Epidemiological, genetical, and physiological studies of the bacterial leaf streak pathogen Xanthomonas campestris pv. translucens (J.J.R.) Dowson
by Hee Kyu Kim

A thesis submitted in partial fulfillment of the requirements for the degree of DOCTOR OF PHILOSOPHY in Plant Pathology
Montana State University

Abstract:
Bacterial leaf streak of barley caused by Xantkomonas campestris pv. translucens has occurred on occasion with severity in irrigated barley and wheat fields in Montana. These studies were undertaken to better understand this pathogen in terms of epidemiology, physiology of parasites and genetics.

A first goal was to develop a selective medium useful for isolation of this pathogen from diseased plant materials and other environments. The medium described herein has high selectivity against Pseudomonas syringae from cereals, and against soil-borne phyto-pathogenic and saprophytic bacteria. This medium may prove useful in the epidemiological studies and survival studies of this pathogen.

This is the first report of this pathogen as an active ice nucleator. Only two other microorganisms, Pseudomonas syringae and Erwinia herbicola, also plant associated bacteria, are known to be ice nucleators. This finding suggests that a new mode of transmission is possible and also points to a useful taxonomic differentiation of the "translucens" groun from the "campestris" group.

A cultivar Oderbrucker (C.I. 4666) described to be resistant 50 years ago, is still resistant to the current field pathogens, Xanthomonas campestris pv. translucens, in Montana, indicating that resistance can be relatively stable as compared to resistance in some other host-parasite systems. Results of greenhouse and field inoculation appear to have no correlation. This may be because this pathogen requires specific environmental conditions, such as frost injury or low temperatures with high moisture, in order for a severe epidemic to occur.

The method of transferring transposon from Escherichia Coli to Xanthomonas campestris pv. translucens is described. Transposon mutagenesis is a useful tool to identify the gene for any particular traits, including traits conferring virulence, etc. Transposon may be used to mark strains of bacterial plant pathogen with antibiotic resistance for epidemiological studies.

In dedication to:

my mother and late father.

EPIDEMIOLOGICAL, GENETICAL, AND PHYSIOLOGICAL STUDIES OF THE BACTERIAL LEAF STREAK PATHOGEN *XANTHOMONAS CAMPESTRIS* PV. *TRANSLUCENS* (J. J. R.) Dowson

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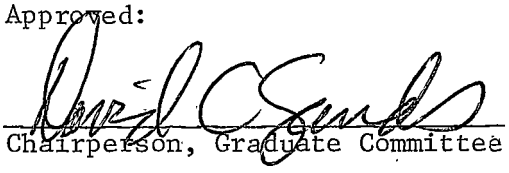
Approved:



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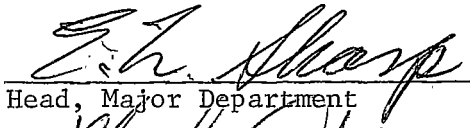

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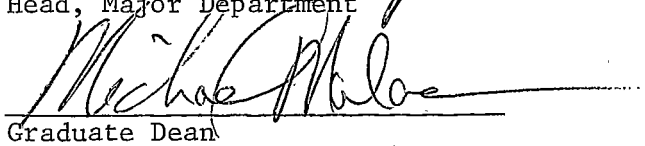

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ABSTRACT

Bacterial leaf streak of barley caused by *Xanthomonas campestris* pv. *translucens* has occurred on occasion with severity in irrigated barley and wheat fields in Montana. These studies were undertaken to better understand this pathogen in terms of epidemiology, physiology of parasites and genetics.

A first goal was to develop a selective medium useful for isolation of this pathogen from diseased plant materials and other environments. The medium described herein has high selectivity against *Pseudomonas syringae* from cereals, and against soil-borne phytopathogenic and saprophytic bacteria. This medium may prove useful in the epidemiological studies and survival studies of this pathogen.

This is the first report of this pathogen as an active ice nucleator. Only two other microorganisms, *Pseudomonas syringae* and *Erwinia herbicola*, also plant associated bacteria, are known to be ice nucleators. This finding suggests that a new mode of transmission is possible and also points to a useful taxonomic differentiation of the "translucens" group from the "campestris" group.

A cultivar Oderbrucker (C.I. 4666) described to be resistant 50 years ago, is still resistant to the current field pathogens, *Xanthomonas campestris* pv. *translucens*, in Montana, indicating that resistance can be relatively stable as compared to resistance in some other host-parasite systems. Results of greenhouse and field inoculation appear to have no correlation. This may be because this pathogen requires specific environmental conditions, such as frost injury or low temperatures with high moisture, in order for a severe epidemic to occur.

The method of transferring transposon from *Escherichia coli* to *Xanthomonas campestris* pv. *translucens* is described. Transposon mutagenesis is a useful tool to identify the gene for any particular traits, including traits conferring virulence, etc. Transposon may be used to mark strains of bacterial plant pathogen with antibiotic resistance for epidemiological studies.

INTRODUCTION

The plant pathogenic bacteria are important economically because they can cause serious disease losses. A study of one of these pathogenic bacteria is of value if it elucidates any aspect of the pathogen that might permit control of the disease. Additionally, the study of bacterial plant pathogens can aid in the study of basic disease mechanisms. In the well documented cases of *Agrobacterium tumefaciens*, *Pseudomonas savastanoi*, *Corynebacterium sepedonicum*, *Pseudomonas syringae*, these species were chosen to elucidate the basic disease mechanisms of tumor induction, gall formation, toxin production and ice nucleation. In this study, the pathogen *Xanthomonas campestris* pv. *translucens* is a fitting choice for study of host specificity phenomena because some of these xanthomonads are host-limited to a single plant species, while others attack two or more species of hosts. Host range differences are distinct and the host specificity of the cultures of this pathogen is a very stable characteristic. It is the purpose of this study to develop an essential tool for epidemiological studies of this pathogen, to investigate new and unique traits of this bacterium and to develop the technique necessary for genetic analysis of its virulence and its host specificity.

Bacterial leaf streak of barley was first observed in 1912 by

Jones, Johnson and Reddy (1917) and the causal agent, after several nomenclatural changes, is now classified as *Xanthomonas campestris* pv. *translucens* (Jones, Johnson and Reddy) Dowson (1939). This pathogen includes a pathover that infects 17 species of the family Gramineae, although no one strain will attack all 17 species. Geographical distribution of this disease is world-wide.

In Montana, this disease has occurred severely but sporadically in irrigated barley and wheat fields and less occasionally on dryland cereals during wet years. This disease is seed-borne and thus, seeds may serve as a primary source of inoculum, even though it is also dissesminated by rainsplash, or wind-driven rain. The bacteria invade plants through the stomata and hydathodes and through wounds.

Plant pathogenic bacteria are often easily isolated from diseased material, however, rapid growth of saprophytic bacteria can obscure the slow growing pathogens, especially when the pathogens are in low numbers. To date, there has been no reliable method for specifically detecting the bacterium when it is greatly outnumbered by saprophytes or by other pathogens. A medium that only permits the growth of this bacterium, excluding all other microbes, would be useful for epidemiological studies and the identification for the causal organism of disease. A few media have been described for isolation of various xanthomonads, but none has yet been useful for

Xanthomonas campestris pv. *translucens*. The medium employed for isolation of this pathogen is Wilbrink's agar (Dowson, 1957), which is not considered to be selective. Therefore, a selective medium designated as KM-1 was developed for isolation of this organism.

This is the first report of *Xanthomonas campestris* pv. *translucens* as an ice nucleating active agent. It is only the third bacterial genus found to be active in ice nucleation, following the discoveries of this property in *Pseudomonas syringae* and *Erwinia herbicola*. With this disease, epidemics appear to be sporadic. The rate of seed-borne transmission is relatively low, with transmission from less than 2 percent of seeds from highly infected plants. An unknown factor in the field may raise these transmission frequencies to result in an epidemic. The data from this study hint that ice nucleation may be a major mechanism by which these bacteria enhance infection leading to severe epidemics.

A transposon is a useful genetic tool to find and to identify the genes that encode any particular traits such as virulence, etc. Transposons are discrete sequences of DNA that are incapable of self-replication and can be inserted into DNA replicons, chromosomes or plasmids. Such insertions of transposons are random events and, in effect, are strongly mutagenic.

In this study, the method by which the transposon can be

transferred from *Escherichia coli* strain 1830 to *Xanthomonas campestris* pv. *translucens* is described.

LITERATURE REVIEW

The genus *Xanthomonas* Dowson 1939 is defined as Gram negative, polarly flagellated, rod-shaped, phytopathogenic bacteria, the colonies of which are distinctively yellow in color (Dowson 1939). All the *Xanthomonas* species recognized at present are plant pathogens and, so far as is known, are found only in association with plants or plant material (Dye and Lelliott 1975). By 30 different cultural, physiological, and biochemical tests, the 209 phytopathogenic *Xanthomonas* cultures comprising 57 recognized species formed a remarkably uniform group which could be easily distinguished from some other yellow pigment producing organisms that were included for comparative purposes (Dye 1962). Therefore it has been suggested that many *Xanthomonas* species could well be regarded as a single species comprising special forms of one species adapted to particular hosts (Dye 1962). With the exception of four of the nomenspecies, *Xanthomonas fragariae*, *X. albilineans*, *X. axonopodis* and *X. ampelina*, all other xanthomonads can be distinguished only from the type species and/or each other by their host range. These other xanthomonads may be regarded as members of a single taxospecies, referred to as the *Xanthomonas campestris* group (Dye 1980, Dye and Lelliott 1975). Young et al. (1978) proposed that all strains except these four nonemspecies be classified as pathovars of *X. campestris*. All

strains have the following characterisitcs: Gram negative rods 0.4 x 1.0 um, single polar flagellum, aerobic, catalase positive, H_2S positive, hypersensitive reaction to tobacco leaves, oxidase negative, hydrolyse Tween 80, nitrite not produced from nitrate, and indole not produced (Dye 1962, 1980, Dye and Lelliott 1975). Most xanthomonads produce "Gum Xanthan", an extracellular polysaccharide slime produced on media containing glucose. Colonies of these species are mucoid, convex, and shiny on nutrient agar or YDC agr (Dye 1980). The pigment from xanthomonads is a non-water soluble carotenoid which in petroleum ether has an absorption spectrum with maxima at 418, 437 and 463 nm (Starr 1977). A few xanthomonads are non-pigmented, i.e., *X. campestris* pv. *manihotis*, or are known to have non-pigmented forms, i.e., *X. campestris* pv. *ricinii*. These pathovars are indistinguishable from *X. campestris* pv. *campestris* except by host reactions. Two varieties of the nomenspecies, *X. phaseoli*, were only distinguishable by production of a diffusible brown pigment in var. *fuscans* and not in var. *vignicola*. Both are now included in *X. campestris* pv. *phaseoli* with no taxonomic differentiation (Dye and Lelliott 1975). To date, 110 nomenspecies of *Xanthomonas* have been characterized and described as a pathovars of *Xanthomonas campestris* group (Dye and Lelliott 1975) in the 8th Bergey's manual of Determinative Bacteriology. This taxonomic lumping was considered

necessary until further research revealed substantive physiological and biochemical differences among pathovars.

Bacterial blight of barley, now generally referred to as bacterial leaf streak of barley, has been recognized as early as 1912 as a widespread disease capable of causing economic loss. In 1917 Jones, Johnson, and Reddy described the causal organism, *Bacterium translucens*, as follows: cylinderical rods rounded at ends, solitary or in pairs; individual rods 0.5 - 0.8 by 1 - 2.5 um, motile by a single polar flagellum; aerobic, no spores, Gram negative and forming yellow colonies on peptone beef agar plates. Inoculation with this bacterium resulted in infection of barley, but negative results were obtained from inoculations on oats, rye, wheat, spelt, emmer, einkorn, and timothy (Jones, Johnson, Reddy 1917). In 1919 Smith, Jones and Reddy described *Bacterium translucens* var. *undulosum* as a causal agent of the black chaff disease of wheat. It is also known to infect spelt, barley, and rye by artificial inoculation. In 1924 Reddy, Godkin, and Johnson described *Bacterium translucens* var. *secalis* as a causal organism of bacterial blight of rye infecting rye only, but otherwise identical to the above two bacteria. In 1936, Hagborg transferred *B. translucens* var. *undulosum* to the genus *Phytomonas* Bergey et al. and changed its rank to that of a forma specialis, in conformity with the International Rules of

Botanical Nomenclature of 1930. In 1939, Dowson transferred the original species to his newly created genus *Xanthomonas*, as X. *translucens* (Jones, Johnson, and Reddy) Dowson. In 1942, Hagborg revised the classification and amended the species description of X. *translucens* to include the five closely related organisms, which are distinguishable chiefly by differences in pathogenic capabilities on wheat, oats, barley, and rye. One of the organisms, X. *translucens* (Jones, Johnson, and Reddy) Dowson, was given new rank as X. *translucens* f. sp. *hordei*; another, *Phytomonas translucens* f. sp. *undulosa* (Smith, Jones, and Reddy) Hagborg, was transferred to the genus *Xanthomonas*; and the third, *Phytomonas translucens* var. *secalis* (Reddy, Godkin, and Johnson) Bergey et al., was transferred to the genus *Xanthomonas* as X. *translucens* f. sp. *secalis*. One of the two of his new forma specialis could infect barley and oats, but not wheat and rye; and thus was named X. *translucens* f. sp. *hordei-avenae*; the other could infect wheat, oats, barley, rye and was named X. *translucens* f. sp. *cerealis*.

In 1945, Wallin and Reddy reported the forma specialis *phleipratensis* from timothy. Bamberg (1936) suggested the existence of strains of the organism differing in pathogenicity. Hagborg (1942) noticed the difference in pathogenic capabilities occurs between different isolates of the same forma specialis of X. *translucens* and suggested the need for recognizing races within the special forms.

Hagborg (1942) considered the *Triticum* spp. to be the natural hosts of f. sp. *cerealis*, which is more pathogenic on wheat. However, Wallin (1946) extended the definition of *X. translucens* f. sp. *cerealis* and discussed the existence of the biological races of this forma specialis. He found that *X. translucens* f. sp. *cerealis* from brome grass (*Bromus inermis*) was more pathogenic on this species than on wheat, considering it a distinct race of the redescribed forma specialis.

From pathogenicity tests, *X. translucens* f. sp. *cerealis* and *X translucens* f. sp. *hordei-avenae* of Hagborg (1942) are not considered valid forma specialis by Fang et al. (1950) who found that neither of these two infects oats. These were included respectively with f. sp. *undulosa* and f. sp. *hordei*. The f. sp. *hordei*, *undulosa*, *secalis* and *phleipratensis* maintained their original description (Jones, Johnson, and Reddy 1917, Smith, Jones, and Reddy 1919, Hagborg 1942, Wallin and Reddy 1945). Fang et al. (1950) redescribed *X. translucens* f. sp.*cerealis* as follows: occurs naturally on smooth brome grass and quack grass; produces water-soaked streaking symptoms on barley, rye, wheat, smooth brome grass and quack grass following wound inoculation, but it is more virulent on grasses. It will infect smooth brome grass, quack grass, and barley by spraying, but with the same inoculation methods it only slightly infects rye and wheat. They found that the f. sp. *hordei* and *phleipratensis* are serologically distinct with intermediate types of f. spp. *undulosa*, *secalis*, and

phleipratensis are serologically distinct with intermediate types of f. spp. *undulosa*, *secalis*, and *cerealis*. Attempts to use form specific phages to identify cultures of X. *translucens* were unsuccessful (Katzelson and Sutton 1953). Moffett and McCarthy (1973) found X. *translucens* isolates from Japanese millet to be less pathogenic on wheat than on the originally recorded host and suggested the possibility of distinct races of X. *translucens* f. sp. *cerealis*. Bradbury (1971) proposed the nomenclature of bacterial streak pathogen of rice (*Oryza sativa* L.) as *Xanthomonas translucens* f. sp. *oryzicola*, a pathotype originally isolated from rice which by artificial inoculation infects only rice.

Six pathotypes of X. *translucens* using forma specialis designation are accepted in the 8th Bergey's manual of Determinative Bacteriology, using the criteria of production of translucent, water-soaked lesions on unwounded plants and the data of Fang, Allen, Riker, and Dickson (1950), Tominaga (1967) and Patel and Shekehwat (1971). The "translucens" group can well be regarded as a large group comprising a number of pathotypes, some with overlapping host ranges within this nomenspecies. These pathovars are detected at the genus level of host specificity. Variations among cultivars have not been analyzed in this way.

Culturally, physiologically, and biochemically indistinguishable

from *X. campestris* except by host range, this pathogen has been recently named a pathovar of the *X. campestris* group, *Xanthomonas campestris* pv. *translucens* (Dye 1962, 1980, Dye and Lelliott 1975, Young, Dye, Bradbury, Panagopoulus, and Robb 1978).

Etiology

The seed-borne nature of this disease was reported by Jones, Johnson, and Reddy 1916, Smith, Jones, and Reddy in 1919. The bacterium penetrates the pericarp causing infection of the plumule through wounds or stomata on the coleoptile. Spreading rapidly through these tissues, it finally reaches the enclosed foliage leaves. By elongation the infected leaf carries the bacteria into the aerial parts of the seedling with water soaked streaks on the primary leaf (Wallin, 1946).

The bacterium infects leaves through stomata, spreading intercellularly (Jones, Johnson, and Reddy 1917, Bamberg 1936). The bacterial invasion is confined only to the thin-walled parenchyma possessing intercellular space, i.e., mesophyll of leaf, chlorenchyma and ground tissue parenchyma of the leaf sheath. In naturally infected leaves, no vascular bundle elements were shown to be infected (Shekhawat and Patel 1978).

This disease was described as a widely occurring disease attacking leaves, leaf sheaths and glumes, early characterized by

water-soaked lesions with bacterial exudates, and later by persistent transparency following the death of the parts invaded. The sporadic occurrence of infection may have been due to the variation in environment. Bamberg (1936) found that low relative humidity of the surrounding atmosphere retarded the growth of the bacterium in culture when the relative humidity was maintained with aqueous solution of sulphuric acid. This retarding effect was noticeable at a relative humidity of 50 percent and was greater as the percentage lowered. The optimum temperature for the growth of this organism on agar has been reported to be in the range of 25 - 30 C (Bamberg 1936), and 24 C (Stewart 1952). The maximum temperature for sustained growth is approximately 40 C with the minimum slightly below 10 C (Bamberg 1936).

The organism is very resistant to dry conditions, surviving for eight months on straw from blighted plants and two years on seed (Jones, Johnson, and Reddy 1917) and 77 days in dry soil (Bamberg, 1936). Moreover, this organism is also resistant to extremely low temperatures ranging from -33 C to 30 C, remaining viable in soil cultures for at least 124 days after December 15, at St. Paul, Minnesota (Bamberg, 1936). On the other hand, Boosalis (1952) reported that *X. translucens* does not survive in non-sterile soil for an appreciable length of time unless it is in or on plant parts. This organism may persist from one season to the next in

seed of barley and wheat, on straw of barley, brome grass and other winter hosts.

Host Range and Epidemiology

The host range encompassed by this translucens group has been reported to include 17 different hosts in the Gramineae: barley (*Hordeum vulgare* L.), foxtail barley (*Hordeum jubatum* L.), wheat (*Triticum aestivum* L.), rye (*Secale cereale* L.), spelt (*T. spelta* L.), Einkorn (*T. monococcum* L.), victory oat (*Avena sativa* L.), smooth brome (*Bromus inermis* Leyss.), cheatgrass brome (*B. tectorum* L.), yellow bristle grass (*Setaria lutescens* (Weisel.) F. T. Hubb.), quackgrass (*Agropryon repens* (L.) Beauv.), orchardgrass (*Dactylis glomerata* L.), water foxtail (*Alopecurus geniculatus* L.), reed canary-grass (*Phalaris arundinaceae* L.), rice (*Oryza sativa* L.) Japanese millet (*Echinochloa crus-galli* var. *frumentaceae*.), and timothy (*Pleum pratense* L.). These studies are referred to in Table 1 of Appendix.

Considering the wide host range data and the epidemiological studies cited above, this pathogen may overwinter on diseased stubble or refuse or on overwintering hosts, becoming a source of spring infection.

The most severe epidemics of this disease occur in relatively warm, wet seasons with the optimum temperature at 20 C. The size of lesion is limited and symptom development is delayed at or below

10 C (Bamberg, 1936). Stewart (1952) observed highest infection at 15.5 - 18 C, moderate infection at 21 C and slight infection at 24 C,with no infection at 27 C, an optimum temperature for growth in culture. A shift of about 5 C cooler than the optimum temperature in culture is more conducive for symptom development. The low relative humidity is probably more often a limiting factor in the development of disease than is low temperature (Bamberg, 1936). Effective transmission of aphids, straw, wind and rain, and by diseased plants coming in contact with healthy plants, occurred only when the host was water-congested (Boosalis, 1952). Field epidemiology studies in Montana using a marked (antibiotic resistant) strain of *Xanthomonas translucens* f. sp. *hordei* indicated that this bacterium is capable of spreading 28 square meters from a single infection locus within 39 days (Hall, Kim, and Sands, 1981).

The distribution of this disease occurs where barley or cereals are grown. *Xanthomonas translucens* was reported as early as 1917 in Colorado, Iowa, Minnesota, Montana, North Dakota, South Dakota, Oregon, Wisconsin and Ohio in the United States (Jones, Johnson, and Reddy), and in Canada, Mexico, Russia, France, Belgium, and Africa (Bamberg, 1936).

Screening of disease resistance

Techniques used for inoculation into cereals include 1) spraying

the bacterial suspension (Jones, Johnson, and Reddy 1917, Fang et al. 1950, Stewart 1952), 2) partial vacuum technique (Boosalis, 1952), and 3) hypodermic introduction of bacterial suspensions by hypodermic syringe (Bamber 1936, Hagborg 1936, Fang et al. 1950). After the hulled barley seed is soaked in bacterial suspension for one hour, 54 percent of the plant is infected. At least 24 hour soaking is needed for non-hulled seeds to yield 50 percent diseased seedlings (Wallin, 1946). Seed and seedling inoculation methods, unaccompanied by wounding, proved to give unsatisfactory results (Bamberg 1936, Hagborg 1936). The inoculation method of forcing inoculum into the leaf roll of young plants or into the leaf sheath of the older plant with hypodermic syringe is generally thought to be the most successful (Bamberg 1936, Hagborg 1936, Fange et al. 1950, Wallin 1946). Hagborg (1936) developed an inoculation method involving minimum injury to the coleoptiles and the enclosed primary leaves. The seedlings were poked with a sharp nichrome needle when they were only 7 - 15 mm above the soil. Then the seedlings were flooded with the bacterial suspension, placed in the greenhouse at 25 C for ten days, and examined for infection.

For an inoculation method to be useful, it must 1) closely simulate natural infection, and 2) result in symptoms closely resembling those that occur in the field. Stewart (1952) reported the barley cultivars, Spartan and C. I. 6240, showed high degrees of

resistance in the field but less under artificial inoculation in the greenhouse. Bamberg (1936) reported that the wheat varieties, Marquis, Mindum and Kubanka, which appeared resistant to natural infection in the field, were rather severely infected when hypodermically inoculated. He suggested that some of the resistance to black chaff under natural condition may be morphological or functional, as it is apparently in some cases of resistance of wheat to stem rust. This type of resistance may be eliminated when hypodermic inoculations are made. Prerequisite to determining resistance are proper inoculation techniques that give reproducible results coinciding closely with field observations.

Chevalier, California, Summit and Oderbrucker (C.I. 4666) were reported to be the most resistant of 53 varieties tested (Jones, Johnson and Reddy 1917). This cultivar Summit is six-rowed and is different from the cultivar Summit recently released. In the epiphytotics of 1944, Oderbrucker (C.I. 4666), a Chevron (C.I. 1111) x Bolivia (C.I. 1257) selection and many selections of the Manchuria germplasm group showed resistance (Dickson, 1956).

Waldron (1929) found an average of 10.5 percent decrease in 1,000 kernel weight of several F_3 families from crosses of susceptible variety Hope with other varieties, when these plants were exposed to infection.

Chemical control

Seed treatment for two hours in formaldehyde solution eliminates the organism from the seed; hot water treatment is also recommended for control of this disease (Jones, Johnson, and Reddy 1917). Mergamma, Agrox, Ceresan, and streptomycin were tested for their effectiveness in controlling this disease. Mergamma gave the highest level of control, however, no chemical treatment eliminated the bacterium completely (Stewart, 1952). Streptomycin was least effective and reduced plant stand counts. Streptomycin and chloramphenicol added to the culture solution increased the resistance of spring wheat Thatcher (C.I. 10003) seedling grown in Shive's four-salt solution with trace elements added (Hagborg, 1956). Reduction in the dry weight per plant by antibiotics was noted, but the resistance of plants to *X. translucens* was obtained apparently by absorption of antibiotics through the root system.

This pathogen is very susceptible to mercurials, and therefore seed treatment with mercuric chloride has been recommended along with crop rotation, field sanitation, clean seed for control (Dickson 1956, Jones, Johnson, and Reddy 1917). The registration and use of mercuric chloride and most organic mercurials has been cancelled. The bactericide cupric hydroxide (Kocide Chemical Corp. Houston,

Texas) has recently been registered and recommended for seed treatment, without evidence of complete control.

Selective Media

A few media have been described for various xanthomonads (Kado and Heskett 1970, Mulrean and Schroth 1981, Schaad and White 1974), but none has been useful for isolation of *Xanthomonas campestris* pv. *translucens*. The medium commonly employed for isolating this pathogen is Wilbrink's agar (Dowson, 1957) which is not considered to be selective. SX agar was described in 1974 by Schaad and White for isolation of *Xanthomonas campestris* from soil. A semi-selective medium for isolation of *Xanthomonas campestris* pv. *juglandis* from walnut was developed by Mulrean and Schroth in 1981. Kado and Heskett in 1970 reported that their D-5 medium selectively favors the growth of *Xanthomonas* spp. and *Agrobacterium tumefaciens* while suppressing the growth of *Pseudomonas* spp.

Ice nucleation

The initiation of precipitation over much of the earth's surface is believed to be caused by the development of ice nucleation in supercooled clouds (Schnell and Vali, 1973). The source, composition and number of small particles initiating freezing events in natural clouds are poorly understood. Ice nuclei are defined as particles initiating the phase transition to ice at temperatures

between 0 and -40 C (Schnell and Vali, 1972). These particles represent a very small fraction, i.e. only one part in 10^{10} - 10^{12} of the total aerosol mass in the atmosphere (Vali and Schnell, 1975).

In 1964, Panagopoulos and Crosse determined frost injury to be a predisposing factor for the development of the symptom of blossom blight of pear caused by *Pseudomonas syringae*, which was found to be present on the leaves and blossoms of pear. In 1974, Sands and Kollas reported *Pseudomonas syringae* to be pear blast causal agent in Connecticut. This disease was reported to have appeared under unusually moist weather conditions at the time of pear blossoming. Typical symptoms were developed after 5 to 6 days of lower temperatures in the -2 C or slightly below during the greentip to one-half inch green period of development, even without frost. Since these discoveries, aerobiologists started to pay attention to the possible role bacteria has in the initiation of precipitation

Recent research has shown that at least the potential exists for biological materials to contribute significant proportions of atmospheric ice nuclei. The transient appearances of ice nuclei active at temperatures of -2 to -5 C had been noticed to accompany the natural decay of the plant leaf material (Schnell and Vali, 1972). In a preliminary attempt to isolate the entity responsible for increased frost damage, Arny et al. (1976) found that water extracts of dried powdered corn leaves caused increased frost sensitivity

when applied as a spray to seedling corn. Addition of either streptomycin or tetracycline at 1,000 ppm to those water extracts eliminated their activity, suggesting the possible role of bacteria in this effect.

In recent years a suggestion was proposed that biogenic ice nuclei, such as leaf-derived nuclei, may constitute a significant fraction of the atmospheric ice nuclei (Schnell and Vali, 1972, 1973, 1976, Vali et al., 1976). Viable bacteria were found from marine fogs and were proven to exhibit ice nucleation activity at temperatures of -4 C in sterile sea water and -2 C in distilled water (Carney, Schnell and Carthy, 1975). Later it was shown that the development of these nuclei results from the worldwide ubiquitous presence of a bacterium *Pseudomonas syringae* (Schnell and Vali 1973, Vali et al. 1976). The evidence of *Pseudomonas syringae* as biogenic ice nuclei and its significance in epidemiology of bacterial blight was further substantiated by Sands, Langhans, de Smet, and Scharen (1981) who isolated this pathogen from raindrops in rainstorms at an elevation from 180 meters to 1,800 meters above the cropland in Montana. These findings give possible meterological implications to the interactions between bacteria and rain. *Pseudomonas syringae* van Hall has been identified as an epiphyte and occasional pathogen (Dowler and Weaver 1975, Panagopoulos

and Cross 1964) and found to be responsible for increased frost sensitivity of corn, bean or lettuce at -5 C or above, while no strains of the *Pseudomonas tabaci*, *P. glycinea*, *P. phaseolicola*, *P. marginalis*, *Erwinia stewartii*, *Escherichia coli* were active (Arny, Lindow, and Upper 1976, Lindow et al, 1975).

Lindow et al. (1978) identified another ice nucleation active bacterium, *Erwinia herbicola* (Loehnis) Dye, which is also active in promoting frost injury of frost sensitive plants at -2.3 C. and below when sprayed with suspension of 10^7 colony forming units (CFU) per milliliter. Both *Pseudomonas syringae* and *Erwinia herbicola* are commonly present as epiphytes on a diversity of plant species from widely separated areas of the United States (Lindow et al, 1975, 1978). Sufficient numbers of ice nucleation active bacteria were present on the leaves to account for the freezing injury to occur (Lindow et al., 1978).

Many species of several genera of phytopathogenic bacteria were tested to determine their ability to act as ice nuclei (Arny et al. 1976, Hirano et al. 1978, Paulin and Luisetti 1978). Positive results have only been obtained with *Pseudomonas* strains, and with one exceptional strain of *Erwinia herbicola* out of about 400 strains. Of 21 strains of *Xanthomonas campestris* pv. *juglandis*, none were ice nucleators. Single strains of *X. campestris* pv. *vitians* and *X. campestris* pv. *maculifoliigardeniae* were also tested negative for

ice nucleation (Paulin and Luisetti, 1978). Attempts were made to use this ice nucleating activity as a taxonomic tool in conjunction with other biochemical and physiological tests (Hirano et al., 1978).

Application of antibiotics such as streptomycin and tetracycline eliminated the ice nucleation activity and prevented the sensitive plants from frost injury (Arny et al. 1976, Lindow et al. 1975). The effects of dyes, antibiotics, and chemical agents on ice nucleating activity of *Pseudomonas syringae* were studied extensively by Maki et al. (1974). The dyes such as methylene blue, crystal violet, and safranin, which combine with the cell wall of bacteria, destroyed the cell activity. Conversely, congo red, which does not combine with the cell wall, did not affect the activity. Both mercuric chloride treatments and heat treatments at 65 C for 5 minutes destroyed the activity. The above observations suggest that ice nucleation activity may be cell associated and, further, may be associated with the cell wall.

History of R factor and transposon

Transfer of bacterial genes can result from the mating of fertility (F) factor, from transduction by lysogenized phage and also from transformation. The latter is defined as genetic recombination brought about by the introduction of purified chromosomes. The

fertility factors can exist either as integral parts of chromosomes or as small free circular chromosomes that multiply once per cell division. Only when the F factor is part of the chromosome can the male cells mate. These F^+ cells are called Hfr (high frequency of recombination) (Watson, 1977).

In addition to the sex factors which carry genes directly involved in the mating, most bacteria contain other freely replicating circular extra-chromosomal elements termed plasmids. Many of the common plasmids are known as resistant transfer factors (RTF) or resistant (R) factors that confer simultaneous multiple resistance to many different antibiotics (Watson, 1977).

Two groups of pili, common pili and sex pili, are known in the Enterobacteriaceae. Sex pili are distinguished from common pili by being of longer length, having terminal knobs, occurring in clusters, and serving as phage absorption sites. Conversely, common pili are short, less than 1.5 u in length, and have no terminal knob, clustering or phage adsorption. Most sex pili are characterized into two groups, typified by F and I pili, respectively, by morphology, phage adsorption and antigenic structure (Lawn et al., 1967). Even though there are certain differences between the two groups of sex pili, I-like pili are usually shorter (0.2 um in length) than F-like pili (20.0 um in length). The knobs at the end of F-like pili, tend to be longer and more complex than those of I-like pili, which are

usually small and undifferentiated (Lawn et al. 1967).

Naturally occurring R factors have been classified into two categories, fi^+ (fertility inhibition$^+$) and fi^-, depending on their ability to inhibit F-mediated conjugation (Watanabe et al. 1964). The fi^- R factors have ability to restrict phage multiplication. The fi^+ R factors determine F-type pili (Lawn et al. 1967) and constitute one class of plasmid (Datta and Hedges 1971). However, the classical fi^- R factors were indicated to be heterogeneous (Lawn et al. 1967, Datta and Hedges 1971). Among 26 fi^- R factors, twenty determined the production of I-type pili, supporting the multiplication of I-pili specific phage, while six did not (Lawn et al. 1967). In the latter group, there was no evidence of production of I-pili. Thus fi^- R factors are classified into four plasmid incompatibility groups called I, P, N. and W, based on the superinfection immunity, surface exclusion and incompatibility (Datta and Hedges 1971, 1972, Datta et al. 1971).

The R factor RP1 which originated in *Pseudomonas aeruginosa* 1822 confers resistance to carbenicillin, neomycin, kanamycin and tetracycline and has a molecular weight of about 40 million daltons, 60 percent GC and a buoyant density of 1.719 g/cm^3 (Grinsted et al. 1972). RP4, an R factor derived from *Pseudomonas aeruginosa* strain S8 and specifying resistance to carbenicillin, neomycin, kanamycin and tetracycline, was mediated by a plasmid of molecular weight about

62 million and the buoyant density 1.710 g/cm^3, 60 percent GC (Saunders and Grinsted, 1972). RP1 and RP4 are very similar to each other by base sequences (Saunders and Grinsted, 1972). RP4, a p-group plasmid, proved to have exceptionally wide host range (Datta et al. 1971, Datta and Hedges 1972) comprising *Escherichia, Salmonella, Shigella* and Proteus group and *Rhizobium* spp. *Agrobacterium* spp. P-group plasmids have been transferred by conjugation from *Pseudomonas aeruginosa* to the plant associated *Rhizobium* spp. (Boucher et al, 1977), plant pathogenic *Erwinia* spp. (Cho et al. 1975), *Agrobacterium tumefaciens* (Levin et al. 1976), *Xanthomonas vesicatoria* (Lai et al. 1977) and also to the phytopathogenic pseudomonads *P. glycinea, P. pisi, P. phaseoloicol* and *P. syringae* (Panopoulos et al. 1975, Lacy and Leary 1976). Plasmid transfer between physiologically capable cells in plants is not limited by the host nor does it require a phytopathogen to phytopathogen relationship (Lacy and Leary 1975).

The wide host range of P-group plasmid implies important economic and epidemiological significance of antibiotic resistance plasmids in the control of plant disease. Recently, Jacob and Grinter (1975) reported that Plasmid RP4 has only one site susceptible to cutting by the restriction enzyme EcoR1. Therefore the P-group plasmid could be a useful tool for genetic study of phytopathogenic bacteria (Boistard and Boucher 1978).

Drug-resistance elements are translocatable, termed transposons and are discrete sequences of DNA incapable of self-replication at a random location. In order to be replicated, it must be inserted into DNA replicons, such as chromosomes or plasmids. When it occurs in a structural gene, the result is generally non-leaky polar mutations (Kleckner et al. 1977). Recently Boucher et al. (1977) reported that when the lysogenic phage Mu was inserted into RP4, the transfer frequency of plasmid from *Escherichia coli* to *Rhizobium meliloti* was reduced from 3×10^{-4} to 2×10^{-9}, about 10^5-fold, possibly due to the suicidal effect of the phage leading to the destruction of the plasmid. Van Vliet et al. (1978) showed that the same principle applies to transfer from *Escherichia coli* to *Agrobacterium tumefaciens*. Those plasmids which do establish in this new host have large deletion of the Mu phage. They reported that the instability of the RP4::Mu system could be utilized to carry transposons into this species to obtain transposon mutagensis. Taking advantage of this suicidal effect of Mu phage, causing inability of Mu-inserted P-group plasmids to become established in recipient cells, Beringer et al. (1978) constructed an *Escherichia coli* strain 1830 harboring a plasmid carrying Mu bacteriophage and the Tn5, transposon coding for kanamycin resistance and also relatively nonspecific in the sites of integration into *E. coli* chromosome (Kleckner et al. 1977). This strain is derived from

a P1 plasmid, pPH1JI, conferring resistance to gentamycin and to very low concentrations of streptomycin; it has a wide host range in which the Tn5 and Mu phage was inserted by Beringer et al. (1978). This strain was found to be Mu immune, unable to produce viable Mu phage. This constructed plasmid was successfully used as a carrier of Tn5 into three *Rhizobium* spp. Owing to the inability of strain K60 of *Pseudomonas solanacearum* to stably maintain P1 plasmid, Boucher et al. (1981) reported that with Tn5 and Tn10 inserted into P1 plasmid, but without Mu phage inserted, he was able to have vehicles which were mutagenic that transposed with relatively high frequencies of ca. 5×10^{-6}. He found that under the same conditions with Tn7 the transposition frequency was low.

By using this transposon that integrates at random, we can determine the frequency of avirulent mutants among the transconjugants. This should permit an approximate estimation of the numerical portion of genome that involves genes for virulence.

Chapter 1

DEVELOPMENT OF A SELECTIVE MEDIUM FOR *XANTHOMONAS CAMPESTRIS* PV. *TRANSLUCENS*

INTRODUCTION

The objective of this study was to develop a selective medium, an essential tool for epidemiological studies of this pathogen. Selective medium development is usually an important step in the scientific study of a bacterial plant pathogen.

MATERIALS AND METHODS

Bacterial strains

The bacterial strains used in this work are listed in Table 1. *Xanthomonas campestris* pv. *translucens*, hereinafter referred to as *X. c. t.* and *Agrobacterium* spp., were maintained on Wilbrink's agar (Dowson, 1957); 10 g of sucrose, 5 g. of Bacto-peptone (Difco Laboratories, Detroit, MI.), 0.5 g. of K_2HPO_4, 0.25 g of $MgSO_4.7H_2O$, 0.05 g. of Na_2SO_3 and 15 g of bacto-agar (Difco) per liter of distilled water. All other strains of *Xanthomonas* were maintained on Nutrient broth (Difco), 5 g of casein hydrolysate (Sigma Chemical Co., St. Louis, MO.), 1 g of yeast extract (Difco), 2.0 g. of K_2HPO_4, 0.5 g of KH_2PO_4, and 15 g of Bacto-agar per liter of distilled water. *Corynebacterium sepedonicum* and *C. insidiosum*

were maintained on yeast glucose calcium carbonate agar; 15 g of glucose, 10 g of yeast extract, 5.0 g of $CaCO_3$, 20 g of agar per liter of distilled water. *Bacillus cereus*, *Erwinia cartovora* var. *carotovora* and *E. carotovora* var. *atroseptica* were maintained on Nutrient agar (Difco Nutrient broth amended with 15 g of Difco agar). *Escherichia coli* was maintained on Luria's agar containing 10 g of casein hydrolysate, 10 g of NaCl, 5 g of yeast extract, 15 g of Bacto-agar per liter of distilled water. *Pseudomonas* spp. were maintained on King's B agar (King et al., 1954). All strains were transferred regularly and stored at 3 - 5 C.

Selective Medium (KM-1)

This selective medium for X. c. t. contains 10 g of lactose (Sigma Chemical Co., St. Louis, MO.), 4.0 g. of D(+) trehalose (Sigma), 0.2 g of thiobarbituric acid (Sigma), 0.8 g of K_2HPO_4 and KH_2PO_4 (Sigma) respectively, 0.03 g of yeast extract (Difco Laboratories, Detroit, MI.), 1 g of NH_4Cl (Mallinckroft Chemical Works, St. Louis, MO.) and 15 g of Bacto agar per liter of double distilled water. Before adding agar, the ingredients were dissolved completely on a hot plate with stirring bar and pH was adjusted to 6.6 with 1 N NaOH solution. After autoclaving these ingredients, cycloheximide (Sigma), dissolved in ethanol, tobramycin (Sigma), dissolved in ethanol-water (1:1, v/v), and ampicillin (Sigma), dissolved in

ethanol-water (1:1, v/v) with addition of 1 pellet of sodium hydroxide (Mallinckroft Inc., Paris, Kentucky) were added aseptically to the final concentration of 100 ug/ml, 8 ug/ml and 1 ug/ml, respectively.

Choice of components of KM-1

Dowson (1939) reported that all the species of his newly created genus *Xanthomonas* utilized lactose as a carbon source. Thus, lactose is one of the carbon sources that appears to be promising as a selective medium component for *Xanthomonas*. Trehalose, a four carbon sugar, is also a good nutritional substrate for the *Xanthomonas campestris* group (Dye and Lelliott 1975). *Pseudomonas fluorescens* also utilizes trehalose (Misaghi and Grogan 1969, Stanier et al. 1966), but is not used by any of the members of the *Pseudonomas syringae* group, *Pseudomonas cichorii* (Sands et al., 1980), or *Pseudomonas putida*, or *Pseudomonas aeruginosa* (Stanier et al. 1966). The combination of these carbon sources provides selectivity against *Pseudomonas syringae*, which has been recognized as a common contaminant in the isolation of *X. c. t.* from diseased material ever since 1936 (Hagborg). Thiobarbituric acid is another component of selective value. Ammonium chloride as a nitrogen source is useful in enhancing the yellowish colony color, a key visual characteristic of these xanthomonads. Yeast extract is a necessary

source of nutritional enrichment, permitting these xanthomonads to grow at reasonably fast rates. The basal medium of KM-1 without antibiotics, exhibits fair selectivity against the fluorescent, oxidase negative, "syringae group" of pseudomonads from cereals.

The key problem in developing the selective medium for *X. c. t.* was that saprophytes grow more rapidly and are, in fact, more resistant to most antibiotics. Most plant pathogenic pseudomonads and other plant pathogenic bacteria grow faster than this pathogen in most of the media. We found that fast growing contaminant bacteria can be suppressed by using diluted concentrations of either penicillin or ampicillin, however, this pathogen is more tolerant of ampicillin than penicillin. In both cases, the mechanism of action of the above two antibiotics inhibits actively growing cells. A search for several other antibiotics with different modes of action was initiated. Tobramycin was found very useful to suppress the fast growing pseudomonads, especially the ubiquitous saprophytic fluorescent, oxidase-positive, nutritionally versatile pseudomonads relatively resistant to other antibiotics. Cycloheximide, a broad spectrum anitfungal agent, was also added.

Characterization of bacterial isolates from the KM-1 selective medium.

Color, shape, and form of colony were initially used to distinguish *X. c. t.* Selected colonies were also transferred to Wilbrink's

agar to observe the rapid development of yellow, mucoid growth, which characterize xanthomonads. These colonies were further confirmed by oxidase test and pathogenicity tests.

Relative colony development of *Xanthomonas campestris* pv. *translucens*.

Single colony culture from Wilbrink's agar was suspended in 5 ml phosphate-buffered saline (PBS) containing 0.85 percent NaCl, 0.57 percent K_2HPO_4 and 0.34 percent KH_2PO_4 (pH 6.8), after adjusting cell suspensions to contain approximately 10^6 colony forming units (CFU) per milliliter as determined by a Klett-Summerson photometric colorimeter with the green filter (Model 800-3, Klett Mfg. Inc., NY., U.S.A.). The serial ten-fold dilutions were made on PBS. Using a spinning turntable, one-tenth of a milliliter of diluted cell suspensions was spread with an L-shaped glassrod over the surface of KM-1 medium and Wilbrink's medium in each of three plates. Plates were incubated at 28° C for 5 - 7 days. Unless otherwise stated, the dilution plating procedure and incubation period are the same throughout this report.

Relative colony development of other *Xanthomonas* nonemspecies

Some strains of *Xanthomonas* spp. other than *X. c. t.* do not grow very well on Wilbrink's agar. Therefore, the relative colony development on KM-1 medium was also compared with nutrient agar

amended with 1 percent glucose.

Spectrum of inhibition

Isolates were first replica plated, and then dilution plated to determine the spectrum of inhibition of this KM-1 medium. The isolates that grew poorly, if at all, on the KM-1 medium by replica plating were selected. Dilution plating was carried out to see how selective this medium was to those bacteria (Table 1).

In order to determine the spectrum of inhibition to soil borne plant pathogenic bacteria and saprophytic bacteria, three strains of *X. c. t.* were mixed with 11 different nomenspecies of bacteria (Table 5). After the dilution plating method was used, the plates were observed in 2 - 3 days for fast growing organisms and again in 5 - 7 days for *X. c. t.* at 28° C.

Selectivity tests

Selectivity of the KM-1 medium was tested by the dilution plating method of various plant samples, such as 1) old samples naturally infected with *X. c. t.*, 2) fresh samples naturally infected, 3) old barley leaf debris not infected, and 4) soil samples from greenhouse. Old barley leaf debris was rinsed with sterile distilled water to remove soil particles and kept in phosphate-buffered saline (PBS) containing 0.85 percent NaCl, 0.57 percent dibasic potassium phosphate (K_2HPO_4), 0.34 percent monobasic potassium

phosphate (KH_2PO_4), for four hours. Old and fresh samples of naturally infected barley were kept in PBS for the same period of time. No surface sterilization was attempted for any plant materials. The 10 grams of the soil sample from the greenhouse were suspended in 100 ml distilled sterile water and stirred vigorously with a stirring bar for 30 minutes. After 10 minutes settling, the supernatant was centrifuged for 20 minutes at 1,000 g force to remove plant materials and soil particles. The supernatant was centrifuged at 3,000 g for 20 minutes. The pellet was suspended in 3 ml PBS and used as the sample for the dilution plating method in which 0.1 ml of each appropriate dilution on PBS was spread on three replicates of both KM-1 medium and Wilbrink's medium.

Shelf life of KM-1 medium

Several packages of this medium were stored at 5° C in a cold room from one week to two months. The selectivity of these KM-1 media were checked at intervals up to two months by the dilution plating method using strains listed in Table 1.

RESULTS

Relative colony development of *Xanthomonas campestris* pv. *translucens*

The average number of colonies of 11 strains of *X. c. t.* developed on KM-1 medium was from 0.91 to 2.13 times greater than on

Table 1. Strains used for evaluation of medium selective for *Xanthomonas campestris* pv. *translucens*.

Nomenspecies and designation	Source[a]
Agrobacterium tumefaciens	1
Agrobacterium rhizogenes	1
Bacillus cereus	2
Corynebacterium insidiosum	2
Corynebacterium sepedonicum	2
Erwinia carotovora var. *atroseptica*	2
Erwinia carotovora var. *carotovora*	2
Escherichia coli	1
Pseudomonas aeruginosa	5
Pseudomonas fluorescens, Pf5	6
Pseudomonas phaseoli, G 50	7
Pseudomonas phaseoli, HB 20	7
Pseudomonas savastanoi	3
Pseudomonas syringae, (8 strains)	10
Pseudomonas syringae pv. *tagetis*	4
Pseudomonas putida	10
Rhizobium phaseoli, 1233	3
Xanthomonas albilineans, 29184	8
Xanthomonas axonopodis, 19312	8
Xanthomonas fragariae, NCPPB 2473	8
Xanthomonas campestris pv. *begoniae*, 077-3382	8
X. campestris pv. *carotae*, Floral-1	8
X. campestris pv. *campestris*, # 73	8
X. campestris pv. *manihotis*, Xm6	8
X. campestris pv. *pelargonii*, 078-1100	8
X. campestris pv. *phaseoli*, 86	8
X. campestris pv. *pruni*, 8D51	9
X. campestris pv. *translucens*, (11 strains)	10
X. campestris pv. *vesicatoria*, # 26	8
X. campestris pv. *vitians*, 068-790, 7D51	9
068-1406, 7D5	9
069-561, 7D42	9

[a]1 = J. M. Jaynes, 2 = R. Davidson, 3 = R. V. Miller, 4 = B. Simmonds; Dept. of Plant Pathology, MSU, Bozeman, MT., 5 = N. M. Nelson, Dept. of Microbiology, MSU, Bozeman, MT., 6 = C. R. Howell, Univ. of TX, College sta., TX., 7 = S. S. Patil, Univ. of HI, Honolulu, HI., 8 = M. Sasser, Dept. of Plant Sci., Univ. of DE, Newark, DE., 9 = C. I. Kado, UC, Davis, CA., 10 = Author.

Wilbrink's medium (Table 2). Generally, more colonies appeared on the KM-1 medium than on rich and non-selective Wilbrink's medium. The size of colonies was from 1.5 - 2.0 to 3.5 - 4.0 mm in 5 - 7 days with yellowish color. All strains of X. c. *t.* grew well on this selective medium when a loopful of cell suspension was streaked and incubated for 5 - 7 days.

Relative colony development of other *Xanthomonas* nomenspecies.

As shown in Table 3, Nutrient agar plus glucose is a good medium for most *Xanthomonas* nomenspecies, whereas few of those grew on Wilbrink's. Single strains each of *Xanthomonas axonopodis*, *Xanthomonas campestris* pv. *campestris* and X. *campestris* pv. *vesticatoria* grew on Wilbrink's medium with colony sizes from 0.5 - 3.0 mm in diameter. Out of 12 nonemspecies, two strains of X. *campestris* pv. *vitians* 7D5 and 7D52 grew poorly or not at all on the Wilbrink's medium, whereas the strain 7D52 of the same species grew on Wilbrink's medium. However, the number of colonies developed on this medium was about half of those on the Nutrient agar.

Whereas one strain each of *Xanthomonas alibilineans* and X. *fragariae* did not grow on KM-1 medium, one strain each of X. *axonopodis* and X. *campestris* pv. *begoniae* did, even though the plating efficiency of these strains was generally low. One strain out of three strains of X. *campestris* pv. *vitians* did not grow at

Table 2. Relative colony development of *Xanthomonas campestris* pv. *translucens* on selective (KM-1) and non-selective (Wilbrink's) medium.[a]

Strain	Colonies per plate[b]		Plating efficiency[c]	Size of Colonies on KM-1
	Wilbrink's	KM-1		
X - 33	326.00 ± 7.07	341.50 ± 0.70	1.04	2.5 - 3.0
X - 40	138.37 ± 16.65	168.00 ± 26.22	1.21	3.5 - 4.0
X - 48	189.25 ± 1.76	235.75 ± 9.54	1.24	1.5 - 2.0
X - 56	389.66 ± 11.93	357.33 ± 6.35	0.91	3.5 - 4.0
X - 58	160.5 ± 11.31	239.0 ± 20.50	1.49	2.5 - 3.0
X - 67	230.75 ± 17.32	416.5 ± 33.23	1.80	1.5 - 2.0
X - 87	170.00 ± 7.07	363.50 ± 45.96	2.13	3.5 - 4.0
X - 90	255.50 ± 7.07	275.25 ± 10.96	1.07	2.5 - 3.0
X - 138	278.00 ± 21.92	329.00 ± 36.76	1.18	2.5 - 3.0
X - 140	265.00 ± 21.16	346.00 ± 21.16	1.30	2.0 - 2.5
X - 153	343.50 ± 16.26	348.50 ± 20.50	1.01	2.0 - 2.5

[a] KM-1; X. c. pv. *translucens* selective medium, Wilbrink's (Dowson, 1957).

[b] Average number of colonies per plate from 3 plates, followed by standard deviation of mean, after 7 days at 28 C.

[c] Plating efficiency = $\frac{\text{Avg. number of colonies on KM-1/plate}}{\text{Avg. no. of colonies in Wilbrink's/plate}}$

Table 3. Relative colony development of other *Xanthomonas* nomenspecies on the selective (KM-1) and non-selective (WA,NAG) medium.

Nomenspecies and designation	Colonies per plate[a]			Plating efficiency[d]	Size of colonies on KM-1
	NAG	WA	KM-1		
Xanthomonas albilineans, 29184	25.00 ± 13.52	0[c]	0	0	0
X. axonopodis, 19312	140.66 ± 22.05	355.66 ± 63.40	29.66 ± 4.04	0.21	2.6 ± 3.2
X. fragariae, NCPPB 2472	77.33 ± 16.01	0	0	0	0
Xanthomonas campestris p.v. *begoniae*, 077-3382	113.66 ± 6.65	-[e]	21.00 ± 2.64	0.18	0.2 - 0.5
X. c. pv. *carotae*, Floral-1	210.66 ± 17.09	-	412.00 ± 56.29	1.96	1.5 - 2.5
X. c. pv. *campestris*, # 73	157.66 ± 6.65	282.00 ± 63.83	180.66 ± 17.24	1.14	3.0 - 3.5
X. c. pv. *manihotis*, Xm6	202.00 ± 24.02	0	296.00 ± 43.00	1.46	3.5 - 4.0
X. c. pv. *pelargonii*, 078-1100	63.66 ± 12.58	-	190.33 ± 32.88	2.98	2.5 - 3.0
X. c. pv. *phaseoli*, 86	97.00 ± 7.00	-	134.00 ± 12.52	1.38	0.7 - 1.2
X. c. pv. *pruni* 8D51	81.33 ± 7.09	-	454.66 ± 46.00	5.59	2.5 - 3.0
X. c. pv. *vesticatoria*, # 26	121.66 ± 18.00	346.33 ± 15.53	293.33 ± 53.25	2.41	0.2 - 0.5
X. c. pv. *vitians*, 068-790, 7D51	191.00 ± 19.00	-	287.33 ± 9.60	1.50	3.0 - 3.5
X. c. pv. *vitians*, 068-1406, 7D5	128.33 ± 12.70	0	127.66 ± 19.13	0.99	2.0 - 3.0
X. c. pv. *vitians*, 069-561, 7D52	135.33 ± 11.23	63.33 ± 10.21	0.66 ± 0.57	0.004	1.0 - 1.5

[a]Average number of colonies per plate from 3 plates, followed by standard deviation, after incumbation at 28 C for 6 - 7 days. NAG: Nutrient agar mended with 1% glucose. WA: Wilbrink's agar.

[b]Very small colonies less than 0.5 mm while colonies of another nomenspecies on NAG were 3.5 - 6.0 mm in diameter.

[c]No colonies developed at all.

[d]Plating efficiency = Avg. number of colonies on KM-1/Avg. number of colonies on NAG.

[e]Microsopic colonies, the sizes being less than 0.1 mm in diameter.

all, while the two strains of this same nomenspecies grew on KM-1 medium. One strain each of *X. campestris* pv. *carotae*, *X. campestris* pv. *manihotis*. *X. campestris* pv. *pelargonii*, *X. campestris* pv. *phaseoli*, *X. campestris* pv. *pruni* and *X. campestris* pv. *vesicatoria* grew on KM-1 medium and the plating efficiency of these nomenspecies was relatively high. However, the growth of *X. campestris* pv. *phaseoli* and *X. campestris* pv. *vesicatoria* was limited and resulted only in small colonies after 5 - 7 days.

Spectrum of inhibition

Most of the pseudomonads listed in Table 1 did not grow on the KM-1 medium by replica plating methods or by streaking a loopful of bacterial cell suspensions. Those pseudomonads and *Agrobacterium* sp. and *Erwinia* spp. that did grow on the KM-1 medium by replica plating grew poorly and did not develop colonies on this medium when the bacterial cell suspensions were spread on by the dilution plating method (Table 4).

When the mixture of the 12 nomenspecies of soil-borne plant pathogenic bacteria and soil-borne saprophytic bacteria was serially dilution plated (Table 5), no colonies appeared on KM-1 medium after 2 - 3 days incubation at 28 C, whereas 376 colonies were developed on the non-selective Wilbrink's medium. Pure colonies of *X. c. t.*, 1.5 - 2.0 mm in diameter appeared in 5 ~ 7 days of

Table 4. Colony development of phytopathogenic and saprophytic bacteria on selective (KM-1) or non-selective medium (Wilbrink's) for *Xanthomonas campestris* pv. *translucens*.

Nomenspecies	Number of colonies[a]		Recovery ratio[c]
	Wilbrink's	KM-1	
Agrobacterium tumefaciens	116.66 ± 26.08	0[b]	0
Erwinia carotovora var. *atroseptica*	19.66 ± 3.51	0	0
Erwinia carotovora var. *carotovora*	283.66 ± 13.20	0	0
Pseudomonas aeruginosa	173.66 ± 14.50	0	0
Pseudomonas fluorescens	283.33 ± 53.72	0	0
Pseudomonas putida	288.33 ± 45.39	0	0
Pseudomonas savastanoi	140.33 ± 11.55	0	0

[a]Average number of colonies per plate from 3 plates, followed by standard deviation, after incubation for 7 days at 28 C.

[b]No colonies developed in 7 - 9 days at 28 C.

[c]Recovery ratio = $\frac{\text{Number of colonies on KM-1 medium}}{\text{No. of colonies on Wilbrink's medium}}$

Table 5. Colony development from a mixed inoculum containing several phytopathogenic and saprophytic bacteria on selective (KM-1) and non-selective (Wilbrink's) medium.

Number of days incubated at 28 C	Number of colonies[a]		Colony size on KM-1 (mm)
	Wilbrink's	KM-1	
2 - 3	376.66 ± 6.65[b]	0	0
5 - 7	602.33 ± 13.65[b]	71.0 ± 11.80[c]	1.5 - 2.0

[a]Average number of colonies per plate from 3 plates, followed by standard deviation.

[b]None of the colonies was recognizable as *X. c. t.*

[c]Apparently pure colonies of *X. c. t.* Bacterial inoculum contained approximately equal CFU's of *Agrobacterium tumefaciens*, *A. rhizogenes*, *Bacillus cereus*, *Corynebacterium insidiosum*, *C. sepedonicum*, *Erwinia carotovora* var. *atroseptica*, *E. carotovora* var. *carotovora*, *Pseudomonas aeruginosa*, *P. fluorescens*, *P. putida*, *P. savastanoi*, *Xanthomonas campestris* pv. *translucens* (3 strains).

incubation. Other colonies of unknown classification less than 0.1 mm in diameter also appeared in further incubation. However, they remained as small colonies less than 0.5 mm in diameter even after 15 days of incubation. Selected colonies of either size were transferred to Wilbrink's agar to confirm the virulence to host and the rapid development of yellow color. All the large colonies were proved to be *X. c. t.*

Selectivity test

No colonies appeared on KM-1 medium from dilution plating of uninfected barley debris and only three small non-yellow colonies developed on this medium from greenhouse soil, while 162 and 93 colonies of the unknown contaminants were developed on Wilbrink's medium, respectively (Table 6).

By taking both old and fresh barley leaf samples from infected plants, it was possible to isolate the *X. c. t.* with plating efficiencies of KM-1, as compared to Wilbrink's of 1.53 and 1.13, respectively (Table 7). On the rich Wilbrink's medium, numerous unknown contaminants, in addition to fluorescent pseudomonads, were encountered. However, on the KM-1 medium only very small colonies started to appear in 3 - 4 days when incubated at 28 C. These colonies remained at less than 0.1 - 0.3 mm in diameter even in 5 - 7 days incubation. *X. c. t.* colonies appeared later

Table 6. Relative selectivity of KM-1 medium when plating non-infected samples.

Samples	Number of colonies[a]	
	Wilbrink's	KM-1
Barley leaf debris	162.0 ± 24.04[b]	0[c]
Greenhouse soil	93.0 ± 2.82 [b]	3.0[d]

[a]Average number of colonies per plate from 3 plates, followed by standard deviation.

[b]Unknown contaminants by dilution plating method.

[c]No colonies appeared after 5 - 7 days at 28 C.

[d]Very small colonies less than 0.5 mm in diameter, not recognizable as xanthomonads.

Table 7. Relative selectivity of KM-1 medium when plating infected samples

Sample	No. of colonies[d] on Wilbrink's X. c. *translucens*	Unknowns	No. of colonies X.c.*t.* on KM-1	Plating efficiency[c]	Size of colonies on KM-1(mm)
Old leaf[a]	73.5± 3.53	415.5 ± 78.48	113.0 ± 16.97[b]	1.53	2.0 - 2.5
Fresh leaf	72.0 ± 29.69	432.5 ± 85.55	81.33 ± 15.69	1.13	2.0 - 2.5

[a]Barley leaf sample stored in laboratory for 7 - 8 months.

[b]Bacterial colonies of unknown classification with diameters < 0.1 mm appeared in 3-4 days growing to < 0.3 - 0.5 mm diameters even after 5 - 7 days incubation at 28 C. The colonies of X.c.*t.* appeared later but developed to large colonies, 2.0 - 2.5 mm in diameter in 5 - 7 days.

[c]Plating efficiency = $\frac{\text{Avg. number of colonies on KM-1 medium per plate}}{\text{Avg. number of colonies on Wilbrink's medium/plate}}$

[d]Average number of colonies per plate from 3 plates, followed by standard deviation.

but developed into large colonies, 2.0 - 2.5 in diameter within 5-7 days at 28 C. Selected colonies of either size were transferred to the rich medium to observe rapid development of yellow color. All the large colonies and a few (1 in 30) of the small colonies were confirmed to be *X. c. t.*

Shelf life

This medium was proved to be still effective as a selective medium even after two months of storage at 5 C.

DISCUSSION

A few selective media have been described for isolation of *Xanthomonas* spp. from nature. None of them is satisfactory for isolation of *X. c. t.* The selective medium designated as KM-1 for this bacterium has been developed to provide sensitive and reliable detection of this bacterium from nature.

Generally, the bacteria comprising the genus *Xanthomonas* grow slower than any of the other plant pathogenic bacteria and the saprophytic bacteria included in this study. The selectivity of this KM-1 medium is mainly based on choice of carbon sources and the use of a combination of antibiotics.

Selectivity and usage of this medium

Other xanthomonads grow on KM-1 medium and, since they are not

believed to be found in cereal grains, do not present problems. Those bacteria capable of growth on KM-1 are: one strain out of three strains of *Xanthomonas campestris* pv. *vitians*, and one strain each of *X. campestris* pv. *carotae*, *X. campestris* pv. *campestris*, *X. campestris* pv. *manihotis*, *X. campestris* pv. *pelargonii*, *X. campestris* pv. *phaseoli* and *X. campestris* pv. *vesicatoria*. The plating efficiency of these nomenspecies, with the exception of *Xanthomonas campestris* pv. *vitians*, was relatively high. The potential use of the KM-1 medium for these nomenspecies remains unknown until many strains representing each species are tested and shown to grow adequately on this medium. One strain of *X. campestris* pv. *pruni* grew very well on this KM-1 medium with a very high plating efficiency. Again, many strains should be tested with this medium along with XPSM medium by Civerolo et al. (1982), in order to determine the potential use of this medium as a selective medium for this nomenspecies.

This KM-1 medium has high selectivity against the soil-borne plant pathogenic baceria, such as *Agrobacterium tumefaciens*, *Agrobacterium rhizogenes*, *Erwinia carotovora* var. *atroseptia*, *Erwinia carotovora* var. *carotovora*, *Corynebacterium insidiosum*, *Corynebacterium sepedonicum* and also the common soil-borne saprophytic bacteria, such as *Bacillus cereus*, *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Pseudomonas putida*. This KM-1 medium may be a useful tool to study the ecology and epidemiology of this pathogen. It has

a relatively long shelf-life of at least two months when refrigerated, high selectivity against soil samples and barley leaf debris, and a high recovery ratio of the several strains of *X. c. t.*, compared to the rich but non-selective medium of Wilbrink's (Dowson 1957).

This medium may be a valuable tool to detect and prove the quantitative distribution of this pathogen on plants, and insects or in raindrops as an ice nucleating active agent. All xanthomonads recognized at present are plant pathogens found only in association with living plants or with plant material (Dye and Lelliott 1975, Dye 1980). This medium could be a tool to detect and identify new saprophytic or avirulent xanthomonads. *X. c. t.* is known as a poor survivor in soil unless it is combined with plant or plant debris (Boosalis, 1952). This medium could reveal a quantitative fluctuation of bacterial cells in the soil, and may, if present, also enable one to find a bacterium-vector relation.

Chapter 2

SCREENING OF DISEASE RESISTANCE

INTRODUCTION

The objective of this study was to develop a method of screening for resistance in barley to bacterial leaf streak caused by *Xanthomonas campestris* pv. *translucens*.

MATERIALS AND METHODS

Field experiments

Field plot experimentation to assay for resistance to bacterial leaf streak of barley was conducted during the summer of 1981 with 54 varieties of commercial barley, 465 lines from the World Barley Collection comprised of 389 Ethiopian, 6 Israeli, 17 USSR, 1 Turkish, 1 Czechosolovakian, 13 Argentinean, 4 Swedish, 12 Jordanian, 1 Iranian, 28 Hungarian, and 4 Bulgarian lines, and another set of 257 Ethiopian lines. Commercial barley varieties were obtained from R. F. Eslick and E. Hockett, USDA, ARS, Department of Plant and Soil Science, Montana State University; the World Collection and Ethiopian lines were obtained from J. Moseman, USDA, ARS, Beltsville, Maryland.

A total of 50 seeds of each of the 54 cultivars and lines were planted at three separate Montana locations where natural infection

has been observed for the last four years. Each variety was planted in 33 cm rows with 3.3 m between rows at the MSU horticultural farm in Bozeman, Montana, on May 30, in Fairfield on May 31, and at the Eastern Montana Research Center, Sidney, Montana, on May 27.

Ten seeds of each of the 465 lines of the World Barley Collection and 257 Ethiopian lines were planted as hill plots, 33 cm apart in both directions on May 27, and June 5, 1981, respectively.

Artificial inoculation was carried out in the experimental plot in Bozeman with a strain X-87 of *Xanthomonas campestris* pv. *translucens*. This strain of f. sp. *undulosa* sensu Hagborg (1942) was chosen for its high virulence levels on wheat and barley. The plots in Fairfield and in Sidney were not inoculated, already containing high levels of natural incidence of this disease. Inoculum for Bozeman plots was grown in Wilbrink's broth at room temperature for four days and diluted to 10^8 colony forming units (CFU) per milliliter, as determined by the Klett-Summerson photoelectric colorimeter with green filter (Model 800-3, Klett Mfg. Co., Inc., N.Y.). One klett unit represents about 10^6 CFU. The plants in the Bozeman plot were wounded by mowing off the top 10 cm of the plants in the evening about 8:30 p.m. June 28, and immediately inoculated by spraying the cell suspension using a back-pack sprayer (Type 40-123, No. 206304, Solo-Kleinmotoren, GMBH, West Germany). Plots were sprinkler irrigated every three days and plants

were observed daily after six days. Symptoms appeared ten days after incoulation. On July 13 the second inoculation was done to avoid the possibility of non-genetically determined escape from infection. The reactions were rated on July 27 by using the following index: 1) No distinguishable symptoms; 2) Water-soaked streak symptoms; 3) Spread of streak symptom to 2nd and 3rd leaves; 4) Streak symptom up to flag leaf but less than 10 percent of flag leaf showing symptoms; and 5) Streak symptom up to flag leaf with more than 10 percent of flag leaf showing sysmptoms. In reading disease severity in a given variety, the worse case was recorded to avoid the possibility of escape.

Greenhouse experiments

Seven cultivars representing four resistant and three susceptible entries were selected based on the field results. Using these seven cultivars (Table 11), various inoculation methods were tested in order to develop an inoculation method that would closely simulate the field observations. The cultivars were planted in metal flats with a mixture of one part sand and three parts soil and grown in the greenhouse.

Spraying cell suspensions without injury to plant

Guttation water appears as a drop on the tip of primary leaves of 7 and 14 day old barley seedlings only in the late evening under

greenhouse conditions. At this hour, fresh bacterial suspensions of 10^6, 5 x 10^7, and 10^8 colony forming units (CFU) were sprayed without injury to the plant. The inoculum concentration was determined by above described turbiditimetric methods.

Introduction of inoculum with hypodermic syringe

Bacterial cell suspensions of 10^6, 10^7, 10^8 CFU were prepared in the same manner as above. A droplet was poked through the basal stem of the 7 and 14 day old seedlings with a hypodermic syringe, gauge 23, another drop was then withdrawn back through the wound. After inoculation, the plants were placed in environmental chambers with the following day and night temperatures; 1) 15.5 and 4.5 C with 14 hrs light and 10 hrs dark, 2) 32.0 and 1.8 C with 14 hrs light and 10 hrs dark, 3) 21.1 and 12.8 C with 12 hrs of light and dark, and 4) 37.8 and 18.4 C (in greenhouse). The reaction was rated after two weeks by the index as follows: 1) a barely visible symptom, followed by water-soaking limited around the wound; 2) water-soaked streaks covering less than 5 percent of leaf area; 3) symptoms typically less than 10 percent of leaf area; 4) symptoms typically less than 25 percent of leaf wounded and slight symptoms on unwounded upper leaves; and 5) typical symptoms with more than 25 percent of the leaf wounded and severe symptom on upper leaves.

RESULTS

Field experiments

The infection of cultivars was less severe at Fairfield than at the other two locations (Table 8). Plants that were resistant in all three locations were Herta, Summit, Oderbrucker, MT 853337. Alpine, Luther, Betzes, Wabet, and Shabet were resistant in two locations tested. Supersusceptible cultivars were California Mariout 67, and Athenais. Reaction of some cultivars or lines, such as Compana, Lion, MT 853231, MT 853244, MT 853260, MT 853287, MT 853290, Suweon 188, Suweon 180, Washonupana, Trebi and Kamiak varied in different locations. With the exceptions of MT 853244 and MT 853287, reactions were always lowest at Fairfield.

In the World Barley Collection and Ethiopian lines respectively, 11 lines and 10 lines were determined to be resistant to bacterial leaf streak when inoculation was performed by wound inoculation with spraying bacterial suspensions under sprinkler irrigation in Bozeman, Montana (Table 9, 10). Of the 646 Ethiopian lines, 19 were resistant. Two out of four Swedish lines were resistant. None were resistant from 17 USSR, 13 Argenenian, 12 Jordanian and 28 Hungarian lines.

Table 8. Relative susceptibility of barley varieties and lines to bacterial leaf streak in the field.

Variety or Lines	Readings[a,d] Fairfield[b,c]	Sidney[b,c]	Bozeman[b,c]
Herta	1	1	1
Athenais	4	5	5
Georgia	-	-	2
Hypana	-	-	5
Summit	1	1	1
C.I. 8159	-	-	4
Compana	1	3	3
Klages	-	-	4
Peatland	-	-	3
Tanekase 105	-	-	5
CM 67	5	5	5
Spartan	-	4	3
Oderbrucker	1	1	1
Lion	1	3	4
MT 853231	1	4	5
MT 853242	4	4	3
MT 853244	3	1	3
MT 853260	1	2	4
MT 853263	4	2	5
MT 853284	4	3	4
MT 853287	4	1	5
MT 853290	1	2	5
MT 853292	5	3	5
MT 853320	4	3	4
MT 853333	5	3	5
MT 853337	2	2	2
MT 853345	5	3	5
Baekdong	4	4	3
Bunong	3	5	2
Kangbori	5	5	5
Olbori	3	5	3
Suweon 188	0	5	2
Suweon 191	5	5	5
Suweon 180	1	5	5
Dongbori #1	5	4	3
Milyang #14	4	4	5
Milyang #11	3	4	2
Suweon 184 (sp. late)	5	-	4
Suweon 184 (sp. early)	5	-	4
Alpine	1	-	1
Washonupana	1	-	4
Nupana	3	4	4
Seashabet	1	-	4
Unitan	4	-	5
Shabet	1	-	2
Betzes	1	-	1
Wabet	1	-	1
Trebi	1	-	3
Steptoe	1	-	2
Robur	1	-	2
Luther	1	-	1
Boyer	3	-	2
Kamiak	1	-	3
Edda II	4	-	5
Average of reading	3.0	3.29	3.67

[a] Index 1) No distinguishable symptoms; 2) Typical water-soaked streak symptoms; 3) Spread of typical symptom to 2nd and 3rd leaves; 4) Typical symptom up to flag leaves, but less than 10% of the flag leaf showing symptoms; 5) Typical symptom up to flag leaves with more than 10% of flag leaf showing symptoms.

[b] Total of 50 seeds of each entry was planted in a single row, 3.3 m long, with 33 cm between rows at MSU Horticultural Farm in Bozeman on May 30, in Fairfield on May 31, and at the Eastern Montana Research Center, Sidney, on May 27, 1981.

[c] In the field plot at Bozeman, each entry was wounded by mowing off the top 10 cm with a rotary lawn mower and inoculated by spraying with fresh bacterial cell suspension (10^8 CFU) of strain X-87 of Bacterial leaf streak pathogen in the evening. Plots at Fairfield and Sidney were not inoculated, but these contained high levels of uniform natural infections.

[d] All field plots were sprinkler irrigated.

Table 9. Resistant selections from world collection of barley.[a,b]

C.I. #	Source	Growth Habit	Index
12558	Ethiopia	Spring	1
12569	Ethiopia	Spring	1
12577	Ethiopia	Spring	1
12595	Ethiopia	Spring	1
12661	Ethiopia	Spring	1
12668	Ethiopia	Spring	1
12776	Sweden	Spring	1
12777	Sweden	Spring	1
12787	Ethiopia	Spring	1
12866	Ethiopia	Spring	1-2
13095	Ethiopia	Spring	1

[a]Ten seeds of each 465 lines of the World Barley Collection were planted as hill plots 33 cm apart in both directions on May 27, 1981, on the Horiticultural Farm of MSU, Bozeman, MT. The plot was sprinkler irrigated.

[b]See footnote of Table 8 for method of inoculation and disease index.

Table 10. Ethiopian barley lines that are selected as resistant to bacterial leaf streak.[a,b]

P.I. No.	Name & Designation	Origin	Species	Growth Habit	Index
382698	Wiebe, G. A. 12-3	107 Ethi	22 Irr	Spring	1
382720	Wiebe, G. A. 24-3	107 Ethi	22 Irr	Spring	1
382732	Wiebe, G. A. 30-3	107 Ethi	22 Irr	Spring	1
382738	Wiebe, G. A. 31-4	107 Ethi	22 Irr	Spring	1
382773	Wiebe, G. A. 38-3	107 Ethi	41 Vul	Spring	1
382830	Wiebe, G. A. 46-3	107 Ethi	22 Irr	Spring	1
383065	Wiebe, G. A. 115-1	107 Ethi	41 Vul	Spring	1
383077	Wiebe, G. A. 119-1	107 Ethi	22 Irr	Spring	1
382511	Wiebe, G. A. 97-1	107 Ethi	17 Dis	Spring	1
382650	Wiebe, G. A. 100-3	107 Ethi	22 Irr	Spring	1

[a]Ten seeds of each of 257 Ethiopian lines were planted as hill plots 33 cm apart in both directions on June 5, 1981 at the MSU Horticultural Farm in Bozeman. This plot was inoculated and sprinkler irrigated.

[b]See footnote of Table 8 for methods of inoculation and indexing disease.

Greenhouse experiments

Spraying bacteria into unwounded plants did not appear to be a reliable method of inoculation. Many plants of a given variety escaped infection and symptom development was too variable.

By wound inoculation with a hypodermic syringe, using 10^7 inoculation levels, severe symptoms developed in all varieties. At the inoculum concentration of 10^6 CFU, slight differences in the degree of symptom development was noted, but infection was not sufficient enough for reliable readings.

Symptom development was influenced by temperature (Table 11) with the minimum threshold temperature for symptom development being 15.5 C. Symptoms were not expressed in any of the seven varieties under the 15.5 C day and 4.5 C night regime even after 30 days. At higher temperatures, symptoms developed, but the readings by disease index did not appear to have any correlation to the results of field experiments (Table 11). The cultivars Luther and Alpine were relatively stable in terms of level of resistance. All four of the varieties selected as resistant were late maturity types, but, inversely, not all of the late maturing types were resistant. The growth of Luther and Alpine appeared to be less responsive to high temperature. Summit and Oberbrucker were resistant in the field experiment, but were rather severely infected in the greenhouse.

Table 11. Effect of temperature on symptom development

Cultivars[a]	Growth Chamber (C)				Field reaction by wound spray inoculation
	15.5-4.5[b]	32.0-1.8[b]	21.1-12.8[b]	37.8-18.4[c]	
Luther	1[d]	2	2	2	1
Alpine	1	2	2	2-3	1
Summit	1	3	4	4	1
Oderbrucker	1	3	4	4-5	1
CM 67	1	2-3	4	5	5
Hypana	1	3	4	4-5	5
Edda II	1	3-4	4-5	5	5

[a] Seven cultivars representing four resistant and three susceptible entries were selected based on the field results (see footnote of Table 8 for disease index) for field results.

[b] Day and night temperatures in growth chamber.

[c] Day and night temperatures in greenhouse.

[d] Inoculation by hypodermic introduction of 2 droplets of 10^6 CFU per ml in greenhouse experiments. The reaction was rated after two weeks in the index: 1) A barely visible symptom, followed by water soaking limited around the wound, 2) Water-soaked streaks covering less than 5% of the leaf area, 3) Symptoms typically less than 10% of leaf area, 4) Symptom typically less than 25% of leaf wounded and slight symptoms on unwounded upper leaves, 5) Typical symptoms with more than 25% of leaf wounded and severe symptom on upper leaves.

DISCUSSION

It is interesting to note that the resistant cultivar Oderburcker (C.I.4666) recognized as early as 1917 by Jones et al. and in 1944 by Dickson (1957) were resistant in all three field locations in Montana. This cultivar was susceptible in greenhouse tests. Luther is a mutant induced from Alpine, both of which were resistant. The cultivar Summit and its derivative MT 853337 were resistant. These findings indicate that the field screening is a reliable method of searching for germplasm sources. Luther and Alpine are good sources of resistance for winter barley, while Summit and Betzes are good sources of resistance for spring barley. Spartan was reported as a resistant cultivar by Stewart (1952), but in the field trial in Bozeman and in Sidney it was rather severely infected. This may be due to either a change of the pathogen since 1952 or to the different environmental conditions. The fact that the disease ratings of MT 853244 and MT 853287 at Sidney were resistant, while at other locations these same entries were susceptible, may be some clue to the presence of physiological races of this pathogen. Disease escape is possible especially when the materials were exposed to only natural infection. However, Sidney appears to be an ideal location for assaying resistance to this disease, since all plants in a row showed consistently uniform infection and no

escaping was noted. Nonetheless, susceptible cultivars might be planted as an end spreader rows or in every third row to give equal exposure to infection in the future experiments. Those lines selected from World Barley Collection, from the Ethiopian lines, as well as the other cultivars selected as resistant, should be further tested in the different epidemic areas, along with susceptible spreader cultivars.

Attempts to develop a screening method for disease resistance in the greenhouse were not successful. Spraying bacterial cell suspensions into unwounded plants was not successful even with post-inoculation incubation in a humidity chamber for 24 hrs. This finding may point to the importance of stomatal opening for a bacterial infection to occur. In the dark humidity chamber, stomata remain closed while carbon dioxide may accumulate around the stomata, preventing the bacteria from invading plants. As soon as the inoculated seedlings are moved to greenhouse bench, the inoculum dries out quickly. In the field situation, dew formed overnight remains until after sunrise and the microenvironment surrounding the stomata changes to aerobic. Then the bacterial cells can invade the plant through open stomata. Symptoms surely can be obtained in the greenhouse and growth chambers at or above 15.5 C, but they appear to have no relationship to the field observations. This fact hampered the genetic analysis of resistant genes from genetic

materials, and at present we must rely on the result of field screening to obtain resistance of barley to this pathogen. Symptom expression appeared to be influenced by temperatures. At 15.5 C or lower, which is well within the temperature range of bacterial growth in culture, the symptoms were not developed in any of the cultivars, regardless of the degrees of resistant. Symptoms developed above this temperature in the greenhouse but results could not be related to those in the field. Several explanations exist for this anomaly. In the greenhouse, the seedlings of Luther, Alpine, Summit and Oderbrucker were susceptible, but they gave a high level of resistance in the field. This may be due to growth stage resistance, as in some cases of wheat to rust. The wheat seedling was reported to be susceptible but older plants were shown to exhibit rather high grades of resistance in the field (Gassner and Straib 1929, Purdy and Allan 1963). Since ultraviolet light is missing in the greenhouse conditions, resistant gene expression may be stimulated by UV light. This may be evidenced by the observation that development of anthocyanin pigmentation was enhanced in the barley cultivar Betzes by ultraviolet illumination (R. F. Eslick, personal communication). Other explanations of the greenhouse/field resistance anomaly exist. In the field situation, resistant cultivars may not permit the epiphytic growth of this pathogen and

thus they may be protected from infection. Alternatively, resistant plants may support the growth of antagonistic microflora, thereby inhibiting the colonization of the pathogen. Greenhouse conditions, generally of low humidity and of low free moisture, may not permit epiphytic growth either of pathogens or saprophytes.

As an effort to incorporate resistant genes into barley, the male sterile facilitated recurrent selection population for the development of broad-based resistance in barley has been developed (Bockelman, Sharp, and Eslick, 1981). Since most of the germplasm sources that are resistant to disease have certain poor agronomic characteristics, the major goal of the recurrent selection population is to develop a germplasm with multigenic resistance combined with good agronomic types, enabling the plant breeder to select for adapted plant types. RSP-5, Rxt, the recurrent selection population for resistance to *X. c. t.* have been screened in epidemic areas over the world. After five cycles of selection and recombination, the percentage of resistant plants in the field is still less than 5 percent in this Rxt population. The screening methods are not yet available for evaluation of the genetic advancement of this population. However, reliable results can be obtained from field screening in locations under intense natural infection such as Sidney, Montana. The new source of resistance should be screened from germplasm of world collection. This Rxt population could be further improved by

1) artificially crossing these resistant gene sources onto the male sterile plants within this population and 2) combining these crossed seeds into the Rxt population in the following recombination cycle. The alternate methods of accumulating resistance genes, evaluation and recombination, may be more successful long range approaches to sustained control of this disease. The final step is to select the fertile resistant plant with good agronomic types in the field and to select lines that the male sterile gene has been eliminated from pedigree method.

Chapter 3

XANTHOMONAS CAMPESTRIS PV. *TRANSLUCENS*, A NEW ICE NUCLEATING PLANT PATHOGENIC BACTERIUM

INTRODUCTION

The objective of this study was to investigate new and unique traits of this bacterium, which may be important factors resulting in severe epidemics, with the goal of obtaining tests useful in taxonomic differentiation of the translucens group from the campestris group.

MATERIALS AND METHODS

Sources of bacteria

Bacterial strains tested are listed in Table 12. All strains of *Xanthomonas campestris* pv. *translucens* are grown at 28 C for four days on Wilbrink's agar, containing 10 g of sucrose, 5 g of Bacto-peptone, 0.5 g of K_2HPO_4, 0.25 g of $MgSO_4 7H_2O$, 0.05 g. of Na_2SO_3 and 15 g of Bacto-agar per liter of distilled water (Dowson, 1957). *Pseudomonas syringae* from wheat and *Pseudomonas fluorescens* were grown at 28 C for two days on King's B agar. The rest of the strains of *Xanthomonas* spp. were grown at 28 C from four to seven days on Nutrient agar amended with 1 percent glucose, containing 8 g of nutrient broth (Difco), 5 g of casein hydrolysate, 1 g of yeast

Table 12. Bacterial strains tested preliminarily for the nucleation activity

Nomenspecies	Source
Pseudomonas syringae, S-2	Author
Pseudomonas fluorescens, Pf5	C. R. Howell, U. of TX.
Xanthomonas albilineans, 29184	M. Sasser, U. of Del.
X. axonopodis, 19312	M. Sasser, U. of Del.
X. fragariae, NCPPB2473	M. Sasser, U. of Del.
Xanthomonas campestris, pv. *begoniae*, 077-3382	M. Sasser, U. of Del.
X. c. pv. *carotae*, Floral - 1	M. Sasser, U. of Del.
X. c. pv. *campestris*, #73	M. Sasser, U. of Del.
X. c. pv. *manihotis*, Xm6	M. Sasser, U. of Del.
X. c. pv. *pelargonii*, 078-1100	M. Sasser, U. of Del.
X. c. pv. *phaseoli*, 86	M. Sasser, U. of Del.
X. c. pv. *pruni*, 8D51	C. I. Kado, UC, Davis, CA.
X. c. pv. *translucens*	Author
X. c. pv. *vesicatoria*, #26	M. Sasser, U. of Del.
X. c. pv. *vitians*, 068-790, 7D51	C. I. Kado, UC, Davis, CA.
068-1406, 7D5	
069-561, 7D52	

extract, 2.0 g of K_2HPO_4, 0.5 g of KH_2PO_4, and 15 g of Bacto-agar per liter of distilled water.

Preliminary testing procedure

The fresh cells of all the bacterial strains were transferred to tubes with 3 ml sterile water and the turbidity of the cell suspension was adjusted to 10^6 and 10^7 colony forming units (CFU) per milliliter as determined by a Klett-Summerson photometric colorimeter with a green filter (Model 800-3, Klett Mfg. Inc. N.Y., U.S.A.). The cell suspensions were exposed at -2C for three hours in the refrigerator in order to determine the ice nucleation activity. The ice nucleation activity was determined by visual observation of ice formation by freezing the bacterial suspensions. No ice formation was observed in the negative controls. *Pseudomonas syringae* strain S-2, from cereals was used as a positive control, while *Pseudomonas fluorescens* and sterile water blanks were used as negative controls. The experiments were repeated for four times with three replicates of each sample.

RESULTS

Laboratory test

As shown in Table 13, all strains of *X. c. t.* tested were ice nucleation active at concentrations of 10^6 and 10^7 CFU as was the known ice nucleator, *Pseudomonas syringae*. No other nomenspecies of

Table 13. Ice nucleation activity of strains tested.

Nomenspecies	Ice Nucleation Activity at 10^6 and 10^7 CFU	
Pseudomonas syringae, S-2	+A	+A
Pseudomonas fluorescens, Pf5	-B	-B
Xanthomonas albilineans, 29184	-	-
X. axonopodis, 19312	-	-
X. fragariae, NCPPB2473	-	-
Xanthomonas campestris pv. *begoniae* 077-3382	-	-
X. c. pv. *carotae*, Floral - 1	-	-
X. c. pv. *campestris*, #73	-	-
X. c. pv. *manihotis*, Xm6	-	-
X. c. pv. *pelargonii*, 078-1100	-	-
X. c. pv. *phaseoli*, 86	-	-
X. c. pv. *pruni*, 8D51	-	-
X. c. pv. *translucens*	+C	+C
X. c. pv. *vesicatoria*, #26	-	-
X. c. pv. *vitians*, 068-790-7D51	-	-
068-1406-7D5	-	-
069-561, 7D52	-	-
Sterile Water	-	-

[a]POSITIVE; Ice formation observed at -2 C in 3 hrs.

[b]NEGATIVE; No ice formation at -2 C in 3 hrs.

[c]See Table 12 for strains studied.

Negative strains were proved to be negative even at higher concentrations (100 - 120 x 10^6 CFU).

This experiment was performed four times with identical results.

Xanthomonas was active. Of the 11 strains of *X. c. t.* all strains were ice nucleation active at -2 C for three hours. One isolate, X-90, was not as active as the others, indicating some variability among the isolates (Table 14). It was noticed that three strains of *Xanthomonas campestris* pv. *vitians* were recorded as negative for ice nucleation activity, confirming the findings of Pauling and Luisetti (1978). All ice nucleation negative strains were further retested at higher concentrations of cell suspensions at approximately 100-120 x 10^6 CFU) and still found to be negative.

DISCUSSION

In relatively recent discoveries it was found that certain plant pathogenic bacteria can initiate physical effects leading to frost injury. Although *Pseudomonas syringae* van Hall and *Erwinia herbicola* (Loehnis) Dye, are reported to be ice nucleators, this is the first report of *X. c. t.* being an ice nucleator.

The severe epidemic of bacterial leaf streak on wheat and barley in Idaho in 1981, which was caused by *X. c. t.*, may have been predisposed by the frost on the 15th - 25th of June in the area of subsequent epidemics (Wesenberg and Sundermann, USDA, ARS, Aberdeen, Res. Sta., Univ. of Idaho, personal communication). This was a casual observation and not supported by direct evidence, ie. the presence of this pathogen on the plant as an inducing factor of

Table 14. Ice nucleation activity of strains of *Xanthomonas campestris* pv. *translucens*.

Strains	Ice nucleation activity 10^6	10^7 CFU
X - 33	++	++[b]
X - 38	++	++
X - 40	++	++
X - 48	++	++
X - 49	++	++
X - 58	++	++
X - 67	++	++
X - 87	++	++
X - 90	+	+[a]
X - 153	++	++
X - 155	++	++
Sterile water	-	-[c]

[a]Ice nucleation activity of this strain was lower than any other strains.

[b]Ice nucleation active at -2 C in 3 hrs.

[c]Ice nuceleation negative at -2 C in 3 hrs.

This experiment was performed four times with identical results.

frost injury to plants. Nevertheless, the possibility is suggested that this organism, seed-borne, or wind-driven by rain, or over-wintering plant debris, had been present as an epiphyte on wheat and barley. One explanation of the development of the severe epidemic is subsequent induction of frost injury. This suggestion is comparable to the findings of Panagopoulos and Crosse (1964), who determined that frost injury was a predisposing factor for the symptom development of blossom blight of pear caused by *Pseudomonas syringae* found to be present on the leaves and blossoms of pear trees. Their observations prompted the research on the ice nucleation activity of plant pathogenic bacteria.

Xanthomonads are probably more closely related to the fluorescent pseudomonads than to any other group of phytopathogenic bacteria, as evidenced by their rRNA cistron similarities and DNA homologies (De Ley, 1978). *Xanthomonas campestris* pv. *translucens* is similar to *Pseudomonas syringae* because it attacks a wide variety of hosts and is moved by wind-driven rain, possibly over long distances. It could be an important factor in the epidemiology and survival of this pathogen, but to date very little evidence has been obtained. Sands et al. (1981) found *Pseudomonas syringae* in raindrops and in ice in rainclouds, but a quantitative measurement of the extent of these ice nucleation bacteria as causal agents is still unknown.

Recently the translucens group of Xanthomonads, culturally,

physiologically, and biochemically indistinguishable from strains in *Xanthomonas campestris* except by host range, has been named a pathovar of the *Xanthomonas campestris* group. Together with the fact that the 17 known hosts are all in the family Gramineae, this finding of ice nucleation activity in the "translucens" group may provide sufficient additional evidence for the need for taxonomic differentiation of the translucens group from other members of the campestris group. Interestingly, all tested "translucens" isolates exhibit ice nucleation activity, whereas not all strains of *Pseudomonas syringae* and *Erwinia herbicola* are ice nucleation active. There is further evidence of a distinct systematic group that many nomenspecies in the campestris group do not grow on Wilbrink's agar (Dowson, 1957), the commonly used medium for the "translucens" group. Most nomenspecies of the members of the campestris group grow on nutrient agar, in which strains of *X. c. t.* grow poorly.

This evidence of *X. c. t.* as a new ice nucleating active agent suggests an important epidemiological aspect of this pathogen. Generally, most bacterial plant pathogens are assumed to be spread by rainsplash within a field. The spread of bacterial plant pathogens over long distances has been attributed to insect vectors and to the movement of infected or infested plant material, including seeds. However, the rate of seed transmission is relatively low, less than 1 - 2 percent (Hall, Kim and Sands, 1981), and the

appearance of disease epidemics is sporadic (Bamberg, 1936). Therefore, there may be some additional modes by which substantial amounts of infection arise in a short period of time, resulting in the severe epidemics as observed throughout wide areas of cereal cropland. This finding of ice nucleation of *Xanthomonas campestris* pv. *translucens* suggests that the following speculations are in order: 1) this pathogen may enhance rainfall; 2) the movements of this pathogen via rainstorms and clouds may be a major method of long-distance dispersal; 3) this pathogen may cause frost damage to the host in a supercooled environment resulting in massive infection; 4) ice nucleation activity may be one reason why this pathogen is such a poor survivor; and 5) ice nucleation following epiphytic growth may be one reason why resistance in the field could not be evaluated in the greenhouse.

Chapter 4

TRANSPOSON MUTAGENESIS OF *XANTHOMONAS CAMPESTRIS* PV. *TRANSLUCENS*

INTRODUCTION

The objective of this study was to develop the method of transferring transposon Tn5 to *X. c. t.* to provide the ground work for marking strains with antibiotic resistance and for genetic analysis of virulence and other traits.

MATERIALS AND METHODS

Bacterial strains

Escherichia coli strain 1830 was provided by J. M. Jaynes and is auxotrophic to l-proline and l-methionine; it harbors a plasmid carrying Mu bacteriophage and Tn5 which codes for resistance to kanamycin and neomycin. This strain was used as transposon donor. These resistant determinants provide genetic markers which permit positive selection for any transpositional event. The recipient strain was *X. c. t.* f. sp. *cerealis* X-58, pathogenic on barley, wheat, oat and rye. The donor strain was maintained on Luria's agar amended with 100 ug/ml each of kanamycin sulfate and neomycin sulfate (Sigma Chem. Co., St. Louis, Mo.) and the recipient strain, susceptible to kanamycin and neomycin, was maintained on Wilbrink's agar.

Media

Succinate Broth (SB) contained 5 g of sodium succinate, 7 g K_2HPO_4, 3 g KH_2PO_4, 1 g $(NH_4)_2SO_4$ and 0.1 g $MgSO_4$ per liter of distilled water and pH was adjusted to 7.0 with NaOH. Luria's broth (LB) contained 10 g casein hydrolysate (Sigma), 10 g NaCl, 5 g yeast extract per liter of distilled water. Luria's agar (LA) was prepared by adding 15 g of Bacto-agar per liter of LB. Nutrient agar (NA) was prepared by adding 15 g of Bacto-agar to 8 g of Difco nutrient broth. Wilbrink's agar (WA) contained 10 g sucrose, 5 g Bacto-peptone, 0.5 g K_2HPO_4, 0.25 g $MgSO_4.7H_20$, 0.05 g Na_2SO_3 and 15 g Bacto-agar per liter of distilled water. Dye's medium contained 1 g $(NH_4)_2HPO_4$, 0.2 g KCl, 0.2 g $MgSO_4.7H_2$), 10 g glucose and 15 g of Bacto-agar per liter of distilled water. All media were autoclaved at 121 C for 20 minutes.

Mating procedure

The donor strain was grown at 37 C overnight in 10 ml of SB amended with 100 ppm each of l-proline, l-methionine, kanamycin and neomycin (SBPMKN) with shaking in 125 ml Erlenmeyer flasks and in 10 ml LB amended with 100 ppm each of kanamycin and neomycin (LBKN) in glass tubes without shaking. The recipient strain was grown for 48 hours in 10 mls of LB in glass tubes at 28 C incubator without shaking or on the same medium for 30 hours with shaking.

Donor and recipient cells were spun down at 5,000 rpm for 20

minutes with a Sorvall 34 rotor (3,020 g) and the pellets were resuspended in 1 ml phosphate-buffered saline (PBS) contained 0.85 percent NaCl, 0.57 percent K_2HPO_4 and 0.34 percent KH_2PO_4. The donor and the recipient cultures were mixed (1:1 v/v) and 0.1 ml of this mixture was collected and spread on a 0.22 um millipore filter disk (Millipore Corp., Bedford, Mass.) on NA or WA from 24 hours to 96 hours at 24 hour intervals at 28 C incubator. The bacterial cells on the filter disks were washed and suspended 3 ml PBS after the appropriate mating time and 0.2 ml of serial dilutions of mixture of bacteria were plated on Dye's medium amended with 100 ppm of kanamycin and neomycin (DKN) which is counterselective for the donor. The putative transpositional mutants were counted 9 - 10 days after plating. Transfer frequencies (Tf) of conjugation were calculated from Tf=colony forming units (CFU) of putative transpositional mutants per milliliter at time t/CFU of donors per milliliter at time zero.

Characterization of putative transpositional mutants

All colonies on the DKN medium were transferred to Wilbrink's agar medium amended with 100 ppm each of kanamycin and neomycin (WKN). All of about 1,200 mutants were distinguished from donor by colony morphology, yellow color on WKN medium and were subjected to virulence test to five plants each of barley (Hypana), wheat

(Winridge, MT 77077), oat (Basin) and rye (Dakold). The virulence was tested by poking two-week old seedlings half way through, and the inoculum of 50 to 80 Klett units was squeezed until the inoculum came out in the top center of the plant.

RESULTS

Optimal conditions for transposon mutagenesis

The cultural conditions, mating time, and the mating medium were critical. Mating on WA was not successful at all, regardless of the cultural conditions and the mating time. NA was a more appropriate medium for mating. Still, no putative transpositional mutants were encountered at mating times of 24, 48, and 72 hours. A mating time of 96 hours on NA at 28 C was essential for successful mutagenesis of this xanthomonad by Tn5 from *Escherichia coli*.

The cultural conditions of donor and recipient prior to mating is also important. The recipient strain grows slower than the donor and requires a lower optimum temperature for growth. Furthermore, shake culture of the recipient strain seems to reduce transfer frequencies. The donor strain grown on LBKN for 24 hours at 37 C and the recipient strain grown on LB for 48 hours at 28 C both without shaking, proved to be the best cultural conditions prior to mating.

Visual observation indicated that the mating mixture on the

Table 15. Transfer frequencies of Tn5[a] to *Xanthomonas campestris* pv. *translucens* under different cultural conditions at 96 hrs mating.

Cultural condition		Transfer frequencies[d]	
Donor	Recipient	NA	WA
LB[b] without shaking	LB with shaking	0.19×10^{-6}	0
LB without shaking	LB without shaking	1.65×10^{-6}	0
SB[c] with shaking	LB without shaking	0.86×10^{-6}	0

[a]Tn5: Transposon coding for kanamycin and neomycin resistance.

[b]LB: Luria's broth, for donor only, amended with 100 ppm of kanamycin and neomycin.

[c]SB: Succinate broth amended with 100 ppm each of l-proline, l-methionine, kanamycin and neomycin.

[d] $Tf = \frac{\text{CFU of transconjugants per ml at time t}}{\text{CFU of donor per ml at time zero.}}$

NA: Nutrient agar, WA: Wilbrink's agar, respectively, on which the millipore filter disks were placed for mating.

This experiment was repeated three times.

millipore filter disk had a considerably more mucoid appearance in the WA medium than in the NA medium.

Host range of mutants

Only one mutant, 38-6, of 1,200 screened in the greenhouse was found lacking virulence to barley. This mutant was confirmed by repeating host range tests five times with two replicates. This mutant was still as pathogenic to wheat, oat and rye as the wild type.

DISCUSSION

Lai et al. (1977) reported transmission of a wide host range R factor plasmid to *Xanthomonas vesicatoria* by conjugation requiring relatively long mating periods. They reasoned that the mucoid slime layer typical of Xanthomonads possibly inhibits the transfer of plasmids.

Mutations induced by transposons are generally found to be strongly polar and non-leady (Kleckner et al., 1977). Therefore, such mutations can provide information on the gene(s) and the organization of operons. This is the first documentation of the transposon mutagenesis in *Xanthomonas* spp. The mating period for transposition of Tn5 into *Xanthomonas campestris* pv. *translucens* was 96 hours which is an unusually long period of time. The fact that mating on WA was not successful at all regardless of cultural

conditions and mating time period of donor and recipient suggests that the sucrose in WA may result in slime production of this recipient, preventing good pilus attachment sites and thus preventing conjugation. The nutrient agar which does not contain sucrose or glucose gave satisfactory frequencies of transfer in 96 hour mating at 38 C. This observation is further supported by the visual observation of more mucoid appearances on mating mixture on millipore filter disks in the WA than that on NA.

The growth conditions prior to mating also affect the transfer frequencies of Tn5. Since the recipient bacterium is aerobic, the cells grow actively by shake culture, hence the more mucoid growth. This may be the reason why the transfer frequency is low when the recipient cells were grown by shaking (Table 15).

The SB medium was reported as a good medium for R factor transmission from *Escherichia coli* J53 to *Xanthomonas vesicatoria* (Lai et al., 1977). *X. campestris* pv. *translucens* did not grow on this SB medium and *E. coli* 1830 did not grow well without shaking on SBPMKN. When the donor was grown on SBPMKN medium with shaking at 37 C and the recipient was grown on LB without shaking, the transfer frequency was relatively low, about half of that when the donor and the recipient cells were grown in LBKN and LB respectively (Table 15). Thus the shake culture, in general, is not considered to be a very good method of culturing both donor and recipient, prior to

mating.

Transposon mutagenesis may be a useful tool to identify the genes coding for virulence to a host and to estimate the relative proportion of genes that affect virulence.

The possible usage of this transposon in plant pathology would be a tool to 1) mark strains of bacterial plant pathogens for epidemiological studies, 2) possibly reduce virulence for use in cross-protection, 3) limit host ranges for use as a biological control agents of weeds, 4) estimate the numbers of genes that affect virulence, and 5) determine the nature of genes that determine virulence.

CONCLUSIONS

A selective medium for isolation of *Xanthomonas campestris* pv. *translucens* (*X. c. t.*) designated as KM-1, is described. The plating efficiencies of this medium for *X. c. t.* are from 0.91 to 2.13 times greater than those of non-selective rich Wilbrink's medium. Some other xanthomonads tested also grow on KM-1 medium. Since they are not believed to be found in cereal crops, they do not present problems. This medium has high selectivity against the soil-borne plant pathogenic bacteria and also the common soil-borne saprophytic bacteria. This medium has a relatively long shelf-life of two months.

This medium may be a valuable tool for the study of the epidemiology of this pathogen in terms of quantitative aspects, such as the rates of seed infestation and seed-borne transmission, the distribution of this pathogen on plants as epiphytes, in soils, on or in insects, or in raindrops as an ice nucleating active agent. Since all xanthomonads species recognized at present are plant pathogens and are found only in association with plant or plant material, this medium may enable discovery and identification of new saprophytic or avirulent xanthomonads.

The cultivar Oderbrucker (C.I. 4666), recognized as resistant since 1917, is still resistant in the field under intense natural

inoculum or artificial inoculation. This fact suggests that the resistance of this cultivar is relatively stable, having lasted for a long period of time. Luther, Alpine, both of winter growth habit, and Summit, Betzes, of spring habit, are good sources of resistance of barley. Ethiopian lines were sources of germplasm for resistance. These sources of resistance should be incorporated into the Male Sterile Facilitated Recurrent Selection Population and further improved. Since screening methods for disease resistance in greenhouse is not available, one must still rely on field screening results. However, the screening method of disease resistance in the greenhouse should be further developed to give the results that coincide with those of field.

This is the first report of this pathogen as an active ice nucleator. Only two other microorganisms, *Pseudomonas syringae* and *Erwinia herbicola* are known to be ice nucleators. All the strains of *X. c. t.* tested were ice nucleation active, whereas not all the strains of the two other ice nucleators were active. This fact may suggest the ice nucleation activity could be one of the additional criteria to the taxonomic differentiation of translucens group from the campestris group. This finding suggests a new mode of transmission by which substantial amounts of infection arise in a short period of time, resulting in the severe epidemics as observed throughout wide areas of cereal cropland. This finding suggests the

following speculations, in order: 1) this pathogen may enhance rainfall, 2) the movements of this pathogen via rainstorms and clouds may be a major method of long distance dispersal, 3) this pathogen may induce severe frost damage to the host in a supercooled environment resulting in massive infection, and 4) ice nucleation activity may be one reason why this pathogen is such a poor survivor in the soil. Future studies should provide the evidences of 1) the presence of this pathogen in raindrops and rainclouds, 2) the epiphytic presence of this pathogen on apparently healthy plant immediately after rainfall, and 3) the induction of frost injury to the plant by which the bacteria invade plant.

This is the first report of transferring the transposon from *Escherichia coli* to plant pathogenic bacteria. The method of transferring Tn5, transposon coded for kanamycin and neomycin resistance, from *Escherichia coli* strain 1830 to *Xanthomonas campestris* pv. *translucens* strain X-58 is described. This strain infects barley, wheat, oat and rye. Of 1,200 trasnpositional mutants screened for virulence to four hosts, one mutant 38-6 was found missing virulence to barley. This mutant was still as pathogenic to wheat, oat and rye as the wild type. This suggests that the gene for virulence to barley is different from genes for virulence to other hosts. More mutants would provide additional information about

the gene for virulence. This technique could be used in plant pathology to: 1) mark strains of bacterial plant pathogens with antibiotic resistance for epidemiological studies, 2) possibly reduce a virulence for use in cross-protection, 3) limit host ranges for use as a biological control agents for weeds, 4) estimate the numbers of genes that affect virulence, and 5) determine the nature of genes that determine virulence.

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APPENDIX

Appendix Table 1. Host range of *Xanthomonas campestris* pv. *translucens* known.

Common name	Scientific name	Annual or perennial	Literature
Barley	(*Hordeum vulgare* L.)	annual	a,d,e,f,l,n
Foxtail barley	(*Hordeum jubatum* L.)	perennial	a
Wheat	(*Triticum aestivum* L.)	annual	a,d,e,f,j,k,l,n
Spelt	(*Triticum spelta* L.)	annual	a,f
Einkorn	(*Triticum monococcum* L.)	annual	a,f
Rye	(*Secale cereale* L.)	annual	a,e,i
Victory oat	(*Avena sativa* L.)	annual	a,e,i
Smooth brome	(*Bromus inermis* Leyss.)	perennial	a,b,d,n
Cheatgrass brome	(*Bromus tectorum* L.)	annual	a
Yellow bristle grass	(*Setaria lutescens* (Weigel.) F. T. Hubb.	annual	a
Quackgrass	(*Agropyron repens* L.) Beauv.	perennial	a,b,d
Orchardgrass	(*Dactylis glomerata* L.	perennial	a,m
Water foxtail	(*Alopecurus geniculatus* L.)	perennial	a
Reed canarygrass	(*Phalaris arundinaceae* L.)	perennial	a
Rice	(*Oryza sativa* L.)	annual	c,h
Japanese millet	(*Echinochloa crus-galli* var. *frumentaceae*)	annual	g
Timothy	(*Pleum pratense* L.)	perennial	0

a = Bamberg, R. H., 1936; b = Boosalis, M. G., 1952; c = Bradbury, J. F. 1971; d = Fang, C. T. et al, 1950; e = Hagborg, W. A. F.,1942; f = Jones, L. R. et al., 1916; g = Moffet, M. L. et al, 1973; h = Patel, P. N. et al., 1971; i = Reddy, C. S. et al, 1924; j = Scharen, A. L., 1964; k = Smith, E. F. et al, 1919; l = Stewart, V. R. 1952; m = Tominaga, T. 1967; n = Wallin, J. R., 1946; o = Wallin, J. R. and C. S. Reddy, 1945.

Appendix Table 2. Pathotypes of *Xanthomonas campestris* pv. *translucens* using the forma specialis designation (From Bergey's manual 8th ed.)

Pathotypes	*Agropyron* spp.	*Bromus* spp.	*Dactylis glomerata*	*Hordeum* spp.	*Oryza sativa*	*Phleum pratense*	*Secale cereale*	*Triticum* spp.
cerealis Hagborg 1942, 317	+[a]	+	-[a]	a[a]	a	-	a/-	a/-
hordei Hagborg 1942, 317	a/-	-	-	-	a	-	a/-	-
oryzicola (Fang et al.[b]) Bradbury 1972, 72					-			
phleipratensis Wallin and Reddy 1945, 939	-	-		-	-	-	-	-
secalis (Reddy et al.[c]) Hagborg 1942, 317	-	-		a/-		-	-	a/-
undulosa (Smith et al.[d]) Hagborg 1942,317	-	-		a/-		-	a	±

[a] + = natural infection; a = artificial infection; - = no infection.

[b] Fang et al. 1957, 119.

[c] Reddy et al 1924, 1040.

[d] Smith et al. 1919, 48.