Survival of Xanthomonas campestris pv. phaseoli and Pectolytic Strains of X. campestris in Bean Debris

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ABSTRACT

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Small populations of Xanthomonas campestris pv. phaseoli, causal agent of common bacterial blight of bean (Phaseolus vulgaris), overwintered in bean debris in no-tillage plots. Common blight bacteria were isolated from debris in these plots collected monthly from October to May of 1985-1986 and October to March of 1986-1987 but not from debris in disked or plowed plots after February of 1986 or 1987. Bean plants inoculated with debris collected from no-tillage plots in May of both years developed common bacterial blight, as did beans planted in no-tillage plots the following growing season. Pectolytic strains of X. campestris, which were not pathogenic on bean, were consistently isolated from bean debris collected throughout the winter and spring of both years and colonized beans and weeds in tillage plots the following growing season. Fatty acid analysis of these pectolytic strains of X. campestris was inconclusive for a pathovar designation. We conclude that bean debris can be a source of inoculum for common blight bacteria and that survival of bacteria is influenced by tillage.

Common bacterial blight of bean (Phaseolus vulgaris L.), caused by Xanthomonas campestris pv. phaseoli (E. F. Smith) Dye, is important worldwide. The pathogen is seedborne, and contaminated seed is the primary source of inoculum (24,27,28). Therefore, planting pathogen-free seed is the primary method of control (27,28). Because the disease occurs in fields planted with certified disease-free seed (24,28), alternative sources of inoculum occur. Bean debris is a possible source of inoculum, but studies conducted to determine its importance in disease epidemiology have yielded conflicting results (13,21,24, 26-28). X. c. pv. phaseoli survived from season to season in surface debris in Nebraska (24) but not in Michigan (21) or Victoria, Australia (26).

Various factors influence whether plant-pathogenic bacteria can survive in crop debris, including geographic area, climate, cultural practices, host genotype, and, possibly, the strain of bacteria (21). Furthermore, the results of any study on overwintering of bacteria depend on the sensitivity of techniques used for bacterial recovery. In this study, we investigated the overwintering of X. c. pv. phaseoli in bean debris in three tillage systems in central Wisconsin.

MATERIALS AND METHODS

Beans were sown at one site on 27 June 1985 (1985 site) and at another site on

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18 June 1986 (1986 site) at the University of Wisconsin Experimental Station in Hancock to evaluate inoculation techniques for X. c. pv. phaseoli (10). Common blight epidemics developed at both sites. On 6 October 1985 and 3 October 1986, three types of tillage plots were created at these sites: moldboard plowed, in which plants were turned 20-40 cm under the soil; disked, in which plants were left on the soil surface or turned 2.5-10.0 cm under the soil: and no-tillage, in which plants were left standing. Tillage plots were replicated three times in a randomized complete block design (nine tillage plots), and each tillage plot was 10×36 m.

Recovery of xanthomonads from bean debris. Beginning in October, before tillage, and at monthly intervals thereafter, ending in May, bean debris samples of approximately two plants were collected from each of five sites selected along a zigzag pattern in each tillage plot. Debris from plowed plots was exhumed from beneath the soil surface, debris from disked plots was collected from the soil surface and from beneath the soil surface, and debris from no-tillage plots was collected as standing plants above the soil surface. Debris from each tillage plot was placed in plastic bags, which were returned to the laboratory in a cooler and stored overnight at 4 C. Debris from each tillage plot was separated into leaf, stem, and pod tissues, and 20 leaflets and 20 2-cm stem and ped sections were weighed and assayed separately. Each sample was ground in 200 ml of 0.01 M potassium phosphate buffer (PB) (pH 7.2) for 1-2 min in a Waring Blendor. Serial dilutions were

prepared, and 0.1-ml aliquots were plated on MXP, a semiselective medium for X. c. pv. phaseoli (3). Xanthomonad colonies on MXP were pale yellow, convex, and mucoid and were surrounded by a zone of starch hydrolysis. Counts were made after incubation for 3-5 days at 24 C. Total xanthomonad population densities were determined for each organ in each tillage plot. For total xanthomonad population densities from debris of each organ separately, and for each year, a repeated measures analysis of variance was performed incorporating the effects of tillage, time (month), and replicate (22).

Pathogenicity tests. Each month (October-May), 15-25 xanthomonad strains isolated from bean debris were randomly selected and their pathogenicity determined on bean. After strains were subcultured, single colonies were isolated on sucrose peptone agar (SPA) (12), then transferred to nutrient broth (Difco) and incubated on a shaker for 24-48 hr at room temperature. Each strain was inoculated onto the adaxial surface of one-half to three-quarter size trifoliolate leaves of 4- to 5-wk-old bean plants (cv. Topcrop) by razor blade (20) or Carborundum cotton swab inoculation methods (4). Control plants were inoculated with nutrient broth plus Carborundum. After 7-10 days in a controlled environment chamber (24 C, 16hr photoperiod, 250 μE·m⁻²·s⁻¹) plants were evaluated for disease symptoms based on the following scale: 0 = symptomless; + = chlorosis and necrosis at the margin of inoculation; ++ = moderate lesion(s), chlorosis, necrosis, and scattered water-soaked spots on the abaxial leaf surface; and +++ = large expanding lesion(s), extensive chlorosis, necrosis, and water-soaking. Isolations were made from leaves of plants with and without symptoms as described previously (10).

Recovery of xanthomonads from plants and development of common blight on beans the following growing season. Beans (cv. Eagle) susceptible to common blight were replanted in tillage plots at the 1985 site on 19 June 1986 and at the 1986 site on 10 June 1987. In early June, before beans were planted, 10 plants of the predominant weed species in each tillage plot were assayed for xanthomonads. Twenty leaves from each weed species from each tillage plot

were weighed and sonicated in 200 ml of 0.01 M PB for 20 min in an ultrasonic cleaner (model B-52, Branson Cleaning Equipment Co., Shelton, CT), then 0.1-ml aliquots were plated on MXP. For each year, 20 xanthomonad strains recovered from weeds were tested for pathogenicity on bean.

After bean plants reached the V1-V2 stage (primary-first trifoliolate stage) (16), 10 primary leaves were randomly selected from plants in each tillage plot and assayed for xanthomonads. For each year, 10 xanthomonad strains recovered

from primary leaves were tested for pathogenicity on bean. Common bacterial blight development was monitored throughout the growing season, and each year isolations were made from leaves of 10 plants with symptoms. Two xanthomonad strains from each leaf were tested for pathogenicity on bean.

Inoculation of bean plants with debris. In 1986 and 1987, bean plants (cv. Eagle) were inoculated in a glasshouse with debris from no-tillage plots collected in May of that year. Debris was ground in a Waring Blendor, sifted

through a No. 32-mesh sieve (<500 mm), and stored in plastic bags at -20 C. The ground debris (1 g per plant) was sprinkled onto one-half to three-quarter size trifoliolate leaves of 4- to 5-wk-old plants that had been wounded by rubbing leaves with a sterile cotton swab immersed in a 1% Carborundum suspension. In another treatment, 1 g of ground debris was incubated in 100 ml of PB on a rotary shaker for 2 hr, 1 g of Carborundum was added, and the suspension was applied onto the adaxial surface of leaves with a sterile cotton

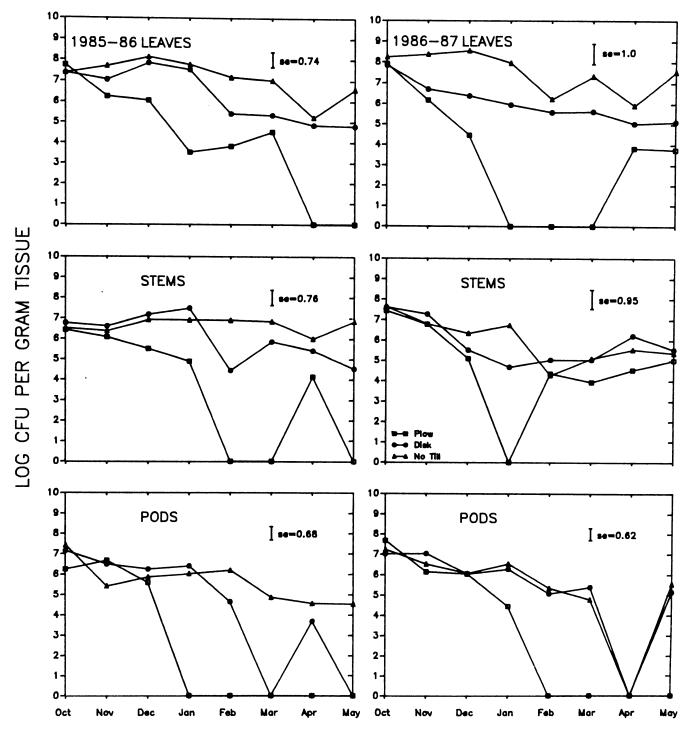


Fig. 1. Recovery of xanthomonads from bean leaf, stem, and pod debris collected from tillage plots at Hancock, Wisconsin, during 1985-1986 and 1986-1987. \blacksquare = Plowed plots, \bullet = disked plots, and \blacktriangle = no-tillage plots. Vertical bars indicate the standard error for making comparisons within a given time point; standard error is based on pooled estimates of variability derived from the repeated measures analysis (22).

swab. Control plants were inoculated with a 1% Carborundum suspension. These treatments were applied to each of 12 plants, and the experiment was set up as a randomized complete block design. After 2-3 wk, plants were examined for symptoms and isolations were made from inoculated and control leaves. Randomly selected xanthomonad strains isolated from leaves were tested for pathogenicity on bean.

Characterization of xanthomonads. The morphological, physiological, and biochemical characteristics of 10 strains of yellow-pigmented bacteria (YPB) recovered from bean debris that were not pathogenic on bean and five known strains of X. c. pv. phaseoli were determined. Flagellation was determined for five strains of YPB and one strain of X. c. pv. phaseoli by electron microscopy (23). Bacteriological tests included Gram staining; cellular morphology; motility; color and slime formation on SPA; growth at 37 C; oxidase, catalase, and urease reactions; aesculin, starch, and gelatin hydrolysis; and litmus milk reaction (6,7). Pectolysis was determined on CVP medium (5). Hypersensitivity was tested on tobacco (cv. Havana 142), with Pseudomonas syringae pv. syringae van Hall (strain B728a) as a positive control (15). Tobacco plants were maintained in a controlled environment chamber (24 C, 16-hr photoperiod, 250 $\mu \text{E} \cdot \text{m}^{-2} \cdot \text{s}^{-1}$), and results were recorded 18-24 hr after inoculation. Xanthomonadin was extracted (14) and identified spectrophotometrically. Fatty acid analyses (19) were performed by Microbial ID, Inc. (Newark, DE) on one strain each of X. c. pv. phaseoli and X. c. pv. phaseoli var. fuscans and 12 strains of YPB.

Table 1. Pathogenicity of xanthomonads isolated from bean debris collected from tillage plots at Hancock, Wisconsin, during 1985-1986 and 1986-1987^a

	No. of strains pathogenic on bean/no. of strains tested b				
Month	1985-1986	1986-1987			
October ^c	17/20	8/25			
November	7/20	5/25			
December	1/20	3/25			
January	2/15	2/25			
February	1/15	4/25			
March	2/20	1/25			
April	1/25	0/25			
May	2/25	0/25			

^a Strains were randomly selected from platings of debris from no-tillage, disked, and plowed plots and cultured in nutrient broth on a shaker for 24-48 hr before being inoculated onto one-half to three-quarter size trifoliolate bean leaves (cv. Topcrop) by razor blade or Carborundrum cotton swab inoculation

RESULTS

Recovery of xanthomonads from bean debris. The limit of detection of xanthomonads in leaf and stem debris was approximately 10^2 cfu/g, whereas in pod debris it was about 10³ cfu/g. For the data analysis, values of 0 were used when no xanthomonad colonies were detected; substitution of values corresponding to the limit of detection did not significantly alter the results of the analysis, however. Xanthomonads were recovered from senescent bean plants in all tillage plots at both sites before tillage, and for any given organ, xanthomonad population densities were not significantly different before tillage (Fig. 1). After tillage, significant treatment effects were observed and depended on the sampling date (Fig. 1). In no-tillage and disked plots at both sites, xanthomonads were consistently recovered from leaf and stem debris throughout the winter and spring, with significantly greater population densities from no-tillage plots at certain sampling dates (Fig. 1). Xanthomonads were consistently isolated from pod debris in no-tillage plots, except in April 1987. In disked plots, recovery from pod debris was significantly lower than that from no-tillage plots for some sampling dates during 1985-1986, whereas during 1986-1987, recovery from pod debris in no-tillage and disked plots was almost identical (Fig. 1). In plowed plots, recovery of xanthomonads from leaf, stem, and pod debris declined over time, particularly for pods, and xanthomonad population densities were significantly lower than those from notillage and disked plots for most sampling dates (Fig. 1).

Pathogenicity tests. With both inoculation methods, strains of X. c. pv. phaseoli caused water-soaked spots on the abaxial leaf surface and disease symptoms that were rated as ++ or +++. Strains with ratings of + (a wound-associated localized necrotic

reaction with no water-soaking) or 0 were considered nonpathogenic on bean. The number of xanthomonads recovered from bean debris that were pathogenic on bean declined after the first sampling date, and by April and May few pathogenic strains were recovered (Table 1). In April and May of 1987, no pathogenic strains were recovered. All pathogenic strains that were recovered in March, April, and May were from notillage debris.

Recovery of xanthomonads from plants and development of common blight on beans the following growing season. Xanthomonads were recovered from six weed species in no-tillage and/or disked plots but not from weeds in plowed plots (Table 2). Weeds from no-tillage plots, particularly common vetch (Vicia sativa L.) and hairy vetch (V. villosa Roth), yielded the greatest number of xanthomonads. All xanthomonad strains from weeds that were tested, except for two from hairy vetch in no-tillage plots in 1986, were not pathogenic on bean.

Recovery of xanthomonads from primary leaves of beans planted in tillage plots the following growing season was 3.4×10^4 and 5.5×10^4 cfu/g of fresh leaf weight from no-tillage plots, 2.2×10^4 and 3.7×10^3 from disked plots, and 9.4×10^3 and 4.1×10^1 from plowed plots in 1986 and 1987, respectively. None of 20 xanthomonad strains tested were pathogenic on bean.

The primary leaves of some bean plants in tillage plots at both sites developed common blight symptoms approximately 4-5 wk after planting, and X. c. pv. phaseoli was isolated from each of 10 diseased leaves sampled in 1986 and 1987. At both sites, diseased plants were found in no-tillage, disked, and plowed plots.

Inoculation of bean plants with debris. After 2-3 wk, some bean plants inoculated with ground debris developed

Table 2. Xanthomonads recovered from weeds growing in tillage plots at Hancock, Wisconsin^a

Tillage		Xanthomonads recovered (cfu/g) ^b			
	Weeds	1986	1987		
No-tillage	Lambsquarter (Chenopodium album L.)	2.2×10^{3}	6.2×10^{2}		
	Medicago spp.	8.8×10^{2}	5.4×10^{3}		
	Ragweed (Ambrosia artemisiifolia L.)	c	1.1×10^{4}		
	Hairy vetch (Vicia villosa Roth)	1.3×10^{4}	7.6×10^{3}		
	Common vetch (V. sativa L.)		3.0×10^{3}		
	Leafy spurge (Euphorbia esula L.)	ND^d	ND		
Disked	Lambsquarter	ND	3.2×10^{2}		
	Ragweed	7.3×10^{2}	9.3×10^{2}		
	Medicago spp.	3.9×10^{3}			
Plowed	Lambsquarter	ND	ND		
	Ragweed	ND	ND		

^a Weeds were selected from tillage plots in early June before beans were planted. Twenty leaves from each species were randomly selected, weighed, sonicated in 200 ml of 0.01 M potassium phosphate buffer, and plated on MXP. Xanthomonads were counted after 3-5 days at 24 C.

^b Strains that induced water-soaking were considered pathogenic.

^c Debris samples were taken before tillage.

^bMeans from three replicates of each tillage treatment each year.

^c Weed species not found.

 $^{^{}d}$ ND = xanthomonads not detected.

common blight symptoms. In 1986, three of 12 plants inoculated with debris shaken in buffer and one of 12 plants inoculated with dry debris developed symptoms, whereas in 1987, only one of 12 plants inoculated with debris shaken in buffer developed symptoms. X. c. pv. phaseoli and xanthomonads that were not pathogenic on bean were recovered from inoculated leaves but not from control leaves.

Characterization of xanthomonads. The morphology and reactions of the YPB from bean debris that were not pathogenic on bean were consistent with those described for Xanthomonas (Table 3), but these strains were differentiated from strains of X. c. pv. phaseoli by their pectolytic ability and colony morphology on MXP and SPA. Accordingly, these YPB will be referred to as pectolytic xanthomonads. On MXP, colonies of X. c. pv. phaseoli and the pectolytic xanthomonads were pale yellow, convex, and mucoid and were surrounded by a zone of starch hydrolysis, but the pectolytic xanthomonads grew faster than X. c. pv. phaseoli. On SPA, colonies of X. c. pv. phaseoli were yellow, convex, and mucoid, whereas colonies of the pectolytic xanthomonads were darker yellow, less mucoid, and smaller than those of X. c. pv. phaseoli. After 3-5 days of growth on SPA, colonies of the pectolytic xanthomonads frequently produced sectors that were light yellow and mucoid and resembled colonies of X. c. pv. phaseoli. The fatty acid composition of the pectolytic xanthomonads was most similar to that of X. campestris, but identification to pathovar was inconclusive (Table 4). One strain, L3, was similar to X. c. pv. phaseoli (Table 4) but was not pathogenic on bean.

DISCUSSION

We conclude that X. c. pv. phaseoli overwintered in bean debris in no-tillage plots on the basis of three separate results: isolation of the pathogen from debris from no-tillage plots, from bean plants inoculated with no-tillage debris, and from bean plants in no-tillage plots the following growing season. However, even though bean plants were heavily infected by X. c. pv. phaseoli by the end of the first growing season at both sites, few strains of X. c. pv. phaseoli were recovered from debris of these plants the following spring, regardless of tillage.

The reduced recovery of X. c. pv. phaseoli from debris was due to poor survival of the pathogen and not simply to increased colonization of debris by pectolytic xanthomonads, because xanthomonad populations in debris did not increase appreciably after the first sampling date. The poor survival of X. c. pv. phaseoli in bean debris was further demonstrated in a separate study (9) in 1987 in which no strains of X. c. pv. phaseoli were detected from among 2,000

xanthomonads recovered from no-tillage debris collected in May and screened with a DNA probe that differentiates X. c. pv. phaseoli from pectolytic xanthomonads associated with bean debris. Evidently, X. c. pv. phaseoli was a poor saprophyte under the conditions of this study, possibly because of sensitivity to antagonistic microorganisms (24) or inability to utilize or survive on senescent plant tissue. Because X. c. pv. phaseoli can survive in dry-leaf inoculum for at least 6 yr (10) and in bean seed for 15 yr (24), sensitivity to antagonistic microorganisms may explain the poor survival of X. c. pv. phaseoli in bean debris. The pathogen may survive longer in standing bean debris because it is less accessible to antagonistic microorganisms.

Our results are similar to those of Schuster and Coyne (24), who found that X. c. pv. phaseoli overwintered in bean debris on the soil surface in Nebraska, but also support the findings of Saettler et al (21) and Wimalajeewa and Nancarrow (26) that X. c. pv. phaseoli does not overwinter in bean debris that is tilled into soil. Although Saettler et al (21) did not recover X. c. pv. phaseoli from standing plants left in the field in Michigan, the methods used and conditions for bacterial survival differed from those in our study.

Standing bean debris and possibly weed debris in or around fields can be the primary source of inoculum for disease outbreaks in bean fields established with certified seed. The short generation time of bacteria allows for small amounts of primary inoculum, such as that surviving in bean debris, to initiate disease under favorable environmental conditions (24). It would be interesting to determine if X. c. pv.

phaseoli could survive in a crop rotation system where the bacteria would have to survive in bean debris for more than one winter or colonize nonhost plants and overwinter in nonhost debris or associated with perennial nonhost plants (2).

An unexpected finding of this study was the efficient colonization of bean debris by pectolytic strains of X. campestris that were not pathogenic on bean. Based on fatty acid analysis, these pectolytic xanthomonads appear to be a heterogeneous group that does not fit into any single recognized pathovar of X. campestris. In another study (8), we were able to differentiate pectolytic xanthomonads from bean debris from X. c. pv. phaseoli, X. c. pv. phaseoli var. fuscans, and other pathovars of X. campestris by restriction fragment length polymorphism analysis of total genomic DNA.

Opportunistic pectolytic strains of X. campestris with unknown pathogenicity, such as those from tomato transplants (11), rotted vegetables (17), and apple buds (18), are difficult to classify in the X. campestris pathovar system (1) and may be incorrectly identified as pathovars of X. campestris. For example, pectolytic xanthomonads isolated from tomato and pepper transplants may be confused with X. c. pv. vesicatoria, which could result in the loss of pathogen-free certification (11). Similarly, pectolytic xanthomonads isolated from bean debris may be incorrectly identified as X. c. pv. phaseoli. Therefore, it is important to differentiate opportunistic pectolytic xanthomonads from X. campestris pathovars. Demonstration of pectolytic ability may be sufficient for differentiation. However, many X. campestris pathovars, such as X. c. pvs. campestris and carotae, are pectolytic (17,25).

Table 3. Characteristics of five strains of Xanthomonas campestris pv. phaseoli and 10 strains of pectolytic xanthomonads isolated from bean debris that were not pathogenic on bean

Characteristics	X. c. pv. phaseoli	Pectolytic xanthomonads		
Yellow mucoid colonies on sucrose peptone agar	+	+		
Gram-negative rods	+	+		
Monotrichous flagellation ^a	+	+		
Growth at 37 C	+	+		
Utilization of glucose	Oxidative	Oxidative		
Utilization of aspargine		_		
Catalase reaction	+	+		
Oxidase reaction		<u>-</u>		
Starch hydrolysis	+	+		
Gelatin hydrolysis	+	+		
Casein hydrolysis	+	+		
Aesculin hydrolysis	+	+		
Acetoin production	_	<u>-</u>		
Urease production	_			
Litmus milk	Proteolytic	Proteolytic		
Pectolysis on crystal violet pectate medium ^b	_	+		
Hypersensitivity on tobacco ^c	_			
Xanthomonadin pigment	+	+		

^a One strain of X. c. pv. phaseoli and five strains of pectolytic xanthomonads from bean debris were viewed

^b Pectolysis was determined after 72 hr, and extensive pitting was observed for (+) strains. ^c Cutlivar Havana 142; strain B728a of *Pseudomonas syringae* pv. syringae produced the expected hypersensitive response.

Table 4. Results of fatty acid analyses of Xanthomonas campestris pv. phaseoli, X. c. pv. phaseoli var. fuscans, and pectolytic xanthomonads isolated from bean debris^a

		Similarity index for X. campestris pathovars								
Strain ^b	X. campestris	phaseoli	phaseoli var. fuscans	raphani	pruni	zinnae	vitians	juglandis	campestris	armoraciae
X. c. pv. phaseoli	0.568	0.568*°	d							
X. c. pv. phaseoli var. fuscans	0.747	• • •	0.747*	•••	•••	•••	•••	• • •	•••	• • •
RD4	0.647				0.601	0.647*			0.576	
RD3	0.773			0.773*	0.604	0.612				
23	0.870			0.870*			0.552	0.552		
RD6	0.735					0.735*	0.719		0.608	
D2L	0.681			0.669	0.681*					0.468
L5	0.621				0.621*			0.439		0.548
P3	0.487					0.323		0.458	0.487*	
S9	0.785							0.785*	0.697	0.617
SI	0.760			0.682				0.760*		0.582
S6	0.725			0.725*	0.560	0.555				
L3	0.579	0.439			0.416	0.579*				
L1	0.668						0.668*	0.586		0.536

^a Fatty acid analysis performed by Microbial ID, Inc., Newark, DE.

Another approach may be to use DNA probes that are pathovar-specific. One such probe has been developed that differentiates *X. c.* pv. *phaseoli* from pectolytic xanthomonads associated with bean debris (9).

The origin of the pectolytic xanthomonads associated with bean debris is unknown, but it is unlikely that they are soil inhabitants because they did not colonize bean plants until late in the first growing season at both sites (Gilbertson, unpublished) and they survived poorly in debris in plowed plots. Furthermore, a greenhouse study was conducted in which bean seeds were planted in soil collected from both sites, and no xanthomonads were recovered from plants growing in these soils (Gilbertson, unpublished). These pectolytic xanthomonads are probably resident epiphytes and/or facultative saprophytes that colonized bean plants during the growing season and by the end of the growing season had extensively colonized senescent bean plants. Because they colonize beans, these pectolytic xanthomonads may be potential biological control agents for common blight.

On the basis of this study, we conclude that X. c. pv. phaseoli can overwinter between bean crops in standing bean debris in Wisconsin and that debris can be a source of inoculum for common blight bacteria. Bean debris infested with X. c. pv. phaseoli may be an important inoculum source in tropical and subtropical regions where more than one crop of beans is planted in the same plot each year. Under these conditions, disposal of bean debris by burning, plowing, or removal may be an effective management tool for common blight. The extensive colonization of bean debris by pectolytic xanthomonads was an unexpected finding that adds to the growing evidence that xanthomonads are not strictly plant pathogens.

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^b RD4, RD3, RD6, D2L, L5, P3, S9, S1, S6, L3, and L1 are pectolytic xanthomonads isolated from bean debris.

c* = Best pathovar fit.

^d Not one of three best-fitting comparisons.

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