D. Don) Spach, *Larix occidentalis* Nutt., *Picea engelmannii* Parry ex. Engelm., *P. glauca* (Moench) Voss, *P. sitchensis* (Bong.) Carr, *Pinus contorta* Dougl. ex Loud., *P. monticola* Dougl. ex D. Don, *P. ponderosa* Dougl. ex Laws, *Pseudotsuga menziesii* (Mirb.) Franco, *Thuja plicata* Donn ex D. Don and *Tsuga heterophylla* (Raf.) Sarg. Three typical isolates T-2789, T-2722 and T-2774 plus the atypical A-0181 isolate were tested for pathogenicity on the various conifers.

Two trees (2 branches/tree) were stab inoculated per isolate. Observations of the inoculation sites were recorded after 5 months as either positive or negative for galling. A positive result was recorded if at least one of the four inoculation sites displayed visible galls. Direct isolations were made onto CPG media from two sites per tree species per isolate. Results were recorded as percent recovery of characteristic bacteria from the two sites. Characteristic bacteria were tested by double diffusion using both whole and purified antisera. Bacteria identified as the inoculated gall-forming bacteria, by immunodiffusion, were back-inoculated into Douglas-fir trees to test for pathogenicity. Four inoculation sites per tree species were used per isolate. Results were recorded as the percent of sites which produced visible galling.

RESULTS

Description of Galls and Isolates:

The Douglas-fir galls were in fairly high numbers within localized marginal growth areas, although a survey of frequency of occurrence was not conducted. The galls were most often found on the tips of branches of mature trees. The galls appeared to be initiated simply along the length of the new shoot (Fig. 1.1a) or at the point where the new year's growth begins (Fig. 1.1b). Older galls were observed in the lower dead branches of 10-15 year old trees. Most of the natural galls were located on branches measuring between 0.2 and 0.8 cm in diameter with the galls measuring between 0.5 and 2 cm in diameter (Fig. 1.1c). The single specimen of the atypical gall collected from the Hope site was taken from a branch measuring approximately 4.0 cm in diameter (Fig. 1.1d). The galls appeared, in general, to be initiated at one point on the branch and to erupt or expand outward enveloping part of the branch.

Two different Douglas-fir gall types were collected. The differences were more apparent when artificially inoculated galls were observed. The more commonly encountered galls were, in general, globose in nature with a rough surface caused by the apparent localized overgrowth of isolated groups of cells (Fig. 1.2a). The distinctiveness of these outgrowths varied within the typical galls both in natural and artificially-inoculated galls (Fig. 1.2b). The surface of these outgrowths were characteristically marked by a single or rarely a cross-shaped
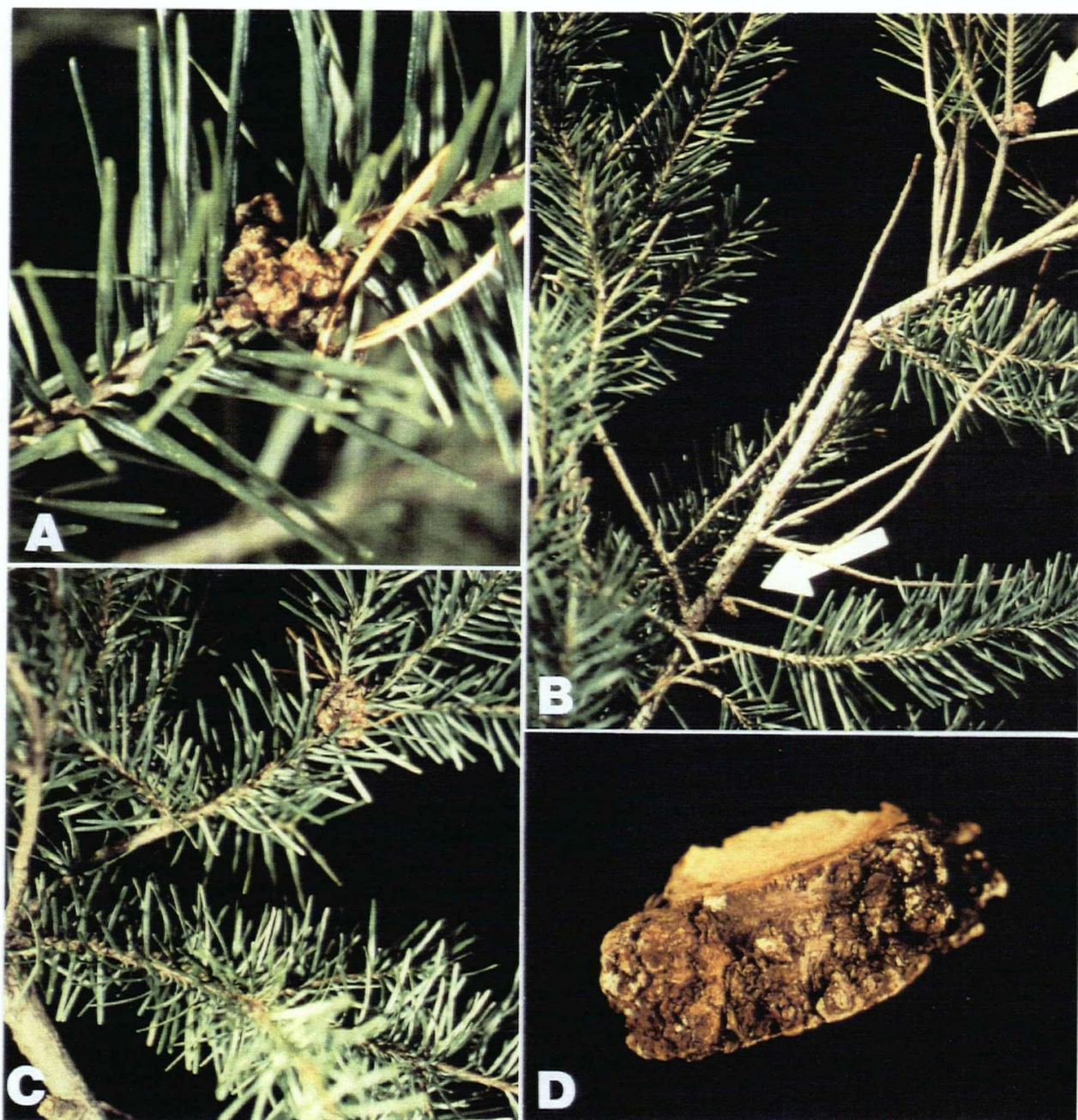
Fig. 1.1. The characteristic location, appearance and size of naturally-occurring galls of Douglas-fir produced by typical isolates (A,B,C) and the atypical isolate A-0181 (D) of the Douglas-fir gall-forming bacteria (actual size).
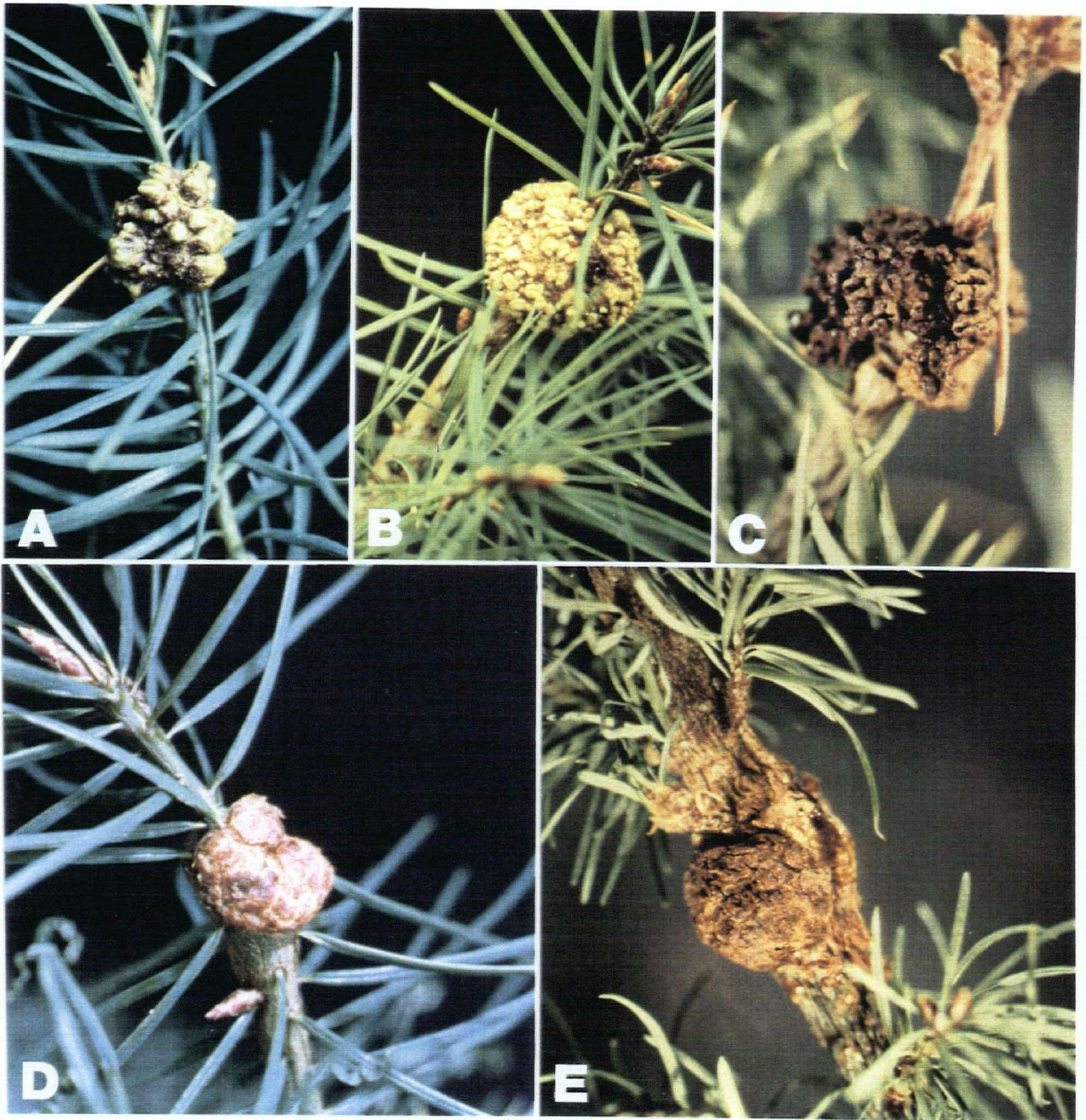
Fig. 1.2. Types of galls formed on Douglas-fir trees artificially inoculated with the two different isolate types of the Douglas-fir gall bacteria. Typical galls formed, after 2 months (A and B) and after 2 years (C), by isolates tentatively identified as *Erwinia salicis*; atypical galls formed, after 2 months (D) and after 2 years (E), by an isolate tentatively identified as *Erwinia herbicola* subsp. *herbicola*.

fissure. As the gall matured, this marking was often less obvious (Fig. 1.2c). Atypical Douglas-fir galls were characterized by being globose to oblong in shape with a smoother, bark-like surface that cracked and peeled slightly as the gall expanded (Fig. 1.2d). The atypical galls appeared to affect the structure of the branch to a greater degree than the typical galls. The atypical galls often distorted the branch both in the resultant direction of growth and in the swelling of the branch below the gall. Douglas-fir galls of both isolates were initially light green in color but as they developed and matured, the galls turned brown and often lost the distinctive markings as noted for the younger galls (Fig 1.2e). The older artificially inoculated galls resembled the galls from which the initial isolations were made.

Galls could be artificially-induced through stab inoculations on most branches of 1-year-old Douglas-fir seedlings as well as along the length of the main stem (Fig. 1.3a). Some galls induced on lateral branches reached a size of 2.0 cm or more in diameter for both of the isolate types but usually galls reached only a size of 0.5 to 1 cm in diameter. The ratio of gall diameter to branch diameter after 4 weeks, was greater than 3.1 and 2.2 for the typical and atypical isolates, respectively. The typical isolates produced faster-growing galls reaching a diameter of 0.5 cm in 4 weeks while the atypical galls took 6 weeks to reach a similar diameter.

The presence of the galling symptoms on the host affected the health and structural integrity of the host tree. Death of
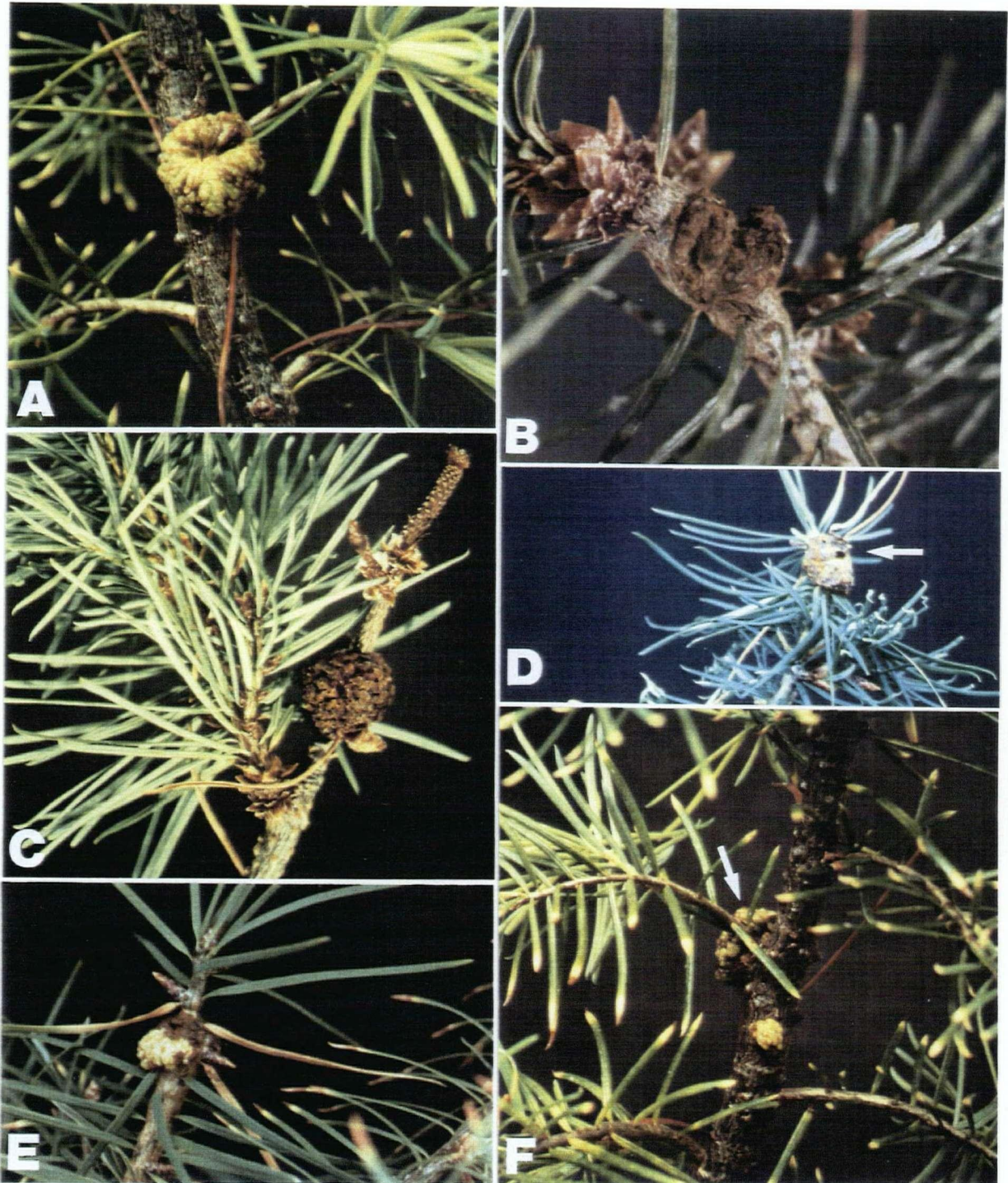
Fig. 1.3. Galls formed on 1- to 2-year old Douglas-fir trees by stab-inoculation with Douglas-fir gall-forming bacteria. (A) gall produced on main stem; (B) galls produced with the atypical and (C) typical isolates causing distortion of inoculated branches; (D) insect feeding activity on an atypical gall; (E) regrowth of gall tissue after insect feeding and (F) apparent spread of gall bacterium from inoculation site (arrow) to new site on a Douglas-fir seedling.

the inoculated branch or simply the tips often occurred after
artificial inoculation. Inoculation of the upper main stem of
young Douglas-fir seedlings, less than 6 months old, often
resulted in death of the seedlings. Well-established trees (1-
year-old or older) did not appear to be severely affected
although the trees sometimes became deformed (Fig. 1.3b). New
branch tips were initiated below the galled tip often distorting
the growth pattern of the tree (Fig. 1.3c).

In artificially-inoculated, greenhouse-grown trees, galls
appeared to stop growth after the first year. The succulent
gall tissue was an attractant to insects (Fig. 1.3d). Galls
often continued to grow after being eaten by insects (Fig.
1.3e). A curious observation of the artificially-induced galls
was that occasionally small galls were produced at locations
other than the inoculation site (Fig. 1.3f). This would suggest
either that the pathogen was transported externally via insects
or internally through the plant's vascular system.

From the two types of galls, two distinct strains of the
gall-forming bacteria were isolated. Simple direct isolation
from young gall tissue resulted in large numbers of the inciting
bacteria. When grown on solid media containing casein
hydrolysate, peptone and glucose, the common strains, typified
by the T-2789 isolate, formed shiny, greyish-white circular
convex colonies with entire margins (Fig. 1.4a). The colonies
have characteristic surface indentations. The atypical A-0181
isolate formed colonies of similar color and translucence (Fig.
1.4b), however the shape of the colonies differed. Young A-0181
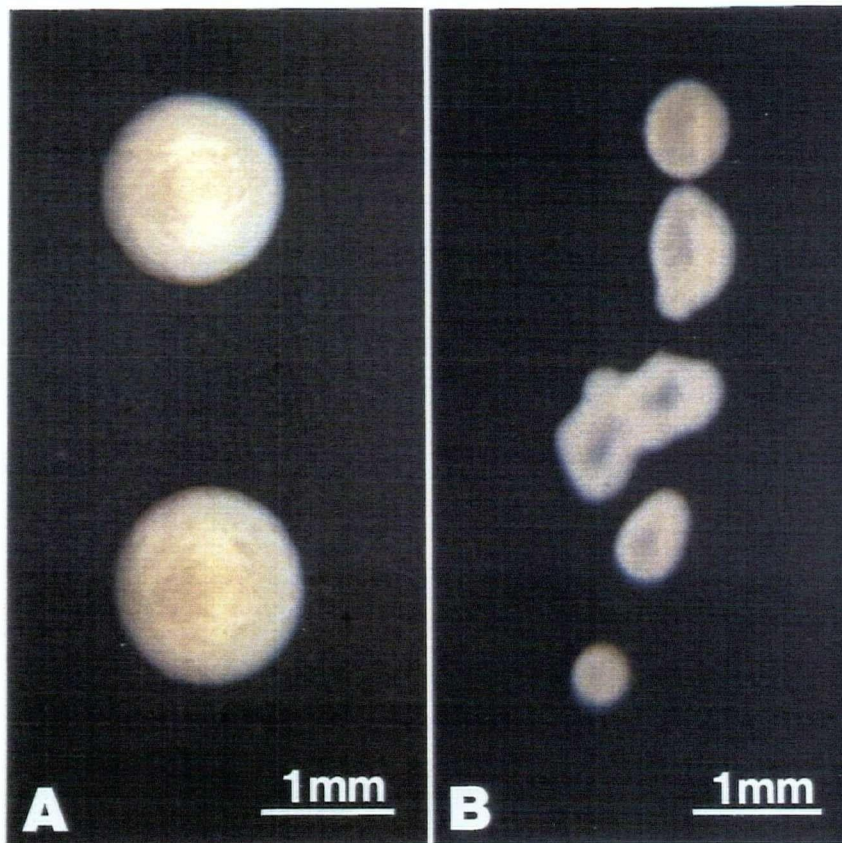
Fig. 1.4. Colonies of the typical (A) and the atypical (B) gall-forming *Erwinia* isolates grown on casein hydrolysate, peptone and glucose medium.

colonies were convex in appearance although as the colonies matured, depressions were formed in the center of the colonies. Extracellular slime was also often produced as the colonies matured causing the coalescence of closely spaced colonies, giving the colonies an elliptical appearance. Colonies of both gall-forming isolates measured between 1.0 to 2.0 mm in diameter after 5-7 days on solid CPG media. Colonies of the atypical isolates grew slightly faster than the typical isolates appearing after approximately 2 days at 21-23°C on solid media.

One important characteristic of the two bacterial isolates was their temperature sensitivity (Table 1.2). The typical isolates were able to survive only at temperatures up to 32±0.5°C in Luria broth. The atypical isolate was able to survive at slightly elevated temperatures of up to 34±0.5°C in Luria broth. The atypical isolate, however, did not survive at room temperature on solid CPG media for more than 10 days. The typical isolates survived under similar conditions for 2 weeks or more. The two isolates differed in their antibiotic resistances (Table 1.3). The typical isolates were resistant to a wider range of antibiotics including ampicillin, erythromycin, kanamycin and tetracycline.

When fatty acid analysis and computer-assisted comparison with a library of authentic strains was used to tentatively identify representative isolates, similarity indices of greater than 0.500 placed both of them unequivocally in the genus *Erwinia* in the family Enterobacteriaceae. The atypical gall-forming isolate A-0181 was positively placed in the *Erwinia*

Table 1.2 Temperature sensitivity of the gall-forming
Erwinia isolates

Temperature (+/- 0.5°C)	Growth in Luria Broth	
	Erwinia T-2789	*Erwinia* A-0181
31	+	+
32	+	+
33	−	+
34	−	+
35	−	−

Table 1.3. Antibiotic resistances expressed by
representative typical and atypical isolates of the
Douglas-fir gall-forming bacteria

Antibiotic	Concentration (µg/ml)	Growth of gall-forming bacterial isolates	
		Typical	Atypical
Ampicillin	30	+	$-^a$
	40	+	−
Carbenicillin	40	+	trace
	50	+	trace
Chloramphenicol	10	−	−
Erythromycin	30	+	−
Fusidic acid	31	+	+
	75	+	$+^a$
Kanamycin	40	trace	−
	50	trace	−
Methicillin	28	+	+
Novobiocin	75	+	+
Oleandomycin	25	+	$trace^a$
Streptomycin	25	−	−
Tetracycline	12.5	+	−

[a]Grown on NA instead of NA/G

herbicola subsp. *herbicola* group. The typical isolates were matched most closely with the *E. salicis* group (Table 1.4).

Antisera Sensitivity:

The cutoff value for ELISA values was chosen to be 0.1 optical density unit (A_{405}), which was greater than twice the background values (Sutula *et al.*, 1986). The lower limits of detection of the whole antisera, as determined by an indirect cell ELISA were 10^{-6} and 10^{-3} for T-2789 and A-0181, respectively. The protein concentrations for the purified IgG fraction were 1.86 mg/ml for A-0181 and 1.0 mg/ml for T-2789. The lower limits of detection for the IgG fractions were 10^{-4} and 10^{-3} for T-2789 and A-0181, respectively (Fig. 1.5).

Specificity -Immunodiffusion:

The whole T-2789 antiserum produced two major precipitin lines with its homologous antigen and each of the typical isolates (Fig. 1.6). One of these lines formed a line of partial identity with the single line of identity produced in reaction with the A-0181 isolate. No precipitin lines were observed with *Agrobacterium tumefaciens*, *Agrobacterium pseudotsugae*, *Erwinia carotovora*, *Erwinia herbicola* subsp. *herbicola* strain 2273, *Escherichia coli*, *Pseudomonas syringae* or *Rhizobium leguminosarum*. Purification of the T-2789 antiserum removed the cross reaction with isolate A-0181 observed with the whole antiserum (Fig. 1.6).

Both the whole A-0181 antiserum and purified A-0181 IgG fraction formed lines of identity only with their homologous antigens (Fig. 1.6). Purification of the A-0181 antiserum

Table 1.4. Tentative identification of Douglas-fir
gall-forming bacterial isolates by analysis of their
fatty acid profiles and computer-assisted comparison
with a library of profiles from authentic strains

Isolate	Possible identities	Similarity index with known strains
T-2739	*Hafnia alvei*	0.635
	Erwinia salicis	0.593
	Erwinia herbicola subsp. *herbicola*	0.544
T-2763	*Erwinia salicis*	0.767
	Hafnia alvei	0.719
	Erwinia herbicola subsp. *herbicola*	0.597
T-2789	*Erwinia salicis*	0.648
	Hafnia alvei	0.644
	Erwinia herbicola subsp. *herbicola*	0.558
A-0181	*Erwinia herbicola* subsp. *herbicola*	0.840
	Enterobacter agglomerans	0.545
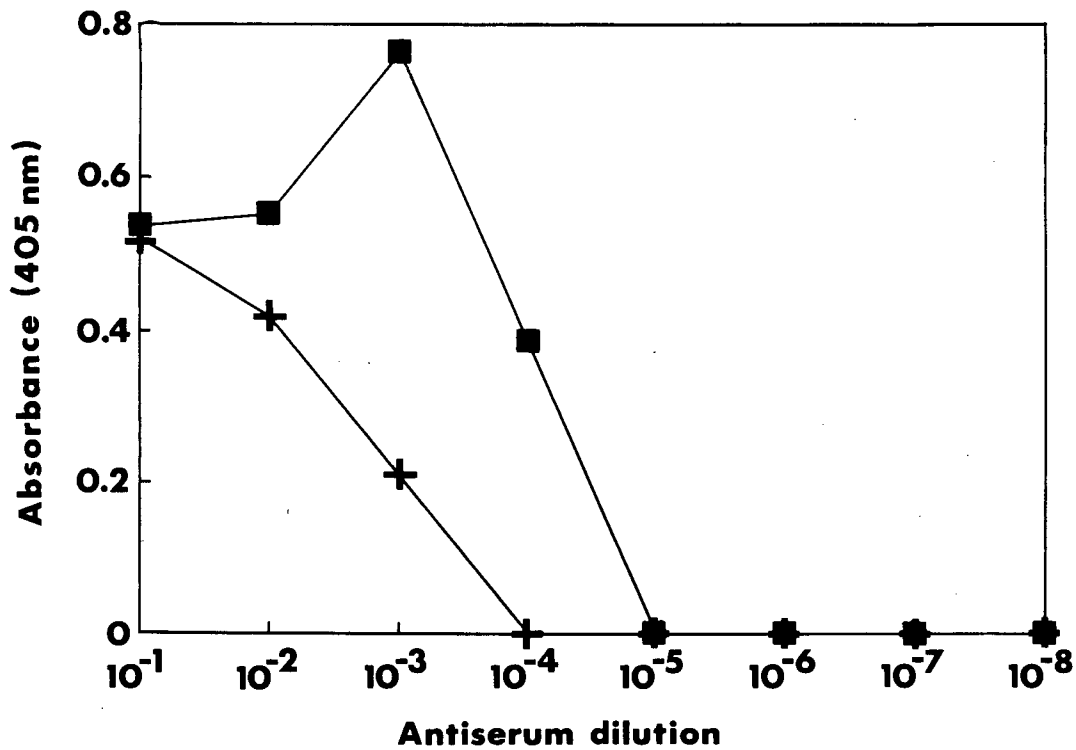
Fig. 1.5. Sensitivity of the IgG fractions of the antisera raised against the typical T-2789 (■) and A-0181 (✚) isolates of the Douglas-fir gall-forming bacteria as determined by an indirect ELISA against their respective homologous antigens. Each point represents the mean of four replicates.
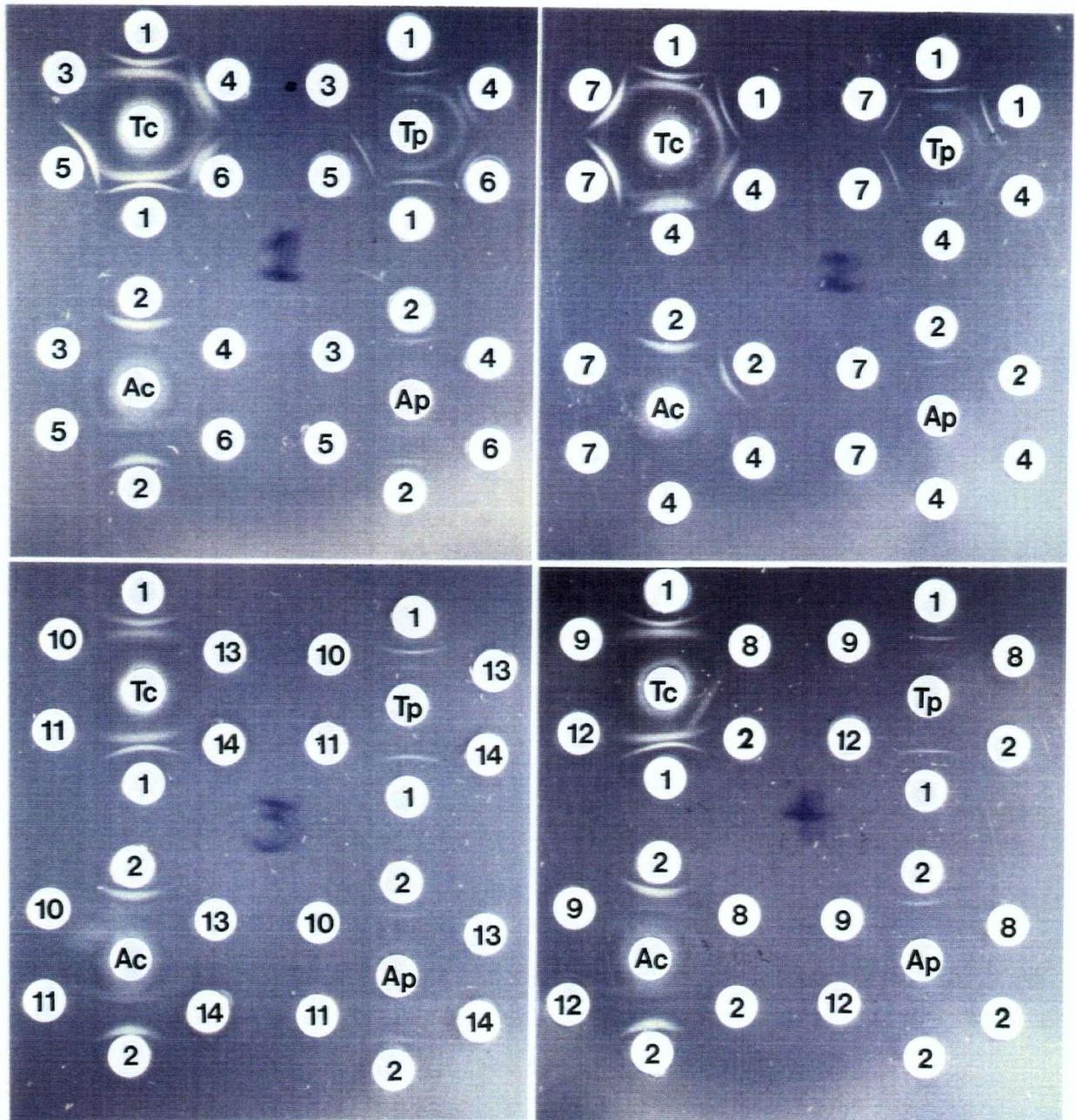
Fig. 1.6. Immunodiffusion patterns produced by reactions
between antisera elicited against the (T) typical T-2789 and the
(A) atypical A-0181 gall-forming *Erwinia* isolates and several
phytopathogenic bacteria. Crude (c) and purified (p) antisera
were tested against (1) *Erwinia* T-2789, (2) *Erwinia* A-0181, (3)
Erwinia T-2721, (4) *Erwinia* T-2722, (5) *Erwinia* T-2739, (6)
Erwinia T-2763, (7) *Erwinia* T-2774, (8) *Agrobacterium
pseudotsugae*, (9) *Agrobacterium tumefaciens*, (10) *Erwinia
carotovora* subsp. *carotovora*, (11) *Erwinia herbicola* strain
2273, (12) *Escherichia coli* strain MC1000, (13) *Pseudomonas
syringae* and (14) *Rhizobium leguminosarum*.

resulted in the reduction of the number of precipitin lines, against its homologous antigen, from two to a single line. No precipitin lines were observed with any of the other phytopathogenic bacteria including *Agrobacterium tumefaciens*, *Agrobacterium pseudotsugae* or even the gall-forming typical isolates.

Specificity -Indirect ELISA:

For the whole T-2789 antiserum, at 1.5 h after substrate addition, a dilution of 10^{-5} detected only the typical isolates T-2789 and T-2722 (Table 1.5). Isolate A-0181 was detected at antiserum dilutions out to 10^{-3}. *Erwinia carotovora* subsp. *carotovora* strain 379 was detected at antiserum dilutions out to 10^{-4} while *Agrobacterium tumefaciens* isolate CH3 and *Rhizobium trifolii* were detected at dilutions out to 10^{-3}. *Pseudomonas syringae* was not detected at any dilution after 1.5 h. Each bacterial spp. tested except for A-0181 and *A. tumefaciens* isolate CH3 was detected with a 10-fold higher dilution of antiserum after 27 h.

For the purified T-2789 antiserum, at 2.5 h after substrate addition, the only bacterial species detected was the homologous antigen, down to a dilution of 5 X 10^6 cells/ml (Table 1.6). A T-2789 cell dilution of 5 X 10^5 cells per ml was detected after 22 h. After 22 h the typical isolate T-2722 was also detected at a concentration of 5 X 10^8 cells/ml.

The A-0181 purified antiserum was also very specific detecting only its homologous antigen, at a dilution of 5 X 10^7 cells/ml, after 2.0 h (Table 1.7). After 27 h an A-0181

Table 1.5. Specificity of whole *Erwinia* T-2789 antiserum in an indirect ELISA. Bacterial concentrations of 5-6 X 10^8 cells/ml were used. The A_{405} values for each bacterial strain were read at two times after substrate addition. Positive ELISA values were above 0.10

Bacterial strain	Time (h)	A_{405} at time indicated for antiserum dilutions				
		10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}
Erwinia T-2789	1.5[a]	0.25	0.25	0.33	0.12	0.00
(grown in LB)	27.5	1.58	1.62	1.87	1.04	0.01
Erwinia T-2789	1.5	0.28	0.25	0.35	0.23	0.00
(grown in NB)	27.5	1.72	1.72	1.95	1.63	0.23
Erwinia T-2722	1.5	0.38	0.39	0.56	0.38	0.00
	27.5	1.98	1.98	1.95	1.63	0.23
Erwinia A-0181	1.5	0.11	0.11	0.00	0.00	0.00
	27.5	0.91	0.98	0.00	0.00	0.00
Erwinia carotovora 379	1.5	0.62	0.50	0.12	0.00	0.00
	27.5	2.00	2.00	1.87	1.04	0.00
Agrobacterium tumefaciens CH3	1.5	1.73	0.32	0.01	0.00	0.00
	27.5	2.00	1.82	0.47	0.01	0.00
Pseudomonas syringae	1.5	0.09	0.00	0.00	0.00	0.00
	27.5	0.81	0.04	0.00	0.00	0.00
Rhizobium leguminosarum	1.5	0.61	0.21	0.00	0.00	0.00
	27.5	2.00	1.53	0.21	0.04	0.00

[a]mean of 4 replications

Table 1.6. Specificity of the purified IgG fraction of the *Erwinia* T-2789 antiserum at a dilution of 10^{-4} in an indirect ELISA. The A_{405} values for each bacterial strain were read at two times after substrate addition. Positive ELISA values were above 0.10

Bacterial strain	Time (h)	A_{405} for cells/ml at time indicated				
		5×10^8	5×10^7	5×10^6	5×10^5	5×10^4
Erwinia T-2789	2.5	0.27[a]	0.24	0.18	0.03	0.00
	22.0	0.85	0.78	0.61	0.15	0.00
Erwinia T-2722	2.5	0.03	0.05	0.00	0.00	0.00
	22.0	0.18	0.04	0.06	0.00	0.00
Erwinia A-0181	2.5	0.00	0.00	0.00	0.00	0.00
	22.0	0.05	0.00	0.00	0.00	0.00
Erwinia herbicola 2273	2.5	0.00	0.00	0.00	0.00	0.00
	22.0	0.04	0.00	0.00	0.00	0.00
Erwinia carotovora 379	2.5	0.00	0.00	0.00	0.00	0.00
	22.0	0.01	0.00	0.00	0.00	0.00
Agrobacterium tumefaciens B-1	2.5	0.00	0.00	0.00	0.00	0.00
	22.0	0.00	0.00	0.06	0.00	0.00
Pseudomonas syringae	2.5	0.00	0.00	0.00	0.00	0.00
	22.0	0.00	0.00	0.00	0.00	0.00
Rhizobium leguminosarum	2.5	0.00	0.00	0.00	0.00	0.00
	22.0	0.00	0.00	0.00	0.00	0.00

[a]mean of 4 replications

Table 1.7. Specificity of the purified IgG fraction of the *Erwinia* A-0181 antiserum at a dilution of 10^{-3} in an indirect ELISA. The A_{405} values for each bacterial strain were read at two times after substrate addition. Positive ELISA values were above 0.10

Bacterial strain	Time (h)	A_{405} for cells/ml at time indicated				
		5×10^8	5×10^7	5×10^6	5×10^5	5×10^4
Erwinia A-0181	2.0	0.52^a	0.25	0.10	0.05	0.05
	27.0	1.59	0.78	0.22	0.06	0.05
Erwinia T-2789	2.0	0.05	0.05	0.05	0.05	0.05
	27.0	0.06	0.05	0.05	0.05	0.04
Erwinia T-2722	2.0	0.06	0.04	0.03	0.03	0.03
	27.0	0.16	0.05	0.03	0.02	0.02
Erwinia herbicola 2273	2.0	0.03	0.01	0.01	0.01	0.01
	27.0	0.07	0.01	0.00	0.00	0.00
Erwinia carotovora 379	2.0	0.01	0.01	0.00	0.00	0.00
	27.0	0.01	0.02	0.00	0.00	0.00
Agrobacterium tumefaciens B-1	2.0	0.07	0.05	0.04	0.05	0.04
	27.0	0.08	0.05	0.04	0.04	0.04
Pseudomonas syringae	2.0	0.06	0.06	0.06	0.06	0.06
	27.0	0.05	0.05	0.06	0.05	0.05
Rhizobium leguminosarum	2.0	0.00	0.00	0.00	0.00	0.00
	27.0	0.00	0.00	0.00	0.00	0.00

[a]4 replications per value

cell dilution of 5 X 10^6 cells/ml was detected. The typical isolate T-2722 was also detected after 27 h at a dilution of 5 X 10^8 cells/ml.

Host Range:

Small growths were observed in just over half of the conifers inoculated with the *Erwinia* isolates (Fig. 1.7). The largest growths and the largest numbers of inoculated bacteria recovered were, however, located on the Douglas-fir trees. The A-0181 isolate was recovered from each conifer species inoculated (Table 1.8). Galling symptoms produced by the atypical isolate, A-0181, were visible on *Abies*, *Chamaecyparis*, *Larix*, *Picea* and *Pinus* spp.

Galling symptoms produced by the typical isolates were most visible on *Abies*, *Larix* and *Picea* spp. Each typical isolate was not recovered from each inoculated tree species. However, when considering the three typical isolates as one group, bacteria characteristic of this group were recovered from at least one inoculated tree per species. Those trees that displayed galling symptoms were not necessarily those from which the inoculated bacteria could be successfully recovered. Characteristic bacteria were recovered from many of the trees that did not produce visible galling symptoms.

All those strains, of both types, identified as the inoculated gall-forming bacteria caused galls when back-inoculated into Douglas-fir, except for isolate T-2722 on *Picea engelmannii*. Two of the cultures of recovered bacteria were lost and hence were not back-inoculated into Douglas-fir.
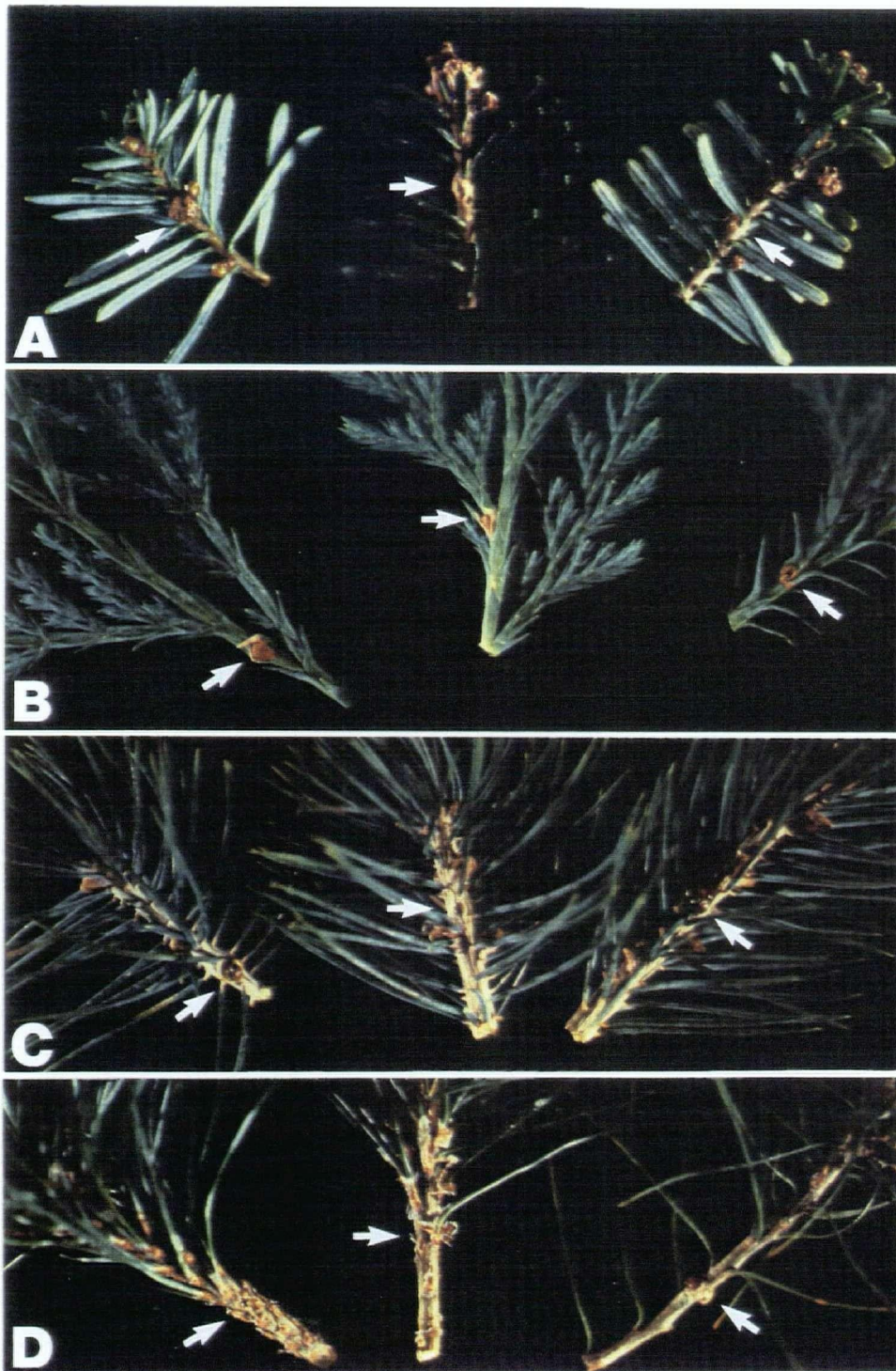
Fig. 1.7. Pathogenicity of gall-forming *Erwinia* isolates on *Abies amabilis* (A), *Chamaecyparis nootkatensis* (B), *Picea engelmannii* (C) and *Pinus contorta* (D). Sites inoculated with: typical isolate T-2789 (left branches), wound-only controls (center branches) and atypical isolate A-0181 (right branches), respectively.

Table 1.8. Pathogenicity of gall-forming *Erwinia* isolates on 14 conifer species. (G) Percentages of observations of galling at the inoculation site are followed by (R) percent recovery of the inoculated bacteria. Percentage of galled sites on Douglas-fir inoculated with the recovered gall-forming *Erwinia* spp. are listed in column P

Conifer species	T-2774			T-2789			T-2722			A-0181			Dry Stab	
	G[a]	R[b]	P	G	R	P	G	R	P	G	R	P	G	R
Family Pinaceae														
Abies amabilis	100	50	100	100	50	100	100	100	100	100	100	100	0	0
Abies grandis	100	50	100	100	50	100	0	0	–	100	100	100	0	0
Abies lasiocarpa	100	100	100	75	0	–	75	50	100	100	50	100	0	0
Larix occidentalis	100	50	100	75	100	100	100	100	100	100	100	50	0	0
Picea glauca	50	100	100	100	50	100	100	100	75	100	100	100	0	0
Picea engelmannii	100	100	100	100	100	100	0	50	0	100	100	100	0	0
Picea sitchensis	75	100	100	50	100	100	100	100	100	100	100	75	0	0
Pinus contorta	50	50	100	75	100	100	100	100	100	100	50	100	0	0
Pinus monticola	0	100	100	0	100	100	50	100	100	75	100	75	0	0
Pinus ponderosa	25	50	100	0	100	50	100	50	50	75	100	100	0	0
Pseudotsuga menzeisii	100	100	50	100	100	100	100	100	100	100	100	100	0	0
Tsuga heterophylla	nd[d]	nd	nd	25	50	50	nd	nd	nd	25	100	50	0	0
Family Cupressaceae														
Chamaecyparis nootkatensis	nd	nd	nd	0	50	nd	nd	nd	nd	100	50	nd	0	0
Thuja plicata	100	0	–	0	50	75	0	0	–	100	50	50	0	0

[a]4 replications
[b]2 replications
[c]4 replications
[d]no data

DISCUSSION

Two different types of bacterial isolates were recovered from Douglas-fir galls collected from southwestern British Columbia. All of the galls collected in this study were similar to those reported by Hansen and Smith in California in the 1930's (Hansen and Smith, 1937), especially those caused by the atypical isolate. At the disease level, many similarities were noted between the Douglas-fir gall disease of California, caused by *Agrobacterium pseudotsugae*, and the B.C. gall disease, the pathogens of which were tentatively identified as *Erwinia* species. Unfortunately, there are no extant cultures of the bacterium, originally named *A. pseudotsugae*, possessing the characteristics first described by Hansen and Smith in 1937. Therefore a direct comparison between the incitants of the two Douglas-fir gall diseases was not possible.

Neither *A. pseudotsugae* nor the gall-forming *Erwinia* isolates caused significant economic damage to their host tree, Douglas-fir. The Douglas-fir galls in this study were collected in mixed forests near lakes or in urban areas. This site yielded the single source of the second type of bacteria isolated. Hansen and Smith collected *A. pseudotsugae* galls in similar areas, also termed as marginal areas where the health of the host was already compromised. With both gall-forming *Erwinia* isolates, the tips of artificially inoculated branches often die. However, due to the characteristic small size of the galls in relation to the size of the host trees observed in the natural environment, little damage was observed. Hansen and

Smith (1937) reported *that* the isolated pathogen was 'not of economic importance but of potential interest to the lumbering industries' as it could deform and even girdle young trees leading to their death. They suggested that if the pathogen was able to invade stands of timber dominated by the host, Douglas-fir, then 'it might readily become an important factor in determining the future composition of the forest'. Fortunately, that pathogen has not become an important factor in the geographical range of Douglas-fir. Because the gall-forming erwinias can similarly cause girdling and death if inoculated in the upper main stem of young Douglas-fir seedlings, they may have the potential to become a problem in tree nurseries.

Methods of transmission of the gall-forming erwinias were not investigated in this study, although the initiation site of the galls within the succulent new year's growth suggests an insect vector. Hansen and Smith (1937) proposed that transmission of *A. pseudotsugae* might be via *Adelges cooleyi*, the Cooley Spruce gall adelgid due to the presence of sites of initiation of gall formation at the sites corresponding to feeding punctures formed by the adelgid. Similar to galls produced by *A. pseudotsugae*, galls produced by the *Erwinia* isolates only developed when inoculated using deep needle stabs. That the succulent gall tissue was an attractive food source for insects, in this study, supports the hypothesis that an insect vector is involved.

One further similarity between the two gall-forming *Erwinia* isolates and *A. pseudotsugae* was the location of the galls on the host plant. The galls of both gall diseases are found close

to the tips of twigs or branches of Douglas-fir trees. The
Douglas-fir galls found in B.C. measured between 0.5 and 2.0 cm
in diameter while *A. pseudotsugae* galls were reported to measure
between 1 millimeter and several centimeters (Hansen and Smith,
1937).

A. pseudotsugae formed galls on artificially inoculated
Douglas-fir in the field a minimum of three months after
inoculation (Hansen and Smith, 1937). Gall growth was limited
to the active growth period of the host. Furthermore the
pathogen could be present in the host for nearly a year before
definite symptoms began to appear. In the present study only
greenhouse inoculations were carried out. However, galls were
formed by the *Erwinia* isolates after less than 2 weeks in young
succulent tissue.

At the microscopic level, the gall tissue organization and
make-up was found to be similar between the diseases caused by
the gall-forming *Erwinia* and *A. pseudotsugae*. Thin-sectioning
of the gall tissues formed by the two types of gall-forming
Erwinia revealed that both gall types were composed of circular
groups of rapidly dividing cells. These cells were about one
half the size of healthy cortical cells (R.J. Copeman,
unpublished). The stab inoculations penetrated through the
stele resulting in unorganized vascular elements extending into
the pith of the young stems. Both bacterial strains appeared to
be localized in the intercellular spaces in the centers of these
groups of dividing cells. These findings are similar to those
in the study by Hansen and Smith (1937).

Douglas-fir was initially thought to be the only host of

the *Erwinia* species as these bacteria were successfully isolated only from Douglas-fir galls. A host range study revealed that the typical and the atypical gall-forming *Erwinia* isolates could also form small galls on species within the genera *Abies*, *Larix*, *Picea* and *Pinus*. The pathogen studied by Hansen and Smith was found to be nonpathogenic on all of the conifers tested, which included Douglas-fir, *Pinus halepensis* (Aleppo pine), *P. lambertiana* (sugar pine), *P. radiata* (Monterey pine) and *Tsuga heterophylla* (coast hemlock). *A. pseudotsugae* strain 180 was found to be avirulent on Douglas-fir in this study. This finding confirms the results of a recent study by De Cleene and De Ley (1981). It also confirms the comment in the eighth edition of Bergey's Manual (Buchanan and Gibbons, 1974) which states that the cultures of *A. pseudotsugae* in the American Type Culture Collection do not display the characteristics as observed by Hansen and Smith in the 1930's.

The galls induced by the *Erwinia* isolates through artificial inoculation were smaller than those produced on Douglas-fir galls in nature and therefore may not be an accurate indication of virulence. The resulting symptoms, however, indicated that the isolates were weakly pathogenic on some conifer species other than Douglas-fir, including *Abies*, *Larix*, *Picea* and *Pinus* spp. Some of the galling symptoms could have been due to a wound response, especially on *Chamaecyparis*, *Tsuga* and *Thuja* spp. from which either low numbers or no bacteria of the typical isolates were recovered. In some cases, the inoculated bacteria were recovered from inoculation sites that were not galled, indicating an epiphytic relationship. A single

gene within the pathogen can be responsible for determining the virulence of the pathogen on a particular host thereby resulting in either a successful infection or an epiphytic relationship (Surico *et al.*, 1985). As the other conifer species tested were not very susceptible to the *Erwinia* spp., this would aid in the explanation of the low incidence of this disease.

In addition to differing in some respects from *A. pseudotsugae*, the two gall-forming *Erwinia* isolate types differed from each other in many respects including resultant gall morphology. Typical Douglas-fir galls collected in this study were rough and irregular in shape with distinctive fissure-like markings present on separate, defined outgrowths that constituted the gall. The artificially-induced atypical Douglas-fir galls were globose and smooth in appearance but did not have any distinct markings on their surface. The galls produced by *A. pseudotsugae* were generally very smooth, like the atypical galls, but had a single distinctive cross-shaped marking covering the entire gall surface, not observed on any of the B.C. galls.

The two gall-forming *Erwinia* isolates differed from each other in colony morphology, temperature sensitivity, growth rate, antibiotic resistances, serological properties and host range. The colonies of both *Erwinia* isolates differed in morphology from those of *A. pseudotsugae* strain 180. Unfortunately, as mentioned before, the cultures of *A. pseudotsugae* available from the National Collection of Plant Pathogenic Bacteria (NCPPB; Hertfordshire, England) do not display the characteristics in the original description. Thus,

not much could be deduced from such comparisons.

With the computer-assisted, fatty acid analysis system (Hewlett Packard) for identification of bacteria, an index of greater than 0.500 on a scale of 0.000 and 1.000 is generally required before a positive identification can be made. An index of 0.500 corresponds to approximately three standard deviations away from the mean fatty acid profile for the library entry (match) in the computer data base (M. Roy, *personal communication*). A difference in similarity indices of greater than 0.100 between the first and second species' choices is usually required for distinguishing between the species and hence a positive identification. Fatty acid analysis is touted as being very reliable for identification purposes, due to the genetic stability of the fatty acids (Anonymous, 1985; Clark *et al.*, 1988). A positive match, with a similarity index of 0.840, was made between the profile of the atypical isolate, A-0181, and that of *Erwinia herbicola* subsp. *herbicola*. The next closest match was at a similarity index of 0.545, producing a difference well above the 0.100 required for a positive match to the first choice of *E. herbicola* subsp. *herbicola*.

The difference between the first choice of *Erwinia salicis* (Day 1924) Chester 1939 and second choice of *Hafnia alvei* Moller 1954 was less than 0.100 for each of the three typical isolates tested. However, the fatty acid profiles of species in the family Enterobacteriaceae are highly similar (M. Roy, *personal communication*) and often the differences in similarity indices between the first and second choices may not reach 0.100. *Hafnia* species, unlike the gall-forming *Erwinia* isolates, can

grow at temperatures up to 40-42°C and are not reported to be associated with plants (Krieg and Holt, 1984). Therefore, the typical isolates were tentatively identified as *Erwinia salicis*, which is a known pathogen of *Salix* sp. (Krieg and Holt, 1984).

The differences in similarity indices, for the typical isolates, between the first choice of *E. salicis* and *E. herbicola* subsp. *herbicola* (the choice for the atypical isolate) measured close to or greater than the 0.100 requirement (for two of the three typical isolates analyzed: 0.170, 0.091, and 0.049) for a distinction between species. Therefore on the basis of fatty acid profiles alone, at least one of the typical isolates is indeed a different species than the atypical isolate.

The identification as *Erwinia* is supported by other observations. The gall-forming *Erwinia* spp. were shown not to produce opines in galled tissues nor do they grow on media that contains octopine or nopaline as the sole carbon and nitrogen source (R.J. Copeman, *unpublished*). They also do not grow on any of the common selective media for *Agrobacterium* and they do not form galls on tomato, raspberry and *Kalanchoe daigremontiana*, common herbaceous hosts of *Agrobacterium tumefaciens*, (Muehlchen, 1985).

Because the Douglas-fir gall-forming bacteria were identified as belonging in the genus *Erwinia*, comparisons could be made with *E. herbicola* f.sp. *gypsophilae*, the pathogen of *Gypsophila*. However, the *Erwinia* isolates were found in this study not to form galls on *Gypsophila*. Furthermore, no serological relationships were observed between the gall-forming

Erwinia and *E. herbicola* subsp. *herbicola* strain 2273.

The two gall-forming *Erwinia* isolate types shared to a degree, a serological relationship as detected by immunodiffusion. When the whole typical T-2789 antiserum was used, a line of partial identity between the typical isolates and the atypical isolate was formed. This was not observed with any of the other phytopathogenic bacteria tested. Purification of the antiserum completely removed the cross reaction with the atypical isolate. Whole atypical A-0181 antiserum or the A-0181 IgG fraction did not cross react with the typical T-2789 isolate nor with any of the other phytopathogenic bacteria when tested by immunodiffusion or by indirect ELISA. A weak reaction was observed with the typical isolate T-2722 with both antisera.

Many similarities were found between the pathogens of the two Douglas-fir gall diseases. As biochemical identification tests were not carried out on the gall-forming *Erwinia* isolate types, a direct comparison and hence a taxonomic relationship to *A. pseudotsugae* could not be made. The identity of *Agrobacterium pseudotsugae* is still in question (De Cleene and De Ley, 1981) because the original *A. pseudotsugae* cultures have been lost and no galls have been found at the original site in California to enable further research. The California bacterium, described as a Gram-negative, plant pathogenic facultative anaerobe, would likely be placed into the genus *Erwinia* today.

Identification of the B.C. isolates by fatty acid analysis placed them unequivocally in the genus *Erwinia*, not in either the *Agrobacterium* or *Pseudomonas* genera, which contain the most

widely recognized plant gall formers. From the observed
characteristics and the results of fatty acid profiles, it would
appear that the two gall-forming isolate types are two different
species of *Erwinia*.

CHAPTER 2

THE MECHANISM OF GALL FORMATION
BY *ERWINIA* SPP. ON DOUGLAS-FIR

INTRODUCTION

Initial experiments to determine the mechanism of gall formation by *A. tumefaciens* focussed on the growth of host tumor tissues. The growth of healthy plant tissues *in vitro* requires an exogenous supply of phytohormones (Parsons *et al*. 1986). Crown gall tissues freed of the inciting bacteria were found to be capable of autonomous growth (Braun 1943). This observation suggested that the pathogen induced the endogenous production of phytohormones in certain host cells. To determine the time frame involved for this bacterial-induced alteration to occur in the host plant cells, a simple heat treatment was used to selectively kill the pathogen but not the host, at specific times after inoculation (Braun, 1943). One to five days after inoculation of *A. tumefaciens* into periwinkle (*Vinca rosea* L.), a high temperature treatment of 46-47°C was applied for 5 days. Host plants were then returned to room temperature for a further 3 months. Galls, similar in size to those produced without the heat treatment, were observed in those plants initially exposed to the high temperature 4 or more days after inoculation (Braun 1943). Cellular alteration, or transformation, therefore occurred within 4 days of inoculation after which time the bacterium's presence was no longer required for gall

development.

No evidence was found to demonstrate that transformation of host plant cells also occurred when inoculated with *P. syringae* pv. *savastanoi* (Comai *et al.*, 1982). The continued presence of the bacteria is required for gall initiation and development. *P. syringae* pv. *savastanoi* has been shown to produce both IAA and cytokinins in culture by detection of the phytohormone enzymes with biochemical tests or by assaying for the actual phytohormones with immunoassays (Comai and Kosuge, 1980; Davis *et al.*, 1985; Mertens *et al.*, 1985). *A. tumefaciens* also produces auxins and cytokinins in culture but at a much lower level than *P. syringae* pv. *savastanoi* (Akiyoshi *et al.*, 1987). The production of higher levels of phytohormones by *P. syringae* pv. *savastanoi* may be necessary for the continued development of the galls as the host plant cells do not produce phytohormones at a level required to sustain such unusual growth (Akiyoshi *et al.*, 1987).

The objective of these experiments was to determine if the continued presence of the gall-forming *Erwinia* spp. was required for gall formation to occur. The design of a heat treatment experiment, similar to that used for the study of *A. tumefaciens*, was chosen for this initial study.

MATERIALS AND METHODS

Bacterial Strains and Host Plants:

The two gall-forming *Erwinia* isolates used in these experiments were the T-2789 and the A-0181 isolates. Bacteria were grown on CPG plates for 5-7 days at 21°C. Isolated colonies were used for stab inoculations.

One to two year old nursery-grown Douglas-fir trees were planted in large styrofoam cups (600 ml) in standard soil mix with 2400 cm³ peat and 200 cm³ Osmocote, a slow release fertilizer (14N-6.0P-11.6K), per 0.16 m³ of sterilized soil. The seedlings were previously stored at 4°C. Trees were used for the heat treatment experiments a minimum of 2 months after transplanting to allow the trees to become established under greenhouse conditions. Trees were fertilized with 20-20-20 once every two weeks at a rate of 0.06 g/L.

Inoculations/Heat treatment:

Four sets of 10 to 12 Douglas-fir trees were selected for their uniformity between sets and labelled. Four branches per tree were labelled and stab inoculated 2-5 cm below the branch tip with sterile pins carrying the bacterial isolate. One bacterial colony was inoculated per branch. A fifth set of trees was stabbed with sterile pins only to serve as a control. All of the trees were placed in a growth chamber at 21°C. At intervals of 2, 4, and 7 days after inoculation, one set of trees was transferred to a second growth chamber. This chamber was set at 35±2°C for the T-2789 experiments or 36±2°C for the A-0181 experiments. The temperature for the second trials with

the atypical isolate was set 2°C higher than in the first trial. Each set of trees was left at the elevated temperature for a period of 8 to 18 days depending on the experiment. Both growth chambers had a 16 h photoperiod of 80 μE m^{-2}sec^{-1} provided by a mixture of cool white fluorescent and incandescent lights.

After the heat treatment, the trees were transferred back to either the 21°C chamber or out into the greenhouse for a further 15 to 18 days for the early trials or for a further 3.5 to 5.5 months for the later trials. The trees were maintained at 21°C for a period of time after the heat treatment to allow for growth at the site of inoculation that may have been hindered at the higher temperatures and also to provide for further growth of the controls to enhance the difference in the treatment responses. The set of wound-only trees and one set of inoculated trees were kept at 21°C for the entire experiment to serve as controls.

Growth Index:

Diameters of the branches at the inoculation sites and just below the inoculation sites were measured, with calipers, upon removal from the high temperature treatment and at the termination of the experiment. Ratios of the two branch measurements were calculated as an index of growth at the inoculation site.

Direct Isolations/Cell ELISA:

The inoculation sites, three per tree, were photographed and then ground up in 0.5 to 1.0 ml of LB in sterile dimple plates. The suspensions were left at room temperature for 15-20 min to permit the release of the bacteria from the host

tissue. For direct isolations, loopfuls of these suspensions were streaked onto CPG plates and incubated at 21°C for 7-10 days.

For the cell ELISA, microtiter plates were sterilized 15 cm below a UV light (General Electric Germicidal G25T8 25 W) for 15 min. For each inoculation site, 25 μl of the suspension was added to each of three wells containing 175 μl of LB. Aliquots (25 μl) of a late log phase culture were added to one row of each microtiter plate to serve as a control on the cell ELISA. The samples were covered and allowed to grow in the wells at 21°C for 90 to 100 h. CPG plates were streaked with loopfuls from selected wells after this incubation period to test for growth and/or contamination.

After the incubation period, an indirect ELISA as outlined in Chapter 1 was performed. Whole antisera was used for the first trials of this experiment for both isolates while IgG fractions were used for the second trials. Whole antisera dilutions of 10^{-5} and 10^{-3} for the T-2789 and A-0181 isolates, respectively, and IgG fraction dilutions of 10^{-4} and 10^{-3} for the T-2789 and A-0181 isolates, respectively, were used. Absorbance values (405 nm) were read after 1.5 and 12.5 h for the first and second trials, respectively.

Statistical Analysis:

Results of the heat treatment experiments were analyzed using the General Linear Model, with differences between treatment means evaluated with Tukey's Multiple Range Test (Statistical Analysis Systems, SAS Institute Inc., Cary, NC, USA).

RESULTS

T-2789 isolate:

None of the wound-only or heat-treated branches formed galls in either trial with the T-2789 isolate (Tables 2.1 and 2.2). Even when the heat treatment was delayed until 1 week after inoculation, no galls were formed. Galls had formed on each inoculated branch of the inoculated control trees kept at 21°C throughout (Fig. 2.1). A minimum of five trees with 3 branches per tree were tested per treatment.

In both trials, the ratios of inoculation site diameter/branch diameter numerically reflected these observations with the inoculated control trees producing significantly higher values than any of the other treatments. In addition to the significant differences between the controls and the heat treatments in each of the trials, the differences between the individual trees were also statistically significant.

Ratios for the wound-only sets and all the heat-treated sets were not significantly different from each other. Ratio means for inoculated control trees, of the second trial, increased 28% over the three months after the heat treatments (Table 2.2). Ratio means for the wound-only and the heat-treated sets increased only 0% and 8%, respectively, over the same time period.

No characteristic bacteria were recovered from any of the wound-only or heat-treated trees by direct isolation onto CPG

Table 2.1. Gall formation by and recovery of the pathogen from heat-treated (35°C for 18 days) Douglas-fir seedlings stab-inoculated with the typical *Erwinia* isolate T-2789. Inoculation sites were indexed for the presence of live bacteria by direct isolation onto CPG media and by a cell ELISA using whole T-2789 antiserum

Inoc- ulated with isolate	Heat treatment		Gall diameter/ stem diameter		Bacteria detected in inoculation site	
	Days at		Days after inoculation		Direct	Absorbance
	21°C	35°C	24 d	34 d	isolation	405nm
−	24	0	1.3c[1]	1.3b	0/10	0.02c
+	24	0	2.5a	3.2a	20/20	0.85a
+	2	18	1.2c	1.3b	0/20	0.08bc
+	4	18	1.4bc	1.4b	0/20	0.08bc
+	6	18	1.4b	1.5b	0/20	0.10b

[1]means in a column followed by the same letter are not significantly different (P=0.05) according to Tukey's multiple range test.

Table 2.2. Gall formation by and recovery of the pathogen from heat-treated (35°C for 12 days) Douglas-fir seedlings stab-inoculated with the typical *Erwinia* isolate T-2789. Inoculation sites were indexed for the presence of live bacteria by direct isolation onto CPG media and by a cell ELISA using the IgG fraction of the T-2789 antiserum

Inoc-ulated with isolate	Heat treatment		Gall diameter/ stem diameter		Bacteria detected in inoculation site	
	Days at 21°C	35°C	Days after inoculation 26 d	108 d	Direct isolation	Absorbance 405nm
–	19	0	1.2b[1]	1.1b	0/16	0.00c
+	19	0	1.4a	2.0a	16/16	0.95a
+	2	12	1.1b	1.2b	0/16	0.00c
+	4	12	1.1b	1.3b	0/16	0.01c
+	7	12	1.1b	1.2b	0/16	0.05b

[1]means in a column followed by the same letter are not significantly different (P=0.05) according to Tukey's multiple range test.
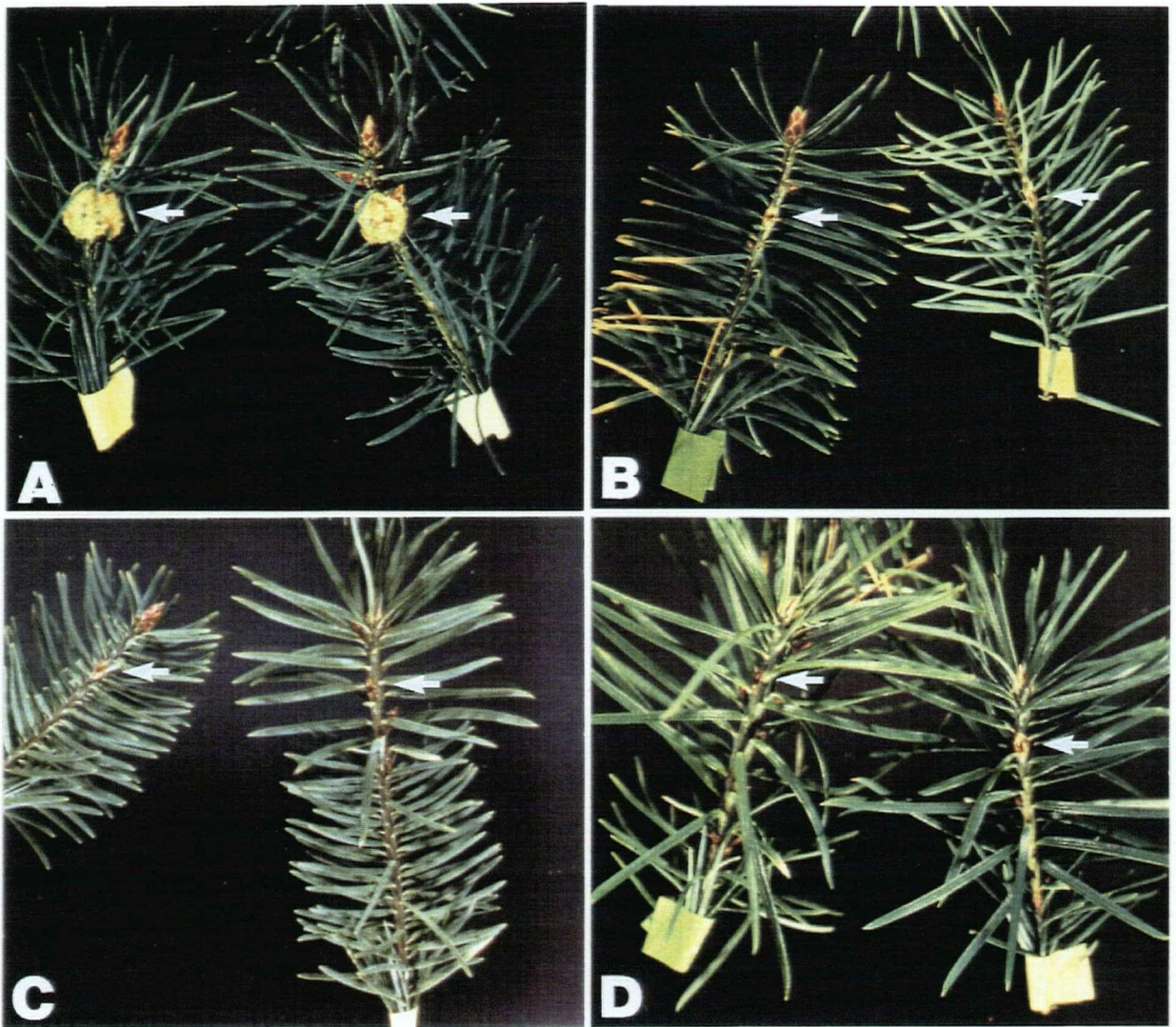
Fig. 2.1. The effect of a high temperature treatment (35±2°C),
applied at various times after inoculation with gall-forming
Erwinia isolate T-2789, on the development of galling symptoms
on Douglas-fir. (A) Inoculated control trees kept at 21°C
throughout the experiment. Inoculated trees (B,C,D) subjected
to the 18-day heat treatment, 2, 4 and 7 days, respectively,
after inoculation.

plates (Tables 2.1 and 2.2). Pure cultures of the inoculated bacteria were recovered in large numbers from each of the inoculated control tree branches.

Cell ELISA results confirmed the direct isolation data that living bacteria were recovered only from the inoculated control trees kept at 21°C throughout (Tables 2.1 and 2.2). Results from the two experiments showed ELISA values for the control trees to be significantly greater than those for the wound-only or any of the heat-treated trees. The mean ELISA value for plants receiving the 7-day heat treatment in the first trial averaged less than 0.10 when the background wound-only value was subtracted. All the wound-only and heat-treated trees in the second trial produced ELISA values less than the cutoff absorbance value of 0.10. None of the CPG plates streaked from the ELISA plate wells of heat-treated branches showed the presence of live *Erwinia* T-2789 bacteria in the microtiter plate wells.

A-0181 Isolate:

Although a high mortality rate was observed in inoculated trees exposed to the 36±2°C heat treatment of both trials, no galls were observed at inoculation sites amongst the survivors (Tables 2.3 and 2.4). A minimum of three trees with four branches per tree were tested per treatment except for the 7-day treatment in the second trial where only two trees survived. Ratios of inoculation site diameter over branch diameter for the inoculated control trees were over 1.5 times greater than those for all of the wound-only and the three heat-treated sets.

Table 2.3. Gall formation by and recovery of the pathogen from heat-treated (36°C) Douglas-fir seedlings stab-inoculated with the atypical *Erwinia* isolate A-0181. Inoculation sites were indexed for the presence of live bacteria by direct isolation onto CPG media and by a cell ELISA using whole A-0181 antiserum

Inoc- ulated with isolate	Heat treatment		Gall diameter/ stem diameter		Bacteria detected in inoculation site	
	Days at 21°C	36°C	Days after inoculation 17 d	29 d	Direct isolation	Absorbance 405nm
-	13	0	1.4b[1]	1.4b	0/5	0.04c
+	13	0	1.6a	2.2a	10/10	0.19a
+	2	10	1.1c	1.4b	2/5	0.21a
+	4	8	1.2bc	1.4b	4/8	0.12b
+	7	6	1.2bc	1.6b	7/7	0.13b

[1]means in a column followed by the same letter are not significantly different (P=0.05) according to Tukey's multiple range test.

Table 2.4. Gall formation by and recovery of the
pathogen from heat-treated (38°C) Douglas-fir
seedlings stab-inoculated with the atypical *Erwinia*
isolate A-0181. Inoculation sites were indexed for
the presence of live bacteria by direct isolation onto
CPG media and by a cell ELISA using the IgG fraction
of the A-0181 antiserum

Inoc-ulated with isolate	Heat treatment		Gall diameter/ stem diameter	Bacteria detected in inoculation site	
	Days at		Days after inoculation	Direct	Absorbance
	21°C	38°C	165 d	isolation	405nm
−	18	0	1.1b[1]	1/16	0.03b
+	18	0	2.2a	10/12	0.71a
+	2	11	1.1b	2/16	0.07b
+	4	11	1.2b	0/12	0.05b
+	7	11	1.2b	0/4	0.03b

[1]means in a column followed by the same letter are not
 significantly different (P=0.05) according to Tukey's
 multiple range test.

Ratios for the wound-only sets and all the heat-treated sets were not significantly different from each other at the end of both trials. Similar to the experiments with the typical T-2789 isolate, differences between the individual trees, in addition to the differences between the controls and the heat treatments, were significant.

In both trials with A-0181, live bacteria were recovered from each inoculated control branch tested using direct isolations. In the first trial, however, live bacteria were also recovered from each of the heat-treated sets but none from the wound-only trees. In the second trial, direct isolation showed survival and recovery of characteristic bacteria from two of the 16 heat-treated branches of the 2-day treatment. Also characteristic bacteria were recovered from one of the wound-only tree branches, presumably due to experimental error. Immunodiffusional analysis confirmed the identity of the recovered A-0181 isolate.

The mean ELISA value for the 2-day heat-treated trees in the first trial averaged above the cutoff absorbance value of 0.10, even when the background wound-only value was subtracted. Furthermore, the average value for the 2-day heat-treated trees was not significantly different from the average inoculated control value when analyzed with Tukey's multiple range test (Table 2.3). Values for the other heat-treated sets and the wound-only set were significantly different from those of the inoculated controls. Mean cell ELISA values for the inoculated control trees, in the second trial, were significantly greater than for any of the other treatments (Table 2.4). The average

ELISA values for the wound-only and the three heat-treated sets in the second trial were all less than the cutoff value and hence were negative. Plate checks on the ELISA assays for both trials showed recovery of bacteria corresponding to the direct isolation data.

DISCUSSION

These heat treatment experiments demonstrated that for both the gall-forming *Erwinia* T-2789 and A-0181 isolates, the presence of live bacteria was necessary for gall initiation and development on Douglas-fir seedlings. Galls only formed on those trees not subjected to the heat treatment and hence only on those trees with live pathogenic bacteria. No evidence was obtained suggesting that transformation of plant cells had occurred.

In order to demonstrate that a transfer of genes to the host plant did take place or alternatively that the bacteria's presence was required for gall formation, it was necessary to kill the inoculated bacteria. The T-2789 and A-0181 isolates are able to grow in liquid culture up to temperatures of 32°C and 34°C, respectively. The combination of temperature and length of time at that temperature which the host trees could withstand was determined empirically. Preliminary experiments involving heat treatment to kill the gall-forming *Erwinia* spp. revealed that the Douglas-fir seedlings would not reliably survive heat treatments of 35-38°C lasting more than about 10 days. Unfortunately each batch of trees and each tree within each experiment reacted differently to the heat treatment. The anticipated high mortality rate was the reason for the use of 10-12 trees per treatment and four inoculation sites per tree.

Differences in temperature and length of heat treatments between experiments were due to the modification of these variables according to the health and survival of the hosts as

well as to the different temperatures required to kill the two isolate types. A higher temperature was required to kill the A-0181 isolate and this is reflected in the resultant higher host mortality rates. The trees were incubated after the heat treatments in the second trials to increase the differences observed between the treatments as well as to allow for the increase in number and hence ease of detection of live bacteria in the heat treated trees by either direct isolation or by cell ELISA.

The initial incubation periods at 21°C of 2, 4 and 7 days were decided upon to encompass the time frame within which potential transformation was expected to occur. Initially, the time required for transformation of host plants by *A. tumefaciens* was experimentally determined to occur within 4 days (Braun 1943). Further research demonstrated that transformation begins approximately 4 h after inoculation and is completed by 8 h after inoculation (Sykes and Matthysse, 1986). Galling symptoms caused by the *Erwinia* spp. are visible within approximately 2 weeks of stab-inoculation. Thus, 7 days was expected to be adequate time to allow transformation by the *Erwinia* spp. to take place if such an event was involved in gall formation. In an attempt to narrow the time frame, in the case of a transfer of genes to the host plant, intervals of 4 and 2 days were also included.

Visual observations and diameter measurements of the inoculation sites were correlated with techniques aimed at the detection of live bacteria at those sites. Ratios of inoculation site diameter over branch diameter reflected the

lack of gall formation on the heat-treated and wound-only trees. Some swelling or growth occurred at the inoculation site of heat-treated or wound-only trees possibly due to wound healing.

Direct isolation was used as a basic selective recovery technique. A cell ELISA was used as a second test to determine the presence of live inoculated bacteria because of its sensitivity and identification properties as a serological assay. Direct isolation from inoculation sites of heat-treated trees demonstrated the bacteriocidal effect of the heat treatment, especially in the T-2789 trials. Live bacteria were recovered by direct isolation from a few of the heat-treated branches inoculated with the A-0181 isolate. This shows the survivability of the A-0181 isolate at temperatures up to 39-40°C. One colony was sufficient to produce a positive result in the direct isolations. The corresponding average ratio measurements and cell ELISA values for these few heat treated branches were not significantly different from the wound-only trees. This would suggest that the small numbers of recovered bacteria were not enough to produce a visible gall. From all but a couple of inoculated control branches, which unfortunately died during the course of the experiments, pure cultures of the isolates were recovered, which corresponds to every inoculation site that formed a gall.

Serological assays are often noted for their sensitivity in detection of the antigen in either low concentration or in mixtures of antigens. The degree of sensitivity of the assay depends in part on the specificity and sensitivity of the antiserum used. From Chapter One, it was demonstrated that the

antisera towards the two *Erwinia* isolates, especially the IgG fractions, were essentially isolate specific. However, the detection levels of the IgG fractions using an indirect ELISA were not very sensitive with 5×10^6 and 5×10^7 cells/ml being the limit of detection of their homologous antigens of the purified T-2789 and A-0181 antisera, respectively. The lower limit for the detection of *Erwinia carotovora* ssp. *carotovora* using a double antibody sandwich ELISA was 10^5 to 10^6 cells per ml (Caron and Copeman, 1984) while the lower limit of detection for *Pseudomonas phaseolicola* using the same assay was 10^4 cells per ml (Barzic and Trigalet, 1982). Direct comparisons to these values cannot be made as the detection assay used in this Chapter was a modification of the basic double antibody sandwich method. An enrichment step, in which the test samples are incubated for a period of time in Luria Broth, was incorporated into the assay to increase the cell concentration in the sample and hence the possibilities of detection. Therefore, the overall detection levels of the cell ELISAs were lower than those indicated in the indirect ELISA specificity tests of Chapter One.

Large numbers of live gall-forming *Erwinia* bacteria were recovered from the inoculated control trees according to the cell ELISA data for both the T-2789 and A-0181 isolates. Background readings were recorded for most of the wound-only and heat-treated trees. In the earlier trials, some of the heat-treated inoculation sites, a few of which were visibly suffering from the effects of the treatment, recorded ELISA values just over the cutoff value of 0.10. These results, when combined

with the diameter ratios and the direct isolation data, suggest that the serological assay was detecting either very low concentrations of the inoculated bacteria and/or dead bacterial cells in the host tissue. The heat-treated branches when tested by ELISA in the later trials, using the purified antisera, did not produce these low positive values. In each of the trials, absorbance readings for the inoculated control trees were significantly greater than those of any of the other treatments in accordance with the visual observations.

Several methods have been used to either investigate the mechanism of gall initiation and development or to determine the successful transformation of plants by *A. tumefaciens*. The design of the heat treatment experiment was chosen in part because it was a simple technique that was used in early experiments with *A. tumefaciens* (Braun 1943). One of the more recent and direct approaches to answer the question of gall formation is to probe for transferred bacterial genes, specifically the phytohormone genes, within the host plant genome. This was not seriously considered due to the extensive analysis that would be required to reach a conclusive answer (Sederoff *et al*. 1986).

Another method, besides the heat treatment, that could answer the question of the mechanism of gall formation is the use of an analogous treatment in a tissue culture system. Culture of tumor tissues and/or detection of phytohormones were not chosen as primary methods of determination due to anticipated problems. Sederoff *et al*. (1986) found that *A. tumefaciens* infected pine callus did not grow on hormone-free

media. The transfer of bacterial genes to the host plant may take place but the expression of the phytohormone genes, in some cases, is not sufficient to allow for autotrophic growth *in vitro*. Recently however, Clark *et al.* (1989) demonstrated, using a tissue culture system, that *E. herbicola* f.sp. *gypsophilae* does not transfer phytohormone genes to the host plant's genome.

The detection of phytohormones in culture is not sufficient to determine the primary producer of phytohormones in the plant-pathogen interaction. *A. tumefaciens* as well as *P. syringae* pv. *savastanoi* produces phytohormones in culture albeit in a considerably lower level (Smidt and Kosuge, 1978; Akiyoshi *et al.*, 1987). Often such investigations can lead to ambiguous results in relation to the question of gall initiation and development. To this end, the heat treatments, modelled after the original *A. tumefaciens* experiments, provided a simple yet conclusive answer to the role of the gall-forming bacterium in the formation of the gall.

In the early heat treatment experiments with *A. tumefaciens*, galls incited with virulent bacteria continued to develop although live bacteria were no longer present, four or more days post-inoculation (Braun, 1943). This suggested that a transformation of the host cells had occurred to warrant continued unregulated growth and division. In the present experiments, the presence of live bacteria is required with both the *Erwinia* T-2789 and the A-0181 isolate for continued gall development to take place. This would indicate that no transfer of genes to the host plant genome occurs, at least not within 7

days of inoculation, as is the case in the *Agrobacterium tumefaciens* system. A mechanism of gall formation closer to that of the *Pseudomonas syringae* pv. *savastanoi* system is likely to be the case with the gall-forming *Erwinia* spp. Thus, the gall-forming *Erwinia* spp. are not potential gene vectors for the genetic improvement of Douglas-fir.

CHAPTER 3

THE RELATIONSHIP
BETWEEN THE PLASMID CONTENT OF THE GALL-FORMING
ERWINIA ISOLATES AND PATHOGENESIS

INTRODUCTION

Plasmids in both *A. tumefaciens* and *P. syringae* pv. *savastanoi* carry genetic determinants that code for the synthesis of auxins and cytokinins, which confer pathogenicity. Thus, it was of interest to determine the plasmid content of the gall-forming *Erwinia*. Bacterial plasmids range in size between 1 kb and greater than 300 kb (Trevors, 1985). Larger sized plasmids often are more difficult to isolate due to the shearing of the DNA during the extraction process. Furthermore, each plasmid has a characteristic copy number under defined conditions. Plasmids present in multiple copies, characteristically the smaller plasmids, are under relaxed control while large (>50 kb) plasmids are under stringent control and hence are in a lower copy number (Broda, 1979). Plasmids are usually found in a double-stranded, covalently closed circular form. However, during the isolation process, one or both strands can be nicked resulting in either an open circular or a linear form. These different forms, along with multimeric forms, can complicate the interpretation of plasmid profiles.

Profiles of plasmid content have been used in bacterial

strain identification (Lazo *et al.*, 1987). Virulent strains of *E. stewartii* contain at least eight plasmids that range in size from 4.1 to approximately 320 kb. A 30 kb plasmid, pEA28, is found in all strains of *E. amylovora*. Conserved plasmids have also been found in *Clavibacter michiganense* subsp. *sepedonicum*, *Curtobacterium flaccumfaciens* pv. *pointsettae* and *Rhodococcus fascians* (Coplin, 1989).

Plasmid DNA is also used for probes in DNA:DNA hybridizations for the identification or differentiation of phytopathogenic strains, as well as for the determination of homology between various DNA fragments (Gilbertson *et al.*, 1989). Plasmid sequences are often in higher copy number than those on the chromosome producing a stronger hybridization signal (Coplin, 1989). Conserved plasmid sequences occur between the plasmid DNA of *A. tumefaciens* and *P. syringae* pv. *savastanoi*. The homology occurs between the genes encoding for enzymes involved in auxin and cytokinin production (Powell and Morris, 1986; Follin *et al.*, 1985). As in this case, DNA homology is often related to common biological function (Von Bodman and Shaw, 1987). In relation to the gall-forming *Erwinia*, conserved DNA sequences between the plasmid DNA of the two types could further explain their relationship.

One of the original methods to determine the function of plasmid genes is to cure the bacterium of the plasmid. Curing a bacterium of its plasmid(s) results in the production of plasmid-free derivatives of the wild-type bacterium. Phenotypes of the cured strains are then compared with those of the wild-type strains. Some methods of curing include the use of a heat

treatment such as used with *Agrobacterium tumefaciens* (Watson *et al.*, 1975), *Erwinia herbicola* (Chatterjee and Gibbins, 1971) and *Xanthomonas campestris* pv. *vignicola* (Ulaganathan and Mahadevan, 1988). Chemical curing agents such as acridine orange, sodium dodecyl sulfate (SDS) and ethidium bromide have also been used to successfully cure bacteria of one or more of their plasmids (Sonstein and Baldwin, 1972). Acridine orange was used for curing the pIAA plasmid from *P. syringae* pv. *savastanoi* (Watanabe and Fukasawa, 1961; Comai and Kosuge, 1980).

The objectives of these experiments included the determination of the plasmid content of the two gall-forming *Erwinia* isolate types. Secondly, experiments aimed at determining the presence of homology between the plasmid content of the two isolate types were carried out. The final objective of this section was to determine the role, if any, of the plasmid DNA of the gall-forming *Erwinia* spp. in the development of galls.

MATERIALS AND METHODS

Bacterial Strains:

The typical isolate, T-2774, and the atypical A-0181
isolate were the main isolates used in these experiments. The
other bacterial strains used and their sources are as outlined
in Table 1.1 in Chapter 1. The chemicals used were from the
Sigma Chemical Company unless otherwise noted.

Total DNA Isolation:

Single bacterial colonies were inoculated into 5 ml of the
appropriate broth and agitated at 200 rpm at room temperature
for 2-4 days, depending upon the bacterial species. Bacterial
cells were harvested by centrifugation at 2100 X g (maximum) for
10 min at 4°C. Pellets were resuspended in 100 μl ice cold
Tris-EDTA-Glucose (Maniatis *et al.*, 1982). A lysing solution
(200 μl) consisting of lysozyme (5 mg/ml), SDS (1%) and
Proteinase K (2μg/ml) was added and gently mixed by tilting the
tube. The cell suspensions were incubated at 60-65°C for 2.5-
3.0 h. The resulting cell lysates were extracted with phenol
and chloroform. The DNA was precipitated with ethanol and
collected by centrifugation. Resuspended DNA samples were
sheared by passage through 22 gauge needles seven to eight
times. An RNase A treatment (20 μg/ml for 10 min at 37°C) was
followed by another ethanol precipitation step to remove the
digested RNA and the RNase.

Plasmid Isolation and Purification:

Several different plasmid extraction protocols were attempted for the gall-forming *Erwinia* isolates: lysis by boiling (Holmes and Quigley, 1981 as outlined in Maniatis *et al.*, 1982), lysis by SDS (Godson and Vapnek, 1973 as outlined in Maniatis *et al.*, 1982) and lysis by alkali (modified Birnboim and Doly, 1979 as outlined in Maniatis *et al.*, 1982; Kado and Liu, 1981; Hardy, 1985). The alkaline extraction protocol as outlined in Maniatis *et al.* (1982) was adopted as the best and simplest procedure to use. For both typical strains and A-0181, minipreps were carried out on 5 ml late log phase LB cultures. For large scale alkaline lysis of A-0181, an initial volume of 1 liter of late log phase LB culture was harvested.

Different methods were used to purify the plasmid DNA from the two *Erwinia* isolates to determine the method with the highest recovery of purified plasmid DNA. Electroelution of the plasmid DNA into dialysis bags, troughs and onto dialysis membranes as well as purification through Low Gelling Temperature agarose (BioRad) and GeneClean (BioRad) were all tested. For the typical isolates, DNA obtained from minipreps was run through agarose gels and gel slices containing the plasmid DNA were cut out. The plasmid bands were then electroeluted into dialysis tubes or onto dialysis membranes using a Biorad Electro-elutor Model 422.

For the A-0181 isolate, large scale plasmid DNA extractions were followed by separation on a cesium chloride (CsCl) gradient. Ethidium bromide was added at a concentration of 0.5 mg/ml. Samples were centrifuged at 170,000 X g (maximum) for 65

h on a Type 65 fixed angle rotor (Beckman). Bands were viewed over a mid-wave U.V. transilluminator (Model TM36 Ultraviolet Products, Inc. San Gabriel, CA) and removed via an 18 gauge needle. Ethidium bromide was removed from the solution by extraction with equal volumes of 1-butanol. This procedure was repeated at least six times or until the aqueous phase no longer displayed a pinkish color. The resulting sample was then concentrated via ethanol precipitation. Further purification of individual A-0181 bands was accomplished by isolation from Low Gelling Temperature agarose or by using GeneClean. DNA concentrations were measured at 260 nm on a Hewlett Packard Spectrophotometer (the absorbance values multiplied by 50 to obtain μg/ml) or by visualization on an agarose gel if the sample concentration was too low.

Restriction enzyme digests were performed in a reaction volume of 20 μl with up to 1.0 μg of DNA and at least two times excess enzyme. Reactions were carried out at 37°C for 2 h. HindIII-digested lambda DNA (fragment sizes: 23.13, 9.42, 6.56, 4.36, 2.32, 2.03, 0.56 and 0.13 kb) was used as DNA size markers.

Samples were run on a Pharmacia horizontal gel electrophoresis apparatus (Model GNA-100) in 0.7% (0.9% for digests) agarose at 60-90 mA for 2 to 2.5 h in 1X Tris-acetate-EDTA (TAE) pH 8.0 (Maniatis *et al.*, 1982) with 0.5 ug/ml ethidium bromide in the gel and in the running buffer. Gels were viewed over a mid-wave ultraviolet transilluminator. Photographs of the gels were taken using a 605 mu narrow band pass filter and Polaroid Type 57 sheet film.

Southern Blots:

DNA samples, digested or undigested, were transferred from agarose gels to Zeta-Probe (BioRad) membranes by an alkaline blotting procedure as outlined for Nytran nylon membranes (Schleicher & Schuell). After photographs were taken, the gels were gently shaken in 2 volumes of 0.25M HCl for 8-10 min to facilitate transfer of the DNA through fragmentation of the DNA strands. Gentle shaking again accompanied two, 15-min incubations in 2 volumes of the blotting solution (0.5M NaOH and 1.5M NaCl). The gel was then placed on a wick made up of three layers of Whatman 3MM paper, saturated with the blotting solution. A piece of Zeta-Probe membrane, soaked for 5 min in the blotting solution and nicked on the top right corner for orientation, was placed on the gel and smoothened out to avoid bubbles underneath. Three layers of Whatman 3MM, saturated with the blotting solution, were placed on the membrane, followed by six layers of dry filter paper. A stack of paper towels, measuring 15-20 cm in depth, was placed on top. The wick was resting on a piece of plexiglass with the ends of the wick soaking in a container full of the blotting solution. The entire apparatus was wrapped in Saran Wrap to avoid excessive evaporation. A piece of glass and a small weight was placed on top of the pile to level the structure. The DNA was then allowed to transfer to the membrane via capillary action for 16-18 h.

Once the DNA was transferred, the paper towels were taken off and then the remaining layers, down to the wick, were carefully turned over. A medium ballpoint pen was used to mark

the lanes of the gel and to date the Zeta-probe membrane. To
ensure the transfer process was complete, the gel was soaked in
0.5 ug/ml of ethidium bromide and viewed over a transillum-
inator. The membrane was soaked in a small volume of 5X SSC
(saline sodium citrate) (Maniatis *et al.*, 1982) for 5 min,
placed between 2 sheets of Whatman 3MM paper, wrapped loosely in
aluminum foil and baked at 60°C for 2 h. Before use in
hybridization, blots were stored at room temperature.

Curing Protocols:

Actively growing cultures of the typical T-2774 or the
atypical A-0181 isolate were diluted 1/1000 into Luria broth and
treated with one of the following curing agents: sodium dodecyl
sulfate (SDS), ethidium bromide or acridine orange at varying
concentrations (Table 3.1). The shake cultures were incubated
at elevated temperatures (28°C for T-2774 and 31°C for A-0181)
for 48 h. One or two successive transfers were made with the
curing agents. Resulting cultures were stored briefly at 4°C or
−80°C before dilution plating.

The potentially cured atypical colonies were probed with
[32]P-labelled plasmid DNA from the atypical A-0181 isolate and the
potentially cured typical colonies were probed with ^{32}P-labelled
plasmid DNA from the typical T-2774 isolate.

Table 3.1. Concentrations of chemical curing agents
added to LB in curing protocols used with the gall-
forming *Erwinia* isolates T-2774 and A-0181

Gall-forming *Erwinia* isolate	Sodium dodecyl sulfate (%)	Acridine orange (μg/ml)	Ethidium bromide (μg/ml)	Temp-erature ($^{\circ}$C)
T-2774	0.0175	60	15	28
A-0181	0.0300	70	40	31

Colony Lifts:

Grid plates or dilution plates of the colonies of interest were grown up for 2 days on nutrient agar (NA) plates and then the colonies were transferred to BioRad CP/Lift membranes according to the accompanying instructions. The membranes were carefully placed on the agar surface for 3-5 min. Identifying patterns were made on the membranes and concomitantly on the plates with an 18 gauge needle. The membranes were placed, colony side up, on 3-5 layers of Whatman 3MM paper saturated with 0.5 M NaOH for 5 min followed by a brief blotting on dry Whatman 3MM paper. This step was repeated once to ensure lysis of the cells. The membranes were then rinsed in 2X SSC and 0.2% SDS briefly to remove cell debris. After blotting dry on Whatman 3MM, the C/P Lift membranes were exposed to UV light (General Electric Germicidal G25T8 25 W) for 5-10 min to ensure covalent linkage of the DNA to the nylon membrane. The membranes were stored, if necessary, at room temperature.

^{32}P Labelling:

Plasmid DNA was labelled using the BRL random primers DNA labelling system. An initial attempt using nick translation (Pharmacia) was not successful. The T-2774 probe was prepared from miniprep DNA that had been purified through electroelution. The A-0181 probe was prepared from CsCl-purified DNA, further purified through GeneClean. Approximately 50 ng of the wild-type plasmid DNA was digested with Pst 1 in a maximum volume of 12 μl. The protocol as outlined by BRL (Bethesda Research Labs), was followed to label the DNA (Feinberg and Vogelstein, 1984).

The labelled DNA was ethanol-precipitated and resuspended in 300 μl H_2O. The counts per minute for total and incorporated ^{32}P-dATP were measured. Two μl samples (1/150th of the total sample) were spotted onto Whatman GF/C glass-fiber discs. To measure total counts, including both incorporated and unincorporated label, the disc was simply placed in a glass scintillation vial, 10 ml of Aquasol was added and counts measured on a Hewlett-Packard Scintillation Counter. To measure incorporated ^{32}P-dATP, the disc containing the sample was rinsed with 15 ml cold 10% trichloroacetic acid (TCA). After two, 15-ml rinses with cold 95% ethanol, the disc was placed in a glass scintillation vial and 10 ml Aquasol added. Counts were measured on the scintillation counter with % incorporation of ^{32}P-dATP calculated from the readings.

Hybridization:

The C/P Lift and Zeta-probe membranes were sealed in plastic sandwich boxes with approximately 20 ml of hybridization buffer (1mM EDTA, 0.5mM $NaHPO_4$ (pH 7.2), 7% SDS). An incubation period, in a shaking water bath set at 65°C, of 5 min for C/P Lift membranes or 2 h for Zeta-Probe membranes, preceded hybridization. The labelled probe was added to the hybridization buffer after a 5 min denaturation period in a boiling water bath. Hybridization was allowed to proceed for 18-24 h at 65°C (shaking) according to the protocol outlined for the BioRad C/P Lift Membranes.

After the hybridization period, the probe was carefully poured into a 50 ml Falcon tube and stored at -20°C. Hybridization was followed by 4, 30-min washes at 65°C. The

first two washing solutions consisted of 1mM EDTA, 40mM NaHPO$_4$

and 5% SDS, while the final two washes consisted of 1mM EDTA,

40mM NaHPO$_4$ and 1% SDS. An initial rinse with the first washing

solution was used to remove a large percentage of unbound label.

The CP/Lift or Zeta-Probe membranes were wrapped in Saran Wrap

and put up on X-ray film with enhancing screens. The X-ray film

was exposed to the hybridized membranes either at room

temperature or at -70°C for between 3 h and 3 days.

RESULTS

Plasmid Content:

For the typical *Erwinia* isolates, a single plasmid band migrating with any isolated chromosomal DNA was observed (lanes 4-8 in Fig. 3.1). Occasionally a different form of the plasmid was seen migrating behind the chromosomal band (lane 9 in Fig. 3.1). The profile for the atypical A-0181 isolate was more complex, consisting of several bands (lane 3 in Fig. 3.1).

Following plasmid purification by CsCl gradient centrifugation, the profile of the atypical A-0181 isolate contained 4 to 5 bands which appeared to consist of different forms of one or possibly two plasmids (Fig. 3.2). The lower band, D (lane 1), was further purified using LGT agarose, and rerun on an agarose gel, showing a high degree of purification (lane 2). Similarly the second band, C, was purified as shown in lane 3. When the top two bands, A and B, were excised and purified together the resulting pattern displayed the four original bands, suggesting that the lower two bands are modified forms of the upper two bands.

Restriction digests for plasmids from both the typical and the A-0181 isolates, are shown in Fig. 3.3. Digests of the T-2789 plasmid using Pst 1 and Hind III suggested a size of approximately 50 kb (lanes 1 and 2). All digests of the fastest moving A-0181 band, Band D, resulted in only partial digests (lane 4 and 5). When run against Hind III-digested lambda DNA, uncut Band D appeared to be between 10 and 20 kb in size (lanes
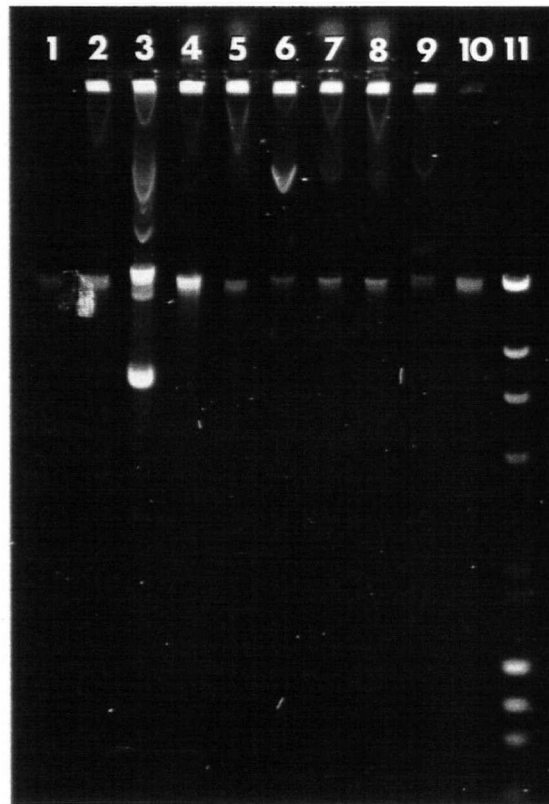
Fig. 3.1. Plasmid DNA profiles of typical and atypical gall-
forming *Erwinia* isolates fractionated on a 0.7% agarose gel and
stained with ethidium bromide. Plasmid DNA was extracted using
the small-scale alkaline lysis protocol as outlined in Maniatis
et al. (1982). Lanes: (1) *Erwinia carotovora* subsp.
carotovora; (2) *E. herbicola* subsp. *herbicola* strain 2273; (3)
atypical isolate A-0181; (4) typical isolate T-2774; (5) typical
isolate T-2789; (6) typical isolate T-2721; (7) typical isolate
T-2739; (8) typical isolate T-2763; (9) typical isolate T-2722;
(10) *Escherichia coli* strain JM101 and (11) HindIII-digested
lambda DNA/HaeIII-digested ØX-174 DNA providing DNA size
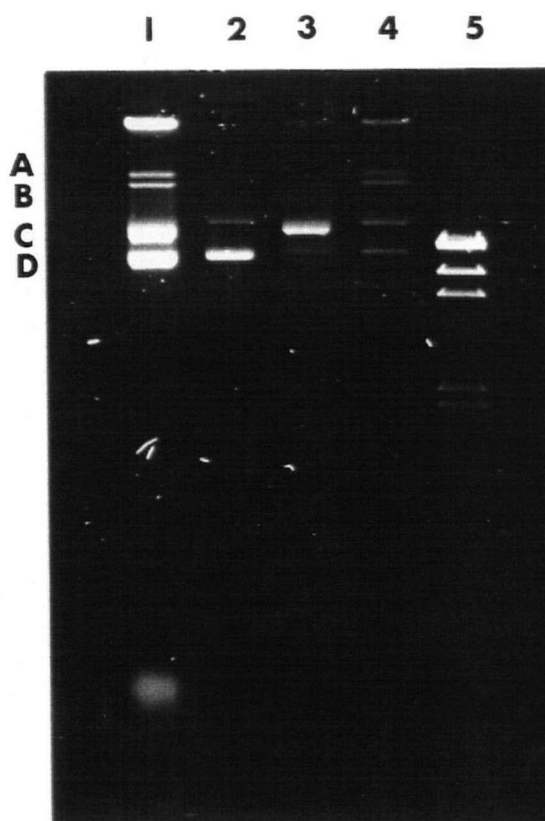standards.

Fig. 3.2.   Plasmid DNA profile of *Erwinia* isolate A-0181
fractionated on a 0.7% agarose gel and stained with ethidium
bromide.   Plasmid DNA was isolated using the large-scale
alkaline lysis protocol as outlined in Maniatis *et al.* (1982)
and purified through cesium chloride gradient centrifugation.
Lanes: (1) Bands A, B, C, D of A-0181; (2) Band D purified
through Low Gelling Temperature (LGT) agarose followed by
phenol/chloroform extraction and ethanol precipitation; (3) Band
C purified through LGT agarose; (4) Bands A and B purified
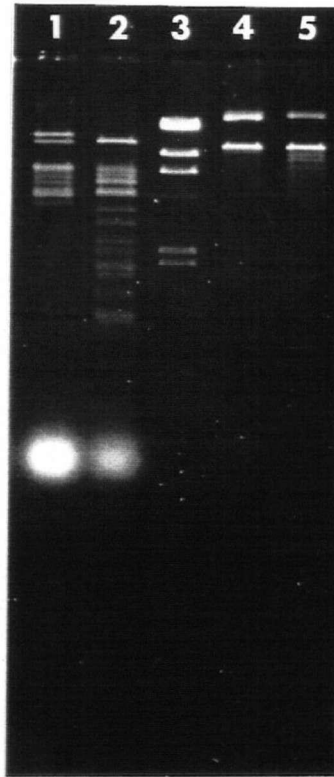through LGT agarose and (5) HindIII-digested lambda DNA marker.

Fig. 3.3. Plasmid DNAs from typical and atypical gall-forming
Erwinia isolates digested with restriction endonucleases,
fractionated on a 0.9% agarose gel and stained with ethidium
bromide. Plasmid DNA was isolated from typical isolates by a
small-scale alkaline lysis procedure (Maniatis *et al.*, 1982) and
from the atypical isolate by a large-scale alkaline lysis
procedure (Maniatis *et al.*, 1982). The atypical plasmid DNA was
further purified through cesium chloride centrifugation and LGT
agarose. Lanes: (1) HindIII-digested typical isolate T-2722;
(2) Pst 1-digested typical isolate T-2722; (3) HindIII-digested
lambda DNA marker; (4) HindIII-digested Band D from atypical
isolate A-0181 (Fig. 3.2) and (5) Pst 1-digested Band D from
atypical isolate A-0181.

1-4 in Fig. 3.1).

Probe Specificity/Homology:

Southern analyses of different bacterial strains using plasmid probes from gall-forming *Erwinia* T-2774 and A-0181 are shown in Figures 3.4, 3.5, 3.6 and 3.7. Lane 6 in Figure 3.4 shows strong hybridization between the typical T-2774 plasmid probe (from a small-scale plasmid DNA preparation) and the total DNA preparation of typical isolate T-2774. Hybridization occurred at a lower degree between the typical T-2774 probe and the A-0181 DNA preparations (lanes 5, 8, 9 and 10). Weak hybridization occurred between the typical probe and the two *Erwinia* spp. (*E. carotovora* and *E. herbicola* subsp. *herbicola* strain 2273) (lanes 3 and 4). No homology was detected between the typical T-2774 probe and the *Escherichia coli* or *Agrobacterium tumefaciens* DNA preparations (lanes 1 and 2). When tested against minipreps of other typical isolates and the atypical isolate A-0181, the T-2774 probe hybridized to each band of each profile of the gall-forming *Erwinia* isolates (Fig. 3.5). No homology was detected between the typical T-2774 probe and the DNA preparations of *Erwinia carotovora* subsp. *carotovora*, *E. herbicola* subsp. *herbicola* strain 2273 and *Escherichia coli* strain JM101 (lanes 1, 2 and 10).

When the CsCl-purified atypical A-0181 plasmid probe was used on similar blots, the hybridization only occurred with the homologous samples (lanes 5, 8, 9 and 12 in Fig. 3.6 and lanes 7 and 13 in Fig. 3.7). The A-0181 plasmid probe hybridized to each plasmid band of A-0181. No hybridization occurred between
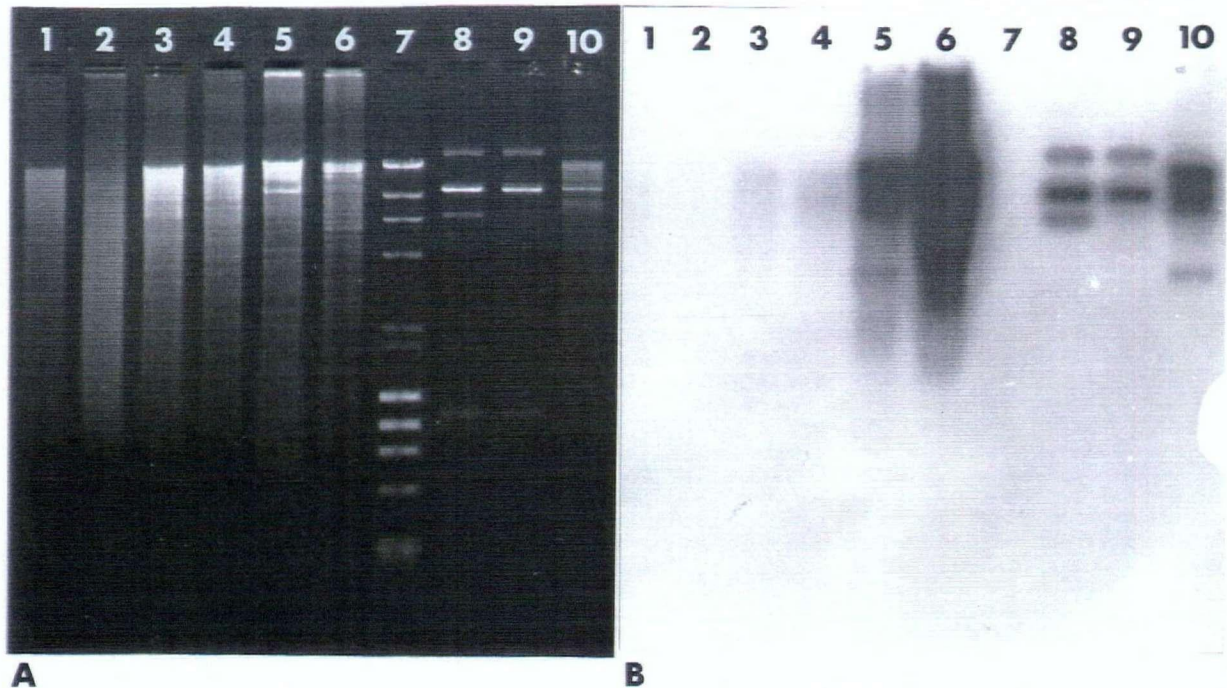
Fig. 3.4. Gel electrophoresis profile and Southern analysis of total genomic and plasmid DNA of various phytopathogenic bacteria probed with plasmid DNA of a typical gall-forming *Erwinia* isolate. A, 0.9% agarose gel stained with ethidium bromide. B, Autoradiogram of Southern blot of DNA transferred to Zeta-Probe membrane and probed with plasmid DNA of typical isolate T-2774 (isolated using the small-scale alkaline lysis procedure as outlined in Maniatis *et al.*, 1982 and purified through electroelution). Lanes: (1) Pst 1-digested total DNA of *Escherichia coli* strain JM101; (2) Pst 1-digested total DNA of *Agrobacterium tumefaciens* strain B-1; (3) Pst 1-digested total DNA of *Erwinia carotovora* subsp. *carotovora*; (4) Pst 1-digested total DNA of *E. herbicola* subsp. *herbicola* strain 2273; (5) Pst 1-digested total DNA of atypical *Erwinia* isolate A-0181; (6) Pst 1-digested total DNA of typical *Erwinia* isolate T-2774; (7) HindIII-digested lambda DNA/HaeIII-digested ΦX-174 DNA; (8) Pst 1-digested plasmid Band D (Fig. 3.2) of atypical *Erwinia* isolate A-0181; (9) Pst 1-digested Band C (Fig. 3.2) of atypical *Erwinia* isolate A-0181 and (10) Pst 1-digested total DNA of atypical *Erwinia* isolate A-0181.
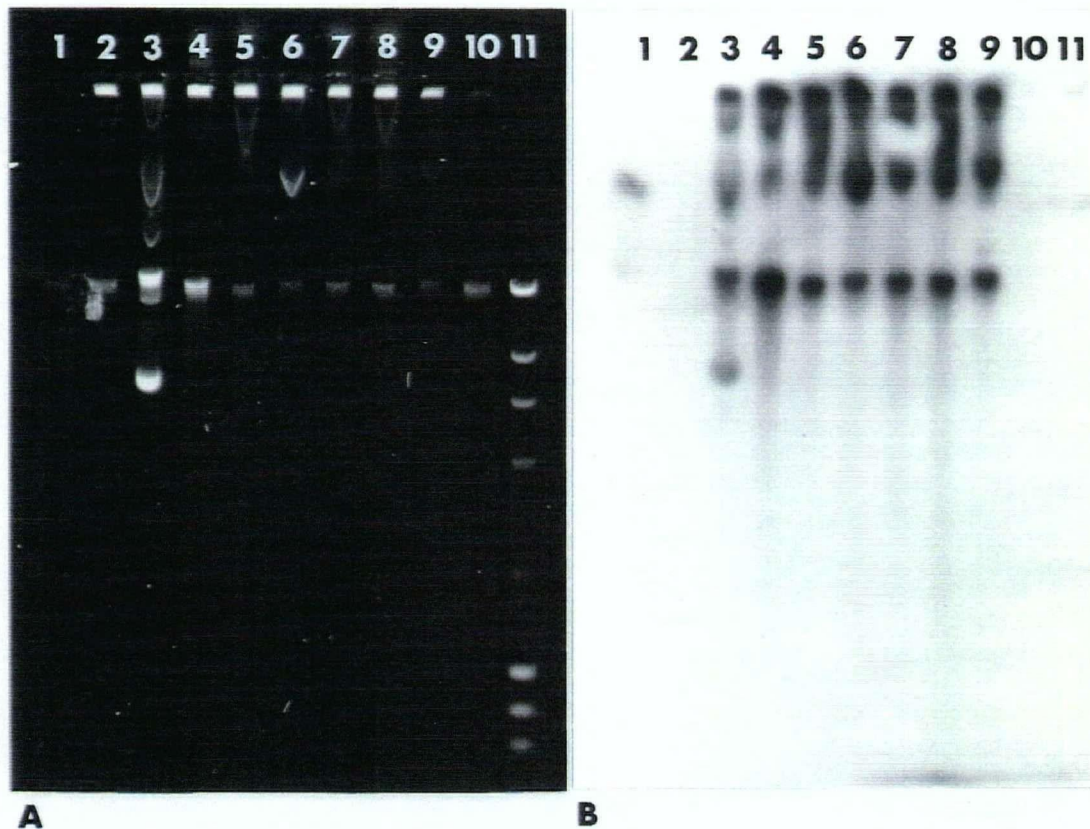
Fig. 3.5. Gel electrophoresis profile and Southern analysis of plasmid DNA of various phytopathogenic bacteria probed with plasmid DNA of a typical gall-forming *Erwinia* isolate. Plasmid DNA was extracted using the small-scale alkaline lysis protocol as outlined in Maniatis *et al.* (1982). A, 0.7% agarose gel stained with ethidium bromide. B, Autoradiogram of Southern blot of DNA transferred to Zeta-Probe membrane probed with plasmid DNA of typical isolate T-2774 (isolated by a small-scale alkaline lysis procedure as outlined in Maniatis *et al.*, 1982 and purified through electroelution). Lanes: (1) *Erwinia carotovora* subsp. *carotovora*; (2) *E. herbicola* subsp. *herbicola* strain 2273; (3) atypical *Erwinia* isolate A-0181; (4) typical *Erwinia* isolate T-2774; (5) typical *Erwinia* isolate T-2789; (6) typical *Erwinia* isolate T-2721; (7) typical *Erwinia* isolate T-2739; (8) typical *Erwinia* isolate T-2763; (9) typical *Erwinia* isolate T-2722; (10) *Escherichia coli* strain JM101 and (11) HindIII-digested lambda DNA/HaeIII-digested ΦX-174 DNA markers.
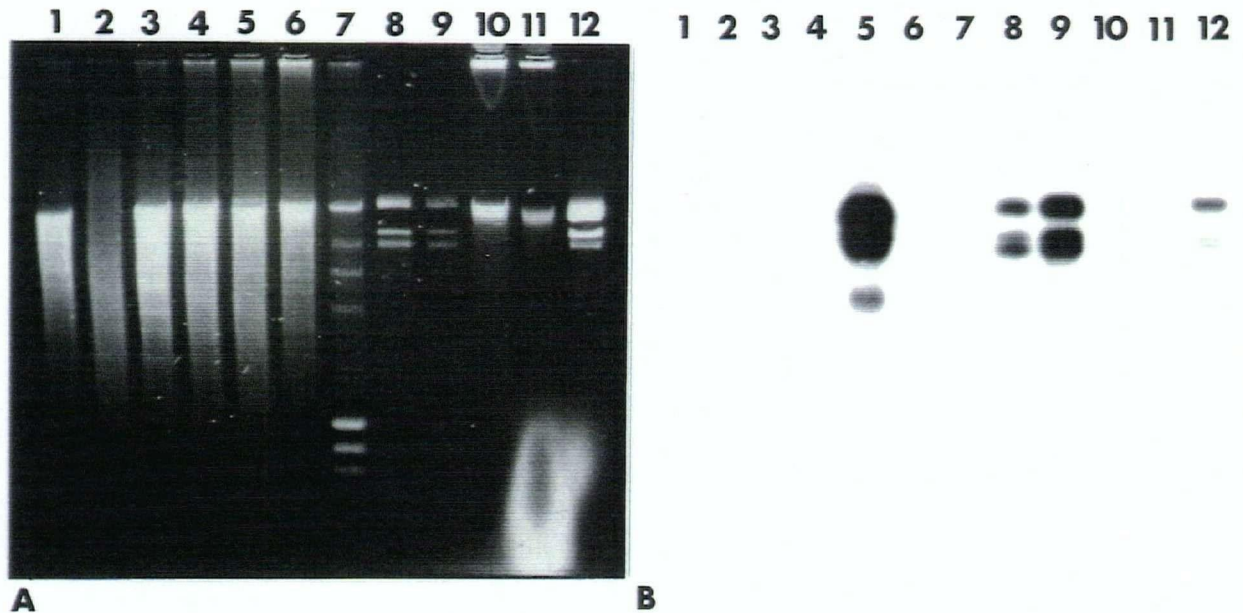
Fig. 3.6. Gel electrophoresis profile and Southern analysis of total genomic and plasmid DNA of various phytopathogenic bacteria probed with plasmid DNA of the atypical gall-forming *Erwinia* isolate. A, 0.9% agarose gel stained with ethidium bromide. B, Autoradiogram of Southern blot of DNA transferred to Zeta-Probe membrane and probed with plasmid DNA of atypical isolate A-0181 (purified through cesium chloride gradient centrifugation and GeneClean). Lanes: (1) Pst 1-digested total DNA of *Escherichia coli* JM101; (2) Pst 1-digested total DNA of *Agrobacterium tumefaciens* strain B-1; (3) Pst 1-digested total DNA of *Erwinia carotovora* subsp. *carotovora*; (4) Pst 1-digested total DNA of *E. herbicola* subsp. *herbicola* strain 2273; (5) Pst 1-digested total DNA of atypical *Erwinia* isolate A-0181; (6) Pst 1-digested total DNA of typical *Erwinia* isolate T-2774; (7) HindIII-digested lambda DNA/HaeIII-digested ΦX-174 DNA; (8) Pst 1-digested plasmid Band D (Fig. 3.2) of atypical *Erwinia* isolate A-0181; (9) uncut plasmid Band D of atypical *Erwinia* isolate A-0181; (10) Pst 1-digested plasmid DNA of typical *Erwinia* isolate T-2774; (11) uncut plasmid DNA of typical *Erwinia* isolate T-2774 and (12) Pst 1-digested plasmid Band D of atypical *Erwinia* isolate A-0181.
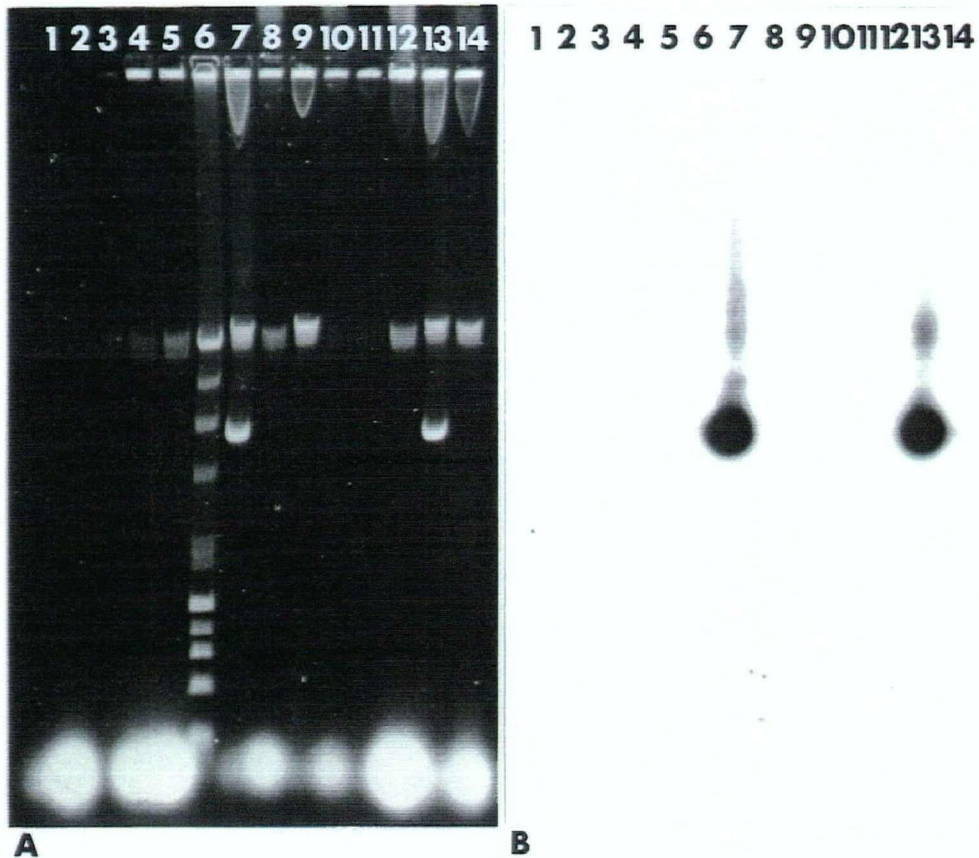
Fig. 3.7. Gel electrophoresis profile and Southern analysis of plasmid DNA of various phytopathogenic bacteria probed with plasmid DNA of the atypical gall-forming *Erwinia* isolate. Plasmid DNA was extracted using the modified small-scale alkaline lysis protocol as outlined in Maniatis *et al.* (1982). A, 0.7% agarose gel stained with ethidium bromide. B, Auto-radiogram of Southern blot of DNA transferred to Zeta-Probe membrane and probed with plasmid DNA of atypical isolate A-0181 (purified through cesium chloride gradient centrifugation and GeneClean). Lanes: (1) *Pseudomonas syringae* pv. *syringae* (cherry strain); (2) *Escherichia coli* strain MC1000; (3) *Agrobacterium pseudotsugae* strain 180; (4) *Erwinia carotovora* subsp. *carotovora*; (5) *E. herbicola* subsp. *herbicola* strain 2273; (6) HindIII-digested lambda DNA/HaeIII-digested ΦX-174 DNA markers; (7) atypical *Erwinia* isolate A-0181; (8) typical *Erwinia* isolate T-2739; (9) typical *Erwinia* isolate T-2721; (10) typical *Erwinia* isolate T-2789; (11) typical *Erwinia* isolate T-2722; (12) typical *Erwinia* isolate T-2774; (13) atypical *Erwinia* isolate A-0181 and (14) typical *Erwinia* isolate T-2763.

the atypical plasmid probe and the typical isolate DNA preparations (lanes 6, 10 and 11 in Fig. 3.6 and lanes 8-12 and 14 in Fig. 3.7).

Curing/Colony Hybridization:

Hybridization occurred between colonies of the typical isolates and the T-2774 probe and to a lesser extent between the colonies of the atypical isolate and the T-2774 DNA probe. No hybridization occurred between *E. carotovora* subsp. *carotovora* and the T-2774 probe (Fig. 3.8).

Hybridization occurred with each potentially cured colony tested of both the typical and atypical isolates, indicating that the plasmids had not been cured from either isolate type (Fig. 3.9). However, because the typical T-2774 DNA probe appeared to contain chromosomal DNA, it is possible that this chromosomal DNA was responsible for the hybridization between the typical DNA probe and the typical colonies. At least 100 colonies per treatment per isolate were tested.
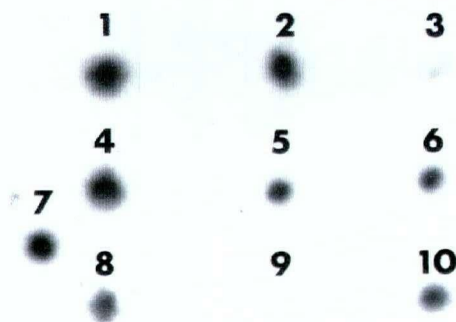
Fig. 3.8. Autoradiogram of colony hybridization of plasmid DNA probe of typical gall-forming *Erwinia* isolate T-2774 with *Erwinia* spp. The DNA for the probe was isolated by a small-scale alkaline lysis procedure as outlined in Maniatis *et al.*, 1982 and purified through electroelution. Colonies of (1) typical *Erwinia* isolate T-2789 (2) typical *Erwinia* isolate T-2774 (3) atypical *Erwinia* isolate A-0181 (4) typical *Erwinia* isolate T-2763 (5) typical *Erwinia* isolate T-2722 (6) typical *Erwinia* isolate T-2721 (7) typical *Erwinia* isolate T-2763 (8) typical *Erwinia* isolate T-2739 (9) *E. carotovora* subsp. *carotovora* and (10) typical *Erwinia* isolate T-2774 were transferred to C/P Lift membranes, lysed and probed with random-primed dAT^{32}P-labelled plasmid DNA.
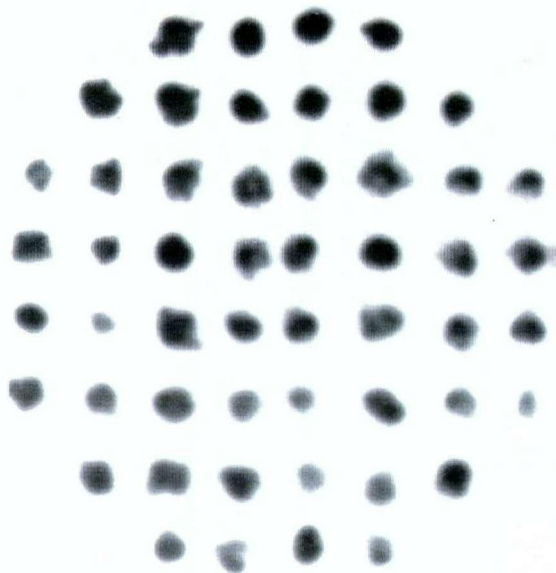
Fig. 3.9. Autoradiogram of colony hybridization of dAT^{32}P-labelled plasmid DNA of atypical gall-forming *Erwinia* isolate A-0181 with potentially cured colonies of *Erwinia* isolate A-0181. The DNA for the probe was purified through cesium chloride gradient centrifugation and GeneClean. Each colony tested hybridized with the plasmid probe.

DISCUSSION

Both the typical and atypical isolates of the gall-forming *Erwinia* carried at least one plasmid. The modified alkaline extraction protocol as outlined in Maniatis *et al.* (1982) was adopted as a rapid and simple procedure to use for routine plasmid isolation. The typical isolates contain one plasmid band while the plasmid profile of the A-0181 isolate contains up to 5 bands which appear to consist of the different forms of one or possibly two plasmids. Large scale plasmid DNA extractions were successful with the atypical A-0181 isolate but not with the typical T-2789 isolate. Problems were encountered when attempting to isolate the plasmid DNA from the typical isolates possibly due to a low copy number and a larger plasmid size. Furthermore, the T-2789 plasmid usually ran with the chromosomal band and rarely in a different form behind the chromosomal DNA hampering attempts to isolate and purify the plasmid DNA.

Assessments including restriction analyses were used to determine the size of the *Erwinia* plasmids. The putative large size of the plasmid of the typical *Erwinia* isolates, and the difficulty of purification of the plasmid, resulted in difficulties of precise estimation of size. In the atypical isolate, the complexity of the plasmid content interfered with defining the size of the plasmid(s). Difficulties could also have arisen from an insufficient amount of DNA following gel purification and the possibility of some contaminants inhibiting the reactions. Thus, restriction digests of the plasmids of both the typical and the A-0181 isolates did not yield

conclusive data regarding the size of the plasmids.

Both the typical plasmid DNA probe (not purified through CsCl gradient centrifugation) and the CsCl-purified atypical plasmid DNA probe were specific to the gall-forming *Erwinia* isolates. No hybridization occurred between either probe and total genomic DNA preparations of *Agrobacterium tumefaciens* and *Escherichia coli*. The non-purified typical T-2774 probe hybridized strongly to the T-2774 total DNA preparation and to a lesser extent to the atypical A-0181 DNA preparation. These extensive hybridization profiles suggest that the non-purified typical plasmid probe may contain some highly repetitive sequences from chromosomal DNA. The presence of chromosomal DNA in the non-purified typical T-2774 plasmid DNA probe is also indicated by the weak hybridization between the non-purified typical T-2774 probe and the *Erwinia carotovora* and *E. herbicola* subsp. *herbicola* strain 2273 total genomic DNA preparations.

There appeared to be conserved plasmid sequences within the various typical gall-forming *Erwinia* isolates when using DNA:DNA hybridization. Furthermore DNA:DNA hybridization profiles showed each plasmid band from the atypical isolate hybridized with the typical T-2774 DNA probe, suggesting the possibility that highly repetitive sequences homologous to chromosomal DNA sequences of T-2774 occur in the atypical plasmids. The CsCl-purified plasmid probe from the atypical A-0181 isolate did not hybridize with any of the typical isolate profiles, indicating that there was no sequence homology between the typical and atypical plasmid types.

If highly repetitive, chromosomally-derived sequences were

present in the A-0181 plasmids, the CsCl-purified plasmid probe would not necessarily detect the chromosomal analogues in the total DNA blot of the typical isolates because of the low concentration of randomly distributed sequences. Thus the sequences in the total DNA blot would be in a very low copy number as compared to the plasmid bands and also may be within different sized restriction fragments, thus 'spread' out over the blot. However, if the repetitive sequences were included in the probe they could pick up common sequences in discrete plasmid bands, as occurred with the A-0181 probe and its homologous blots. These results may indicate that there are chromosomal-like repetitive sequences in the atypical gall-forming *Erwinia* plasmids, maybe resulting from integration of the plasmids into the chromosome and subsequent excision of some of the chromosomal DNA with the plasmid DNA (Hardman and Gowland, 1985b).

Curing of the Ti plasmid from *A. tumefaciens* was originally achieved through the use of a heat treatment (Watson *et al.*, 1975). Curing of the pIAA plasmid from *P. syringae* pv. *savastanoi* was achieved using an acridine orange treatment (Comai *et al.*, 1982). For both of these pathogens, the plasmid-free strains were not able to induce galling on the host plants indicating that genes essential to the pathogenicity of the pathogen were located on the plasmids. Plasmid-free strains were not however produced from the atypical gall-forming *Erwinia* isolates using the curing protocols. Therefore, no correlation between plasmid presence and pathogenicity could be determined for the atypical *Erwinia* isolates.

It is often difficult to rid bacterial pathogens of plasmids via curing protocols. Some explanations for this difficulty include the fact that not all plasmids, especially large plasmids, are susceptible to the curing agents (Hardman and Gowland, 1985a). Large plasmids share many chromosomal replication characteristics and also may integrate into the chromosome thus avoiding the effects of the curing agents (Broda, 1979).

Other methods that could have provided insight into the role(s) or function(s) of the gall-forming *Erwinia* plasmids in gall formation include the transfer of the *Erwinia* plasmids into suitable hosts via conjugation with or without a helper plasmid (Hamamoto and Murooka, 1987). *Escherichia coli* or other *Erwinia* spp., such as *E. carotovora* could serve as recipients in the case of gall-forming *Erwinia* isolates. This method would require that either characteristic antibiotic markers or nutritional markers be used to screen potential recipients. Another method to determine if the plasmid genes are involved in pathogenicity involves the direct cloning of portions of the plasmids (Mahillon *et al.*, 1988) again into suitable hosts such as *E. coli* or *Erwinia carotovora*. This method would require the expression of the *Erwinia* genes within the new host organism and a method of determining the expression of virulence genes. The use of transposon mutagenesis to produce mutant derivatives of the original pathogen (Mills, 1985; Salch and Shaw, 1988) was considered but not adopted due to the complex nature of such a study, ie. in determining the site of mutation and relating it to a change in pathogenicity.

The experimental approach of curing was chosen to relate plasmid DNA to pathogenicity because resources for such experiments were available and useful antibiotic markers for screening procedures were not found in the gall-forming *Erwinia* isolates. Had cured strains been obtained, they would have been inoculated back into Douglas-fir to see if the phenotype had been altered. Because they were not, the role of the plasmid DNA, in these isolates, in pathogenesis remains unresolved.

GENERAL DISCUSSION

The continued presence of gall-forming bacteria, isolated from B.C. Douglas-fir galls, was required for gall formation to occur. This would indicate that an interkingdom transfer of genes to the plant host does not occur as in the crown gall system. Rather, the gall-forming bacteria, identified as *Erwinia* species, were found to emulate the system of *P. syringae* pv. *savastanoi*.

A presumptive next step would be to conclude that the gall-forming *Erwinia* isolates produce and secrete both auxins and cytokinins, again similar to *P. syringae* pv. *savastanoi*. In the *P. syringae* pv. *savastanoi* system, the host plant cells surrounding the invading bacterium are affected by the exogenous supply of hormones and respond with cell division and cell elongation (Comai *et al.*, 1982). A continued supply of phytohormones from the pathogen induces the unregulated growth and division of plant cells characteristic of tumor tissues. As the gall-forming *Erwinia* spp. produce galls similar in structure to those produced by *A. pseudotsugae*, which in turn resembled those produced by *P. syringae* pv. *savastanoi*, the presumption that they also produce and secrete phytohormones like *P. syringae* pv. *savastanoi* is not without basis.

The ratio of excess cytokinins to auxins determines the form of the plant gall. A high cytokinin to auxin ratio results in the development of shoots from the tumor tissue, while a low cytokinin to auxin ratio results in the development of root structures. An intermediate ratio of the two phytohormones

results in an unorganized tumor. Most of the galls produced by the *Erwinia* spp. fit into the unorganized category. However, a few of the galls consisted of distinct outgrowths resembling the beginnings of teratomatous growths. Variations in the cytokinin to auxin ratio produced by the invading pathogen itself could explain this phenomenon.

The function of the plasmid genes or simply their involvement in pathogenicity could not be determined. It was hypothesized that plasmid genes could encode for phytohormone enzymes, since a common denominator of phytohormone production is likely for all of the phytopathogenic gall formers. Similarities in regions of DNA involved in pathogenicity exist between the two oncogenic phytopathogens, *A. tumefaciens* and *P. syringae* pv. *savastanoi*, and could also exist with the gall-forming *Erwinia herbicola* f.sp. *gypsophilae* and the Douglas-fir gall-forming *Erwinia* spp. Therefore, an elegant method to determine the role of the *Erwinia* plasmids in pathogenicity would be to probe the plasmids directly with the phytohormone genes of either *A. tumefaciens* or *P. syringae* pv. *savastanoi*. This option to directly determine the function of the plasmid genes would be interesting to investigate in a future study.

The two gall-forming Douglas-fir isolates differed in many respects from *A. tumefaciens*, *Pseudomonas* spp. and *Erwinia herbicola* f.sp. *gypsophilae*, the three most common gall-forming bacterial phytopathogens, as well as from *A. pseudotsugae strain 180*. The two isolates also differed from each other in a number of characteristics, although both isolate types were classified in the genus *Erwinia* by fatty acid analysis. The unexpected

finding of two distinct isolate types of the gall-forming

bacteria dictated that parallel studies should be conducted on

the two isolate types throughout this thesis. The discovery of

two isolate types also required description and tentative

identification of the two isolate types although identification

of the pathogen was not an objective of this study. The

variation in characteristics of the two isolate types lead to

speculation on the classification of the two isolate types as

two different species.

The two gall-forming *Erwinia* isolate types varied slightly

in colony morphology (convex versus concave surfaces), the upper

temperatures limiting growth (the atypical isolate was able to

grow at a higher temperature) and their resistances to

antibiotics (the typical isolate was resistant to a wider range

of antibiotics), growth rate (atypical colonies reached a size

of 1 mm in diameter on CPG at 2 days and typical colonies

reached a similar size at 4 days). The outer appearance of the

galls produced by the two *Erwinia* isolate types differed

slightly although the inner cell organization was similar (R.J.

Copeman, *unpublished*). Plasmid profiles of the two *Erwinia*

isolate types (Chapter 3) suggested that each type contains at

least one plasmid, with the typical isolates containing a larger

plasmid than the atypical isolate. The plasmid probe of the

typical isolate hybridized to the plasmid bands of the atypical

isolate but the reverse was not observed indicating no homology

occurring between the plasmid DNA of the two isolate types.

Plasmid profiles sometimes are used in species identification

but variations occur within many species thus making

determinations of relationships difficult via this character
alone.

A serological relationship between the two isolate types
could be demonstrated by immunodiffusion when using the whole
typical T-2789 antiserum but not when the purified typical T-
2789 antiserum was used. However, as with plasmid profiles,
serological relationships should not be used alone in describing
bacterial strains. Cross reactions have been observed for
Erwinia spp. with other Enterobacteriaceae when using polyclonal
antisera to sort bacterial strains into serogroups (De Boer,
1987). Differences have also been observed within a species
with respect to serological traits (De Boer *et al.*, 1979) which
might explain the lack of identity between the two isolate types
when using the atypical antisera.

The host ranges of the typical and atypical gall-forming
Erwinia isolates were not identical. The atypical isolate
formed galling symptoms on more conifer species than the typical
isolates. It is not likely that the two isolate types are
different pathovars of the same species because the
infrasubspecific ranking of pathovar refers to strains that are
similar with respect to biochemical and bacteriological
characteristics but different with respect to pathogenicity on
one or more plant hosts (Krieg and Holt, 1984).

Fatty acid profiles also differed for the two *Erwinia*
isolate types. Identification of the isolates achieved by fatty
acid analysis indicated that the two isolate types were in fact
two different species. A positive match was made for the
atypical isolate, while the 'goodness of fit' for two of the

three typical isolates analyzed was not indisputable. However,
from their observed and experimentally determined
characteristics, the two gall-forming isolate types appear to be
two different species of *Erwinia*. Further testing should be
done to clearly define the relationship between the two gall-
forming *Erwinia* isolates.

The gall-forming bacterial pathogens isolated from Douglas-
fir trees in B.C. do not appear at a high frequency, although as
stated before, a survey was not conducted on this aspect of the
organism. Furthermore, little damage was inflicted on the host
plant by the gall-forming *Erwinia* isolates. There have been
several reports of bacterial galls on conifers (R.S. Hunt,
personal communication) but it is not known if the incitant was
isolated or the identity determined. The host range of the
gall-forming Erwinias is very limited, with *Pseudotsuga* and some
Abies, *Larix*, *Picea* and *Pinus* spp. constituting the majority of
susceptible hosts. The potential for spread to surrounding
forested areas is therefore very small, although the presence of
an insect vector is unknown.

Due to the infrequently observed natural damage and the
limited host range of the gall-forming erwinias, these pathogens
appear to be limited threats to the forest industry in terms of
decreased timber quality. Thus, although the gall-forming
Erwinia isolates provide no scientific advantage in terms of
providing a potential gene vector, they, on the other hand, are
not an economic threat to the trees in terms of disease. The
gall-forming *Erwinia* isolates could serve very well as
additional models both in the study of phytohormone production

by bacteria in plant-pathogen interactions and in the origin of phytohormone genes in bacterial phytopathogens.

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