Erwinia alni, a New Species Causing Bark Cankers of Alder (*Alnus* Miller) Species

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The causal agent of an undetermined disease of black alder (*Alnus glutinosa*) and Italian alder (*Alnus cordata*) was identified as an *Erwinia* species. This alder bacterium induces dark brown necrotic cankers on alder plants; these cankers are often longitudinally elongated and occur in the bark of the trunks and also in the bark of branches, twigs, and suckers. A dark watery liquid often exudes from small cracks in the cankers and stains the bark surface. Disease symptoms were produced on the trunks of 2-year-old black and Italian alder trees after artificial inoculation of selected strains of bacteria obtained from typical bark cankers (G. Surico and L. Mugnai, *Inf. tore Fitopatol.* 12:41–43, 1992). Morphological examination of the pathogen by electron microscopy showed that it is a motile rod-shaped organism. All of the strains examined were gram negative, oxidase negative, and facultatively anaerobic with fermentative metabolism and had the general characteristics and fatty acids of members of the family *Enterobacteriaceae*. Strains PVFi 20^T (T = type strain), PVFi 23, PVFi 25, and PVFi 27 were chosen for further characterization. These four strains exhibited 96 to 100% DNA homology in hybridization experiments performed at 40 and 50°C. They were most closely related to *Erwinia nigrifluens* (levels of homology 40°C, 49 to 65%). Phenotypic differentiation from *E. nigrifluens*, which induces a similar disease on Persian walnut but is nonpathogenic on alder, is based on positive reactions by the alder strains for acetoin (Voges-Proskauer reaction), endoglucanase activity, and acid production from maltose and negative reactions for esculin hydrolysis and acid production from raffinose, melibiose, sorbitol, and inositol. The fatty acid profiles of the alder strains were quantitatively different from those of all previously validly named plant-pathogenic species belonging to the *Enterobacteriaceae*. On the basis of data described above, the name *Erwinia alni* is proposed for the new organism.

A bacterial disease of black alder (*Alnus glutinosa* (L.) Gaertn.) and Italian alder (*Alnus cordata* Desf.) has been observed in several locations in Tuscany and Liguria (Italy) over the last few years (30). The symptoms of this disease are the same as those observed by Moriondo in 1958 (21) on black alder and resemble those caused by *Erwinia nigrifluens* on Persian (English) walnut (34) and by *Erwinia quercina* on oaks (*Quercus agrifolia* Née and *Quercus wislizenii* A. DC.) (14).

At first diseased alder plants have small necrotic cankers in the bark of the trunk and also in the bark of branches, twigs, and suckers. These cankers are slightly sunken, dark brown, and irregularly circular and appear to be water soaked. They usually develop around lenticels or are localized at nodes with dead twigs or on leaf scars. As the infection progresses, the necrosis spreads laterally, becoming deeper and reaching the cambium and sometimes the first layers of wood, which then turn tan to brown. Cankers may eventually girdle and kill a branch or tree.

Another symptom is exudation in spring and summer of a dark watery liquid from small cracks in the cankers, which flows down and stains the bark surface black. This exudation is similar to the exudation caused by *Erwinia nigrifluens* on walnut. More detailed descriptions of the symptoms have been given elsewhere (30, 31).

We previously reported the isolation from diseased tissues of gram-negative, fermentative, rod-shaped bacteria which reproduced the disease in inoculation tests performed with Ital-

ian alder and black alder but were avirulent on walnut and oak (30, 31). In the same experiments, *Erwinia nigrifluens* NCPPB 564^T (T = type strain) and *Erwinia quercina* NCPPB 1852^T did not produce symptoms on alder. In this study we determined the taxonomic position of six strains associated with bark cankers of alders by performing phenotypic tests and DNA hybridization experiments. Our results indicate that these strains are members of a new species in the genus *Erwinia*, for which the name *Erwinia alni* is proposed.

MATERIALS AND METHODS

Bacterial strains and growth conditions. The alder strains used in this study were isolated by us from bark cankers on Italian alder trees growing in Tuscany (strains PVFi 20^T, PVFi 21, and PVFi 22) and on black alder trees growing in Liguria (strains PVFi 23, PVFi 25, and PVFi 27) in 1990 and 1991. The strains used in DNA hybridization studies are listed in Table 3. For comparison, *Erwinia nigrifluens* NCPPB 564^T, *Erwinia quercina* NCPPB 1852^T, *Erwinia carotovora* subsp. *carotovora* NCPPB 2577, and *Pseudomonas syringae* pv. *syringae* Y 37 were included in some experiments. Strains of *Erwinia amylovora*, *Erwinia rubrifaciens*, and *Pantoea agglomerans* were also used in the fatty acid analysis. Most of the reference strains were obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB); the only exception was strain Y 37, which was obtained from G. L. Ercolani, Istituto di Microbiologia agraria e tecnica, University of Bari, Bari, Italy. The strains were stored on slopes of nutrient agar (NA) containing 2% (vol/vol) glycerol at 4°C and were subcultured every 2 weeks. All cultures were incubated at 27°C. For long-term storage the bacteria were suspended in 15% glycerol and kept in liquid nitrogen.

Morphological, biochemical, and physiological characterization. A total of 71 phenotypic features were examined; 12 cultures (6 isolates and 6 reisolates) of the bacterium associated with the alder bark canker disease were included in this analysis. Cultures of *Erwinia nigrifluens* NCPPB 564^T, *Erwinia quercina* NCPPB 1852^T, *Erwinia carotovora* NCPPB 2577, and *Pseudomonas syringae* pv. *syringae* Y 37 were also examined for comparison. Cells and flagella were observed by phase-contrast light microscopy and electron microscopy. Colony characteristics and pigmentation were determined by using streaked cultures on the following six media routinely used to differentiate commonly phytopathogenic genera (23): Difco NA, NA containing 5% sucrose (NSA), yeast extract-dextrose-calcium

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carbonate agar (YDC) (35), King's medium B (15), Miller-Schroth medium (19), and the high-sucrose medium of Crosse and Goodman (9). The tests used to determine oxidase and catalase activities, tobacco hypersensitivity, potato soft rot ability, acid production from carbohydrates in medium C of Dye, the mode of utilization of glucose (oxidation-fermentation test), production of hydrogen sulfide from cysteine, production of urease, pectate degradation (in Hildebrand's medium), and the Voges-Proskauer reaction were tests described by Dickey and Kelman (10) and Schroth and Hildebrand (25). The tests used to determine hydrolysis of gelatin, starch, and esculin, nitrate reduction, and production of indole were performed by using the methods of Lelliott and Stead (17). The tests used to determine β -galactosidase activity, citrate and malonate utilization, phenylalanine deaminase activity, casein hydrolysis, lysine and ornithine decarboxylase activities, the methyl red reaction, and motility were the tests described by Smibert and Krieg (26). Arginine dihydrolase activity was detected by the methods of Møller (20) and Thornley (32), and 5- and 10-day-old cultures in Difco nutrient broth (NB) containing 0.2% $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ and 0.5% CaCl_2 or no supplement were examined for endospore formation as described by Lelliott and Stead (17). Growth factor requirements were determined after incubation for 8 days on minimal medium (10.5 g of K_2HPO_4 per liter, 4.5 g of KH_2PO_4 per liter, 1.0 g of $(\text{NH}_4)_2\text{SO}_4$ per liter, 0.5 g of sodium citrate $\cdot 2\text{H}_2\text{O}$ per liter, 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ per liter, 5 g of glucose per liter, 15 g of agar per liter) containing 0.5% yeast extract or no supplement. Salt tolerance was determined in NB containing 2 and 5% NaCl; data were recorded daily for 7 days. The growth in NB at 5, 25, 32, 36, and 42°C and the growth on NSA in anaerobic jars (Becton, Dickinson and Co., Cockeysville, Md.) at 27°C were recorded after 7 and 14 days of incubation of static cultures. Endoglucanase production was assayed by the cup-plate technique (36) by using the medium of Andro et al. (3) containing 0.5% (wt/vol) carboxymethyl cellulose.

The inocula for biochemical and physiological tests were prepared from 24- to 48-h cultures grown on NA containing 2% (vol/vol) glycerol at 27°C.

Preparation of high-molecular-weight DNA. Cells were grown in NB at 27°C for 24 h with shaking and DNA was isolated by the method of Giovannetti et al. (12).

DNA base compositions. The G+C contents of strains PVFi 20^T, PVFi 23, PVFi 25, and PVFi 27 were determined spectrophotometrically by the thermal denaturation method and were calculated by the method of Schildkraut and Lifson (24). Two to five values were determined for each strain, and these values were averaged.

DNA hybridization. DNA-DNA hybridization was carried out under optimal and stringent conditions (40 and 50°C respectively) by using the filter method as described by Hernandez et al. (13). DNA stock solutions were prepared in TE buffer (10 mM Tris, 1 mM EDTA; pH 8), and the concentration was adjusted to 20 $\mu\text{g ml}^{-1}$. A 25- μl portion of each DNA solution was diluted in 475 μl of 0.4 M NaOH–10 mM EDTA (pH 8), and three to six replicates were prepared for each strain.

DNAs of *Erwinia nigrifluens* NCPPB 564^T, *Erwinia salicis* NCPPB 2535, and *Erwinia alni* PVFi 20^T and PVFi 27 were labelled with digoxigenin by the random primer method, using a nonradioactive DNA labelling and detection kit (Boehringer, Mannheim, Germany) as recommended by the manufacturer. DNA samples were blotted onto a Zeta-Probe nylon blotting membrane, prehybridized, hybridized, and washed by using the formamide protocol described in the Zeta-Probe instruction manual (Bio-Rad). The optimal temperature of renaturation (T_m) (40°C) was calculated by using the following equation: $T_m = 81.5 + 16.6(\log M) + 0.41(\text{G+C content}) - 0.72(\text{formamide concentration})$, where M was the sodium ion molarity (2) and the formamide concentration was 50%. The hybridization membranes were scanned with an LKB Ultrascan II laser densitometer, and the average absorbance value for each strain was used to evaluate the degree of hybridization. The results of heterologous hybridization reactions (reactions involving DNAs from different strains) were compared with the results of homologous hybridization reactions (reactions in which labelled DNA and unlabelled DNA from the same strain were used).

DNA-DNA hybridization experiments were performed with representative members of the family *Enterobacteriaceae*.

Fatty acid analyses. Qualitative and quantitative analyses of the cellular fatty acid compositions of strains PVFi 20^T, PVFi 23, and PVFi 27 were performed by using the method of Stead (28). Strains were cultured for 24 h on Trypticase soy agar at 28°C prior to fatty acid determinations. The fatty acids were identified and quantified by using the Microbial Identification System software (MIDI, Newark, Del.). The profiles obtained were compared with the profiles in a commercial library containing data for a wide range of bacteria (MIDI Aerobe Library) and a self-generated plant pathogen library containing data for all previously validly named plant-pathogenic species belonging to the *Enterobacteriaceae*, based on the strains in the NCPPB. The data in this library were generated by using generation software (MIDI).

RESULTS AND DISCUSSION

Phenotypic characteristics of the alder strains. The alder strains had the biochemical characteristics of the family *Enterobacteriaceae* (6) (Table 1). They were gram negative, oxidase negative, catalase positive, facultatively anaerobic, and fermentative as determined by the oxidation-fermentation test. The cells were rod shaped (0.5 to 0.8 by 1.2 to 2.3 μm), did not form spores, and were motile by means of peritrichous flagella (Fig. 1). The cells occurred singly or rarely in pairs.

On NA after 2 days, the colonies were round, slightly raised, butyrous, and 1 mm in diameter; after 5 days the colonies were whitish and 2 to 2.5 mm in diameter. On NSA after 2 days, the colonies were radially striated, low, domed, round with smooth surfaces and entire margins, mucoid, and 1 to 1.5 mm in diameter. After 5 days on NSA, the colonies were umbonate with narrow translucent margins (only one isolate produced colonies that had wrinkled margins), whitish, and 2 to 2.5 mm in diameter. No diffusible pigments were observed on either NA or NSA. On Miller-Schroth agar after 5 days, the colonies were reddish orange, round with entire margins, smooth, domed, and 1 mm in diameter; the color of the substrate changed from blue-green to reddish. On YDC agar after 5 days, the colonies were white, circular, raised with slightly undulate margins, and 3 mm in diameter; no clearing of calcium carbonate occurred around the colonies. On King's medium B after 5 days, the colonies were 2 to 2.5 mm in diameter, circular, slightly convex, and yellowish with smooth surfaces and slightly undulate margins; no fluorescent pigments were produced. On the high-sucrose medium, isolated colonies were 0.5 to 1 mm in diameter after 4 days and 3 to 4 mm in diameter after 10 days, circular with entire margins, domed, and shining with smooth surfaces. All strains grew well in NB at 25°C, produced uniform turbidity, and did not form a pellicle. The alder bacteria did not induce a hypersensitive response in tobacco leaves and did not rot potato tuber slices. None of the strains produced polygalacturonase or pectate lyase when they were tested on pectate gels at pH 5 and 8. The alder strains were not capable of hydrolyzing casein, gelatin, starch, or esculin within 12 days after inoculation. All of the strains produced H_2S from cysteine within 24 h. The methyl red test and the Voges-Proskauer reaction were positive after 10 days of incubation. The test for endoglucanase activity was positive after 3 days of incubation. Urea was slowly hydrolyzed within 4 to 5 days.

All strains grew at 25, 32, and 36°C but not at 5 and 42°C. The results of other tests are shown in Table 1.

Fatty acid composition. All of the fatty acid profiles contained more than 50,000 U of peak area, so all of the major fatty acids present were detected. For each profile, more than 99% of the total peak area was accounted for by named fatty acids. The profiles were typical of members of the *Enterobacteriaceae* (1, 5, 33). They contained only two hydroxy fatty acids, 14:0 3OH and an as-yet-unknown hydroxy fatty acid with an equivalent chain length of 14.503 (28a). The other major fatty acids included the saturated fatty acids 12:0, 14:0, and 16:0, the monounsaturated fatty acids 16:1 w7c and 18:1 w7c, and the cyclopropane fatty acid 17:0 cyclo. The minor fatty acids included 18:0 and the cyclopropane acid, 19:0 w8c (Table 2). A principal-component analysis (Fig. 2) clearly showed that the profiles of three *Erwinia alni* strains clustered together but were clearly different from the profiles of the 60 other strains included in this analysis (including *Erwinia amylovora*, *Erwinia herbicola*, *Erwinia nigrifluens*, *Erwinia quercina*, and *Erwinia rubrifaciens* strains). In a much larger study of more than 300 strains representing all previously validly named plant-pathogenic members of the *Enterobacteriaceae* (28a), the *Erwinia alni* strains formed a discrete cluster. When the *Erwinia alni* profiles were compared with the profiles in two fatty acid profile libraries, one of which (the NCPPB library) contained all previously validly named and several invalidly named plant-pathogenic species belonging to the *Enterobacteriaceae*, none of the *Erwinia alni* profiles closely matched the profile of any

TABLE 1. Physiological and biochemical characteristics of six *Erwinia alni* strains, selected *Erwinia* species, and *Pseudomonas syringae* pv. *syringae*^a

Characteristic	<i>Erwinia alni</i> PVFi 20 ^T , PVFi 21, PVFi 22, PVFi 23, PVFi 25, and PVFi 27 ^b	<i>Erwinia</i> <i>quercina</i> NCPBP 1852 ^T	<i>Erwinia</i> <i>nigrifluens</i> NCPBP 564 ^T	<i>Erwinia</i> <i>carotovora</i> NCPBP 2577	<i>Pseudomonas</i> <i>syringae</i> Y 37 ^c
Anaerobic growth	+	+	+	+	—
Motility	+	+	+	+	+
Growth on Miller-Schroth medium	+	—	(+)	+	—
Catalase activity	+	+	+	+	+
Oxidase activity	—	—	—	—	—
Fluorescent pigments produced on King's medium B	—	—	—	—	+
Pink diffusible pigment produced on YDC	—	—	—	—	—
β-Galactosidase activity	—	—	—	+	—
Tobacco hypersensitivity	—	—	—	(+)	+
Potato slice rot	—	—	—	+	—
Pectate liquefaction (pH 5 and 8)	—	—	—	+	—
O-F test (fermentation) ^e	+	+	+	+	—
Growth factors requirement	—	+	—	—	—
Voges-Proskauer reaction	+	+	+	+	—
Methyl red test	(+)	—	+	+	—
Endoglucanase activity	+	+	—	+	—
Indole production	—	—	—	—	—
Lysine decarboxylase activity	—	—	—	—	—
Ornithine decarboxylase activity	—	—	—	—	—
Blue pigment produced on YDC	—	—	—	—	—
Yellow pigment produced on NA	—	—	—	—	—
Utilization of:					
Citrate	—	+	—	+	+
Malonate	—	—	—	—	+
Tolerance to:					
2% NaCl	+	+	+	+	+
5% NaCl	(+)	(+)	(+)	+	(+)
Growth at 36°C	(+)	(+)	+	+	—
Hydrolysis of:					
Esculin	—	—	(+)	—	—
Casein	—	—	—	+	+
Gelatin	—	—	—	+	+
Starch	—	—	—	—	—
H ₂ S produced from cysteine	+	+	+	+	—
Urease activity	(+)	—	+	—	+
Nitrate reduction	—	—	—	+	—
Phenylalanine deaminase activity	—	—	—	—	—
Arginine dihydrolase activity ^f	—	—	—	—	—
Acid production from:					
Glucose	+	+	+	(+)	+
Galactose	+	+	+	+	+
Fructose	+	+	+	(+)	(+)
Mannose	+	+	+	+	+
Xylose	+	—	+	+	+
L-Arabinose	+	—	+	+	+
Sucrose	+	+	+	+	+
Lactose	—	—	—	+	—
Raffinose	—	—	+	+	+
Melibiose	—	—	+	+	+
Trehalose	+	—	+	+	(+)
Maltose	+	—	—	—	—
Erythrose	—	—	—	—	—
Melezitose	—	—	—	—	—
Salicin	+	+	+	+	—
Glycerol	+	+	+	+	+
Mannitol	+	+	+	+	(+)
Sorbitol	—	+	+	+	(+)
Inositol	—	—	+	+	+
Adonitol	—	—	—	—	—
Dulcitol	—	—	—	—	—

^a NCPBP, National Collection of Plant Pathogenic Bacteria, Harpenden, England; PVFi, Istituto di Patologia e Zoologia forestale e agraria, University of Florence, Florence, Italy.

^b The pathogenicity of *Erwinia alni* strains was tested by Surico and Mugnai (30, 31).

^c This bacterium was supplied by G. L. Ercolani, Faculty of Agriculture, University of Bari, Bari, Italy.

^d +, positive reaction; —, negative reaction; (+), slow positive reaction.

^e O-F, oxidation-fermentation.

^f Determined by the methods of Thornley (32) and Møller (20).

FIG. 1. Electron micrograph of *Erwinia alni* PVFi 20^T (= NCPPB 3934^T = ICMP 12481^T). The cell was 0.6 by 1.6 μ m.

other species. An *Erwinia alni* entry comprising 11 profiles of selected strains was added to the NCPPB library. On the basis of their profiles these strains were all identified as *Erwinia alni* strains that had similarity indices of >0.75 ; the values for the next nearest neighbor were less than one-half this value. Thus, the fatty acid profiles of the alder strains clearly typical of the *Enterobacteriaceae* but also clearly different from the fatty acid profiles of all previously validly published species. The differences were mainly differences in the amounts of the fatty acids present and not differences in the types that occurred.

The differences in the *Erwinia alni*, *Erwinia amylovora*, *Erwinia herbicola*, *Erwinia nigrifluens*, *Erwinia quercina*, and *Pantoea agglomerans* profiles are shown in Table 2.

DNA base composition. *Erwinia alni* PVFi 20^T, PVFi 23, PVFi 25, and PVFi 27 had DNA G+C contents of 50.1 to 50.7 mol%. The base composition of the *Erwinia alni* type strain, strain PVFi 20, was 50.5 mol% (mean of four values). These values fall within the G+C content range of the genus *Erwinia* (50 to 58 mol%) (16).

DNA-DNA hybridization. The levels of relatedness between labelled *Erwinia nigrifluens*, *Erwinia salicis*, and *Erwinia alni*

FIG. 2. Principal-component analysis of fatty acid profiles of *Erwinia* strains. Multiple runs were processed for the three *Erwinia alni* strains along with occasional duplicate runs for strains of each of the other species. Symbols: $\blacklozenge$, *Erwinia alni* (multiple runs of strains PVFi 20^T, PVFi 23, and PVFi 27); +, *Erwinia nigrifluens* (3 strains); *, *Erwinia quercina* (3 strains); $\square$, *Erwinia amylovora* (26 strains); $\circ$, *Erwinia herbicola* (26 strains); $\triangle$, *Erwinia rubrifaciens* (2 strains). The strains were obtained either from the NCPPB or from the Central Science Laboratory diagnostic clinic. Complete fatty acid profile data for these and other *Erwinia* strains will be published elsewhere.

DNAs and unlabelled DNAs from these and other species belonging to the *Enterobacteriaceae* are shown in Table 3.

At the optimal hybridization temperature (40°C), the labelled DNAs from *Erwinia alni* PVFi 20^T and PVFi 27 exhibited high levels of similarity (range, 96 to 100%) with the

TABLE 2. Total cellular fatty acid contents of *Erwinia alni*, *Erwinia quercina*, *Erwinia nigrifluens*, *Erwinia amylovora*, *Pantoea agglomerans*, and *Erwinia herbicola*

Fatty acid class	Mean % (SD)					
	<i>Erwinia alni</i> (n = 3) ^a	<i>Erwinia quercina</i> (n = 3)	<i>Erwinia nigrifluens</i> (n = 3)	<i>Erwinia amylovora</i> (n = 26)	<i>Pantoea agglomerans</i> (n = 2)	<i>Erwinia herbicola</i> (n = 26)
Saturated straight-chain fatty acids						
12:0	6.99 (0.18) ^b	0.54 (0.04)	3.00 (0.10)	4.87 (0.19)	3.28 (0.39)	3.48 (0.38)
14:0	2.01 (0.06)	10.59 (0.82)	6.16 (0.12)	4.72 (0.35)	5.47 (0.16)	5.44 (0.56)
16:0	33.46 (0.12)	33.02 (0.96)	32.22 (0.61)	35.70 (0.78)	29.91 (1.71)	29.39 (0.97)
18:0	0.51 (0.02)	0.19 (0.11)	0.35 (0.05)	0.39 (0.18)	Tr ^d	0.35 (0.16)
Unsaturated fatty acids						
16:1 w7c	17.11 (1.98)	25.64 (3.07)	26.97 (1.47)	22.81 (1.19)	28.73 (1.69)	25.58 (2.78)
18:1 w7c	18.84 (0.47)	10.42 (2.11)	14.83 (0.87)	9.14 (0.58)	12.76 (1.85)	16.56 (3.53)
Cyclopropane fatty acids						
17:0	16.26 (1.78)	8.76 (2.26)	6.40 (1.53)	10.92 (1.37)	9.34 (1.21)	8.20 (3.11)
19:0 w8c	1.53 (0.37)	1.29 (0.32)	0.57 (0.17)			Tr
Hydroxy fatty acid 14:0 3OH	8.62 (0.04)	8.79 (0.29)	8.50 (0.25)	8.69 (0.45)	8.22 (0.46)	8.51 (0.45)
Unknown 14.503 ^c	0.68 (0.04)	0.66 (0.15)	0.81 (0.11)	0.75 (0.13)	0.56 (0.29)	0.82 (0.16)

^a n is the number of strains tested.

^b The means were based on multiple runs of each *Erwinia alni* strain. For the other taxa, duplicate runs of at least one strain were processed. The strains were obtained either from the NCPPB or from confirmed diagnoses made at the Central Science Laboratory diagnostic clinic. Complete data for the fatty acid profiles of these and other *Erwinia* strains will be published elsewhere. The *Erwinia alni* strains used were PVFi 20^T, PVFi 23, and PVFi 27.

^c Equivalent chain length relative to the lengths of known fatty acids.

^d Tr, trace.

TABLE 3. Levels of DNA-DNA similarity among *Erwinia*, *Enterobacter*, and *Pantoea* strains under optimal (40°C) and stringent (50°C) conditions

Source of unlabelled DNA	% Relative binding at 40°C (SD)				% Relative binding at 50°C (SD)	
	<i>Erwinia alni</i> PVFi 27	<i>Erwinia alni</i> PVFi 20 ^T	<i>Erwinia</i> <i>nigrifluens</i> NCPBP 564 ^T	<i>Erwinia</i> <i>salicis</i> NCPBP 2535	<i>Erwinia</i> <i>nigrifluens</i> NCPBP 564 ^T	<i>Erwinia alni</i> PVFi 20 ^T
<i>Erwinia alni</i> PVFi 27	100	>100	58 (1.8)	60 (4.3)	7 (0.4)	98 (13.5)
<i>Erwinia alni</i> PVFi 20 ^T	99 (3.9)	100	55 (1.8)	54 (6.1)	9 (1.3)	100
<i>Erwinia alni</i> PVFi 23	98 (7.7)	>100	64 (2.8)	42 (2.8)	10 (3.5)	>100
<i>Erwinia alni</i> PVFi 25	96 (3.3)	>100	64 (1.2)	45 (4.0)	10 (3.1)	>100
<i>Pantoea ananas</i> pv. <i>ananas</i> (<i>Erwinia ananas</i>) NCPBP 1846 ^T	22 (0.7)	14 (1.8)	9 (2.5)	43 (1.6)	6 (1.0)	6 (0.6)
<i>Pantoea ananas</i> pv. <i>uredovora</i> (<i>Erwinia uredovora</i>) NCPBP 800 ^T	25 (0.5)	10 (0.6)	19 (0.9)	33 (2.4)	6 (1.6)	3 (0.7)
<i>Enterobacter cancerogenus</i> (<i>Erwinia cancerogena</i>) NCPBP 2176 ^T	24 (1.3)	11 (2.0)	19 (0.9)	28 (3.9)	3 (0.2)	4 (0.2)
<i>Erwinia carotovora</i> subsp. <i>carotovora</i> NCPBP 2577	34 (0.4)	22 (3.9)	34 (2.3)	51 (9.4)	6 (0.6)	7 (1.1)
<i>Erwinia chrysanthemi</i> NCPBP 402 ^T	28 (2.9)	42 (2.2)	24 (3.4)	31 (2.2)	5 (1.7)	6 (1.0)
<i>Erwinia cypripedii</i> NCPBP 3004 ^T	14 (4.5)	11 (0.9)	16 (0.7)	28 (1.0)	3 (0.6)	4 (0.7)
<i>Erwinia herbicola</i> NCPBP 1268	13 (1.2)	15 (1.4)	14 (0.7)	23 (2.7)	4 (1.0)	6 (1.2)
<i>Erwinia mallotivora</i> NCPBP 2851 ^T	21 (2.3)	8 (1.0)	15 (1.8)	24 (2.0)	4 (0.6)	5 (0.4)
<i>Erwinia nigrifluens</i> NCPBP 564 ^T	65 (9.9)	ND ^a	101 (4.5)	36 (2.0)	93 (11.1)	11 (1.1)
<i>Erwinia nigrifluens</i> NCPBP 565	49 (6.1)	51 (5.4)	100	35 (7.1)	100	21 (4.8)
<i>Erwinia quercina</i> NCPBP 1852 ^T	35 (0.6)	18 (1.3)	22 (3.2)	43 (1.6)	7 (2.2)	11 (1.3)
<i>Erwinia quercina</i> NCPBP 3077	35 (2.2)	13 (0.9)	20 (2.9)	35 (2.5)	4 (0.3)	4 (0.4)
<i>Erwinia rubrifaciens</i> NCPBP 2020 ^T	50 (7.1)	40 (10.0)	29 (6.2)	56 (6.7)	5 (0.2)	17 (3.9)
<i>Erwinia salicis</i> NCPBP 2532	37 (1.7)	16 (1.5)	30 (2.3)	82 (5.3)	4 (1.2)	6 (1.0)
<i>Erwinia salicis</i> NCPBP 2535	53 (2.2)	30 (4.4)	38 (3.5)	100	7 (1.7)	12 (1.9)

^a ND, not determined.

DNA of other *Erwinia alni* strains and low levels of similarity (less than 37%) with most of the other *Erwinia* strains.

The levels of relatedness between *Erwinia alni* and *Erwinia nigrifluens* NCPBP 564^T and NCPBP 565 were between 49 and 65%. Similar values were obtained with *Erwinia alni* when DNA from *Erwinia nigrifluens* NCPBP 564^T was used as the probe.

The levels of hybridization between labelled DNA from *Erwinia salicis* NCPBP 2535 and unlabelled DNAs from *Erwinia alni* strains varied between 42 and 60%. On the other hand, *Erwinia salicis* exhibited 51 and 56% DNA relatedness to *Erwinia carotovora* subsp. *carotovora* and *Erwinia rubrifaciens*, respectively. *Erwinia rubrifaciens*, a bacterium which was found by Wilson and coworkers (35) to be similar to *Erwinia nigrifluens* and which causes phloem canker of Persian walnut trees, exhibited 40 and 50% homology to *Erwinia alni* PVFi 20^T and PVFi 27, respectively.

The levels of DNA homology between two *Erwinia alni* strains and *Erwinia quercina* NCPBP 1852^T and NCPBP 3077, the other organisms which (together with *Erwinia nigrifluens*), were initially suspected to be closely related to *Erwinia alni*, were less than 35%.

Under stringent conditions (50°C in this study) the levels of homology between labelled DNA from *Erwinia nigrifluens* NCPBP 564^T and unlabelled DNAs from *Erwinia alni* strains were much lower than the values obtained at the optimal temperature, while the levels of homology with strains belonging to the same species remained high (Table 3). When DNA from *Erwinia alni* PVFi 20^T was used as the probe, the levels of homology with *Erwinia alni* strains were around 100% but the levels of homology with *Erwinia nigrifluens* were 11 and 21% and the levels of homology with *Erwinia salicis* were 6 and 12%. The levels of homology with strains of other species belonging to the genus *Erwinia* were less than 17% (Table 3).

The results obtained in the DNA-DNA slot hybridization experiments, which were performed under optimal and strin-

gent conditions, suggested that *Erwinia alni* PVFi 20^T, PVFi 23, PVFi 25, and PVFi 27 belong to the same genomic group and are not closely related to the other *Erwinia* species tested.

Taxonomic position of the alder strains. The results of the initial studies on *Erwinia alni* PVFi 20^T, PVFi 21, PVFi 22, PVFi 23, PVFi 25, and PVFi 27 isolated from alder trees indicated that these organisms belonged to the family *Enterobacteriaceae* as described by Brenner (6). The alder strains were initially classified in the genus *Erwinia* on the basis of the description of Lelliot and Dickey (16). In this study the alder strains were compared with type strains and reference strains belonging to the genus *Erwinia*, including strains of *Erwinia nigrifluens* and *Erwinia quercina*, the bacterial species which cause (on walnut and oak, respectively) diseases which are similar in some respects to the alder bark canker observed in Italy. Selected strains of the genera *Pantoea* and *Enterobacter* were also used in various parts of this study.

The results of an analysis of 73 characteristics, including fatty acid data and nucleic acid base compositions, supported the initial assignment of the alder strains to the family *Enterobacteriaceae*, which includes, in addition to the genus *Erwinia*, two other genera which contain phytopathogenic bacteria, the genera *Enterobacter* and *Pantoea* (4, 7, 11). However, *Erwinia alni* PVFi 20^T and PVFi 27 exhibited only 10 to 25% DNA binding at the optimal hybridization temperature to *Pantoea ananas* pv. *ananas* NCPBP 1846^T, *Pantoea ananas* pv. *uredovora* NCPBP 800^T, *Enterobacter cancerogenus* NCPBP 2176^T, and *Erwinia herbicola* NCPBP 1268. Moreover, the average G+C content (50.4 mol%) of the alder strain DNAs examined so far differs from the G+C contents of phytopathogenic species belonging to the genus *Enterobacter* (52 to 61 mol% [8, 22]) and from the G+C contents of most of the phytopathogenic members of the genus *Pantoea*, including *Pantoea agglomerans* ATCC 27155^T (55.6 mol%) and *Pantoea dispersa* ATCC 14589^T (57.8 mol%) (11). Therefore, since there are no phenotypic differences between the genera *Er-*

winia and *Pantoea* (18) and since the levels of DNA homology were highest with strains currently placed in the genus *Erwinia* and generally lowest with strains recently transferred to the genera *Pantoea* and *Enterobacter*, we propose that the alder strains should be placed in the genus *Erwinia*. These organisms represent a distinct new taxon which may have quarantine significance within the European Union and for which a name is required.

The alder strains were found to be phenotypically and genetically highly related, indicating that they are members of a single species. One of the alder strains, PVFi 27, exhibited average levels of homology to *Erwinia nigrifluens* NCPPB 564^T, *Erwinia rubrifaciens* NCPPB 2020^T, and *Erwinia salicis* NCPPB 2535 at 40°C of 65, 50, and 53%, respectively. However, these average homology values are below the minimum level (70%) accepted for species delineation (27, 29), and the average homology values in hybridization experiments performed at 50°C were 11, 17, and 12%, respectively. These low relatedness values, coupled with the results of physiological tests and a fatty acid analysis, suggested that the alder strains are different from all previously described *Erwinia* species and should be recognized as members of a separate species. Therefore, we propose the name *Erwinia alni* for this organism. The taxonomic description below summarizes the properties of this organism.

Description of *Erwinia alni* sp. nov. *Erwinia alni* (al'ni. L. fem. n. *alnus*, alder; L. gen. n. *alni*, of alder, referring to the plant from which the organism was first isolated). Cells are gram negative, oxidase negative, catalase positive, and fermentative (producing acid but not gas from glucose). The cells are 0.5 to 0.8 by 1.2 to 2.3 µm, have rounded ends, occur singly or rarely in pairs, and are motile by means of peritrichous flagella. Abundant growth occurs in NB and on NA, YDC, Miller-Schroth medium, and other common laboratory media. Colonies on NSA are umbonate with translucent margins, whitish, and 2 to 2.5 mm in diameter after 5 days of incubation at 27°C. The organism grows at 25, 32, and 36°C but not at 5 and 42°C. No diffusible pigments are produced. The methyl red test and the Voges-Proskauer reaction are weakly positive. Growth factors are not required. None of the strains produces polygalacturonase or pectate lyase on pectate gels at pH 5 and 8. The strains do not induce a hypersensitive response in tobacco leaves and do not rot potato tuber slices. After inoculation, pathogenic strains typically cause bark canker disease of alder. They do not produce arginine dihydrolase, lysine and ornithine decarboxylases, phenylalanine deaminase, β-galactosidase, and indole. Casein, gelatin, starch, and esculin are not hydrolyzed. Nitrate is not reduced. Hydrogen sulfide is produced from cysteine. Citrate and malonate are not utilized. All strains hydrolyze carboxymethyl cellulose and are slightly positive for urease activity. Acids are produced from glucose, galactose, fructose, mannose, xylose, arabinose, sucrose, trehalose, maltose, salicin, glycerol, and mannitol oxidatively, but are not produced from lactose, raffinose, melibiose, erythrose, melezitose, sorbitol, inositol, adonitol, and dulcitol. Good growth occurs in the presence of 2% sodium chloride.

The major fatty acids include 14:0 3OH, 12:0, 14:0, 16:0, 16:1 w7c, 18:1 w7c, 17:0 cyclopropane, and 19:0 w8c cyclopropane.

The G+C content of the DNA is 50.1 to 50.7 mol% (as determined by the thermal denaturation method).

Description of the type strain. The type strain of *Erwinia alni* is strain PVFi 20. This strain has all of the characteristics described above for the species. Its DNA G+C content is 50.5 mol%. *Erwinia alni* PVFi 20^T was isolated from Italian alder in Tuscany as the causal agent of the bark canker disease of alder. After artificial inoculation, this strain typically produces the

symptoms of bark canker disease of alder. The type strain has been deposited in the NCPPB, Harpenden, United Kingdom, as strain NCPPB 3934 and in the International Collection of Micro-organisms from Plants, Auckland, New Zealand, as strain ICMP 12481.

ACKNOWLEDGMENTS

We thank M. Durante, A. Vaughan, L. Bonuomo, E. Bruno, S. Barcaccia, J. Wilson, and A. Aspin for their help with some of the experiments.

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