“*Erwinia nulanidii*” Is a Subjective Synonym of *Erwinia persicinus*

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The organism named “*Erwinia nulanidii*” was isolated in 1979 from bean seeds and was described in 1981, but the name was never validated. The results of biochemical tests and membrane protein profile and DNA relatedness studies indicated that this name is synonymous with *Erwinia persicinus*, a validly published name for a species previously isolated from, but not shown to be pathogenic for, tomatoes, bananas, and cucumbers. Pathogenicity tests revealed that all *E. persicinus* strains, including an “*E. nulanidii*” strain, were pathogenic for bean pods and seeds.

In 1981, Schuster et al. described a new, pink-pigmented species of bacteria isolated from beans (10) and named the organism “*Erwinia nulanidii*.” This name appeared just after the Approved Lists of Bacterial Names (13) was published, and it was never subsequently validated by publication in the International Journal of Systematic Bacteriology. In 1990, Hao et al. (5) described the pink-pigmented species *Erwinia persicinus*, which was isolated from tomatoes, bananas, and cucumbers. *E. persicinus* was shown to be distinct from all other validly published erwiniae, including the pink-pigmented species *Erwinia rhapontici* (9) and *Erwinia rubrifaciens* (18). “*E. nulanidii*” was not included in the study of Hao et al. because they were not aware of its existence.

This study was undertaken to recharacterize “*E. nulanidii*” in order to validate it as a new species. On the basis of the biochemical test, membrane protein profile, and DNA relatedness results which we obtained, it is clear that “*E. nulanidii*” is a synonym of *E. persicinus*.

MATERIALS AND METHODS

Bacterial strains. “*E. nulanidii*” NCPPB 3375 (= Pinky A = IBSBF 793 = ICMP 8932 = ATCC 49742) was obtained from the National Collection of Plant Pathogenic Bacteria. The type strain of *E. persicinus* (strain ATCC 35998 [= HK 204 = CDC 9108-82 = AJ2716 = IAM 12843 = JCM 3704]) and strains HK 200, HK 201, and HK 203 were obtained from K. Komagata, Tokyo, Japan. The strains were maintained on glucose-yeast extract-calcium carbonate agar or on nutrient agar. To prepare DNAs, strains were cultivated in nutrient broth at 30°C to the late logarithmic or stationary phase. The DNAs of all other organisms used in DNA hybridization studies (Table 1) were prepared previously in the DNA Hybridization Laboratory, Investigation and Surveillance Laboratory Section, Emerging Bacterial and Mycotic Diseases Branch, Centers for Disease Control and Prevention, Atlanta, Ga.

Phenotypic characteristics. Oxidation-fermentation of D-glucose was tested by the method of Hugh and Leifson (6). Tests for production of H₂S from cysteine, production of reducing substances from sucrose, production of indole, reduction of nitrate to nitrite, and acid production from carbohydrates (using C medium) were performed by the methods of Dye (3). Lipolysis of Tween 80 was tested by the method of

Sierra (11), hydrolysis of esculin was tested by the method of Sneath (14), and oxidase production was tested by the method of Kovacs (7). The presence of protopectinase on potato, carrot, onion, and pepper slices and tyrosinase production were determined by the methods of Lelliott et al. (8). Liquefaction of sodium polypectate was determined by the method of Skerman (12), and utilization of sodium salts of organic acids was determined by the method of Wilkie (17). All other tests were performed as described by Edwards and Ewing (4) or Schuster et al. (10). The oxidase test was read immediately after Kovács reagent was added. All other test preparations were incubated at 28°C, and the results were recorded after 2, 5, or 14 days.

Pathogenicity tests. The pathogenicity of “*E. nulanidii*” NCPPB 3375 and the pathogenicity of *E. persicinus* strains were determined with bean pods (*Phaseolus vulgaris* cv. Carioca) and the fruits of tomatoes (*Lycopersicon esculentum* cv. Angela), cucumbers (*Cucumis sativus* cv. Aodai), and bananas (*Musa* sp. cv. Nanica). Each pod or fruit was artificially inoculated by placing 1 drop of a bacterial suspension containing approximately 10⁸ CFU/ml on the surface and then puncturing the surface with a sterile needle. Five samples of each plant were inoculated with each strain. Bananas were detached from the plant and maintained in a laboratory at room temperature. Other plants were maintained for 48 h in a humid chamber in a greenhouse. Symptoms were recorded 5 days after inoculation.

PAGE of membrane proteins. Polyacrylamide gel electrophoresis (PAGE) of membrane proteins was performed by the method of Thaveechai and Schaad (15), and the gels were stained with silver nitrate by the method of Blum et al. (1). A 12% sodium dodecyl sulfate (SDS)-polyacrylamide gel was used. The protein patterns were visually compared and analyzed by using a scanning densitometer.

DNA hybridization. The methods used for DNA purification and DNA hybridization on hydroxyapatite have been described by Brenner et al. (2). DNA from “*E. nulanidii*” NCPPB 3375 was labeled in vitro with [³²P]dCTP, which was provided in a nick translation reagent kit (Bethesda Research Laboratories, Inc., Gaithersburg, Md.). An incubation temperature of 60°C was used to determine optimal DNA relatedness values, and 75°C was used to assess DNA relatedness under stringent conditions. The percentage of relative binding was determined by normalizing the amount of labeled DNA bound to hydroxyapatite in heterologous reactions to the amount bound in homologous control reactions, in which both the labeled and unlabeled DNAs were from “*E. nulanidii*” NCPPB 3375. The

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TABLE 1. Levels of DNA relatedness between "*E. nulanidii*" and other erwiniae

Source of unlabeled DNA ^a	Labeled DNA from " <i>E. nulanidii</i> " NCPBP 3375 ^b		
	Relative binding ratio at 60°C	% Diver- gence	Relative binding ratio at 75°C
" <i>Erwinia nulanidii</i> " NCPBP 3375	100	0.0	100
<i>Erwinia persicinus</i> HK 200	87	0.0	89
<i>Erwinia persicinus</i> HK 201	83	0.0	87
<i>Erwinia persicinus</i> ATCC 35998 ^T	79	0.0	88
<i>Erwinia persicinus</i> HK 203	78	0.0	81
<i>Erwinia rhapontici</i> ICPB ER 106C	66	11.0	32
<i>Erwinia rhapontici</i> ATCC 29283 ^T	63	10.5	33
<i>Erwinia rhapontici</i> ICPB ER 106A	60	11.0	29
<i>Erwinia amylovora</i> ICPB EA 178	42		
<i>Erwinia herbicola</i> ATCC 33243 ^T	40		
<i>Erwinia milletiae</i> ATCC 33231 ^T	38		
<i>Erwinia cypripedii</i> ICPB EC 155	36		
<i>Pantoea ananas</i> ATCC 33244 ^T	33		
<i>Erwinia uredovora</i> CDC 9574-82	30		
<i>Pantoea stewartii</i> ATCC 8199 ^T	29		
<i>Erwinia psidii</i> 435-84 ^T	29		
<i>Erwinia mallotivora</i> NCPBP 2851 ^T	28		
<i>Erwinia chrysanthemi</i> ATCC 11663 ^T	26		
<i>Erwinia carotovora</i> ATCC 15713 ^T	25		
<i>Erwinia tracheiphila</i> ATCC 33245 ^T	24		
<i>Erwinia carotovora</i> subsp. <i>betavascularum</i> ATCC 8061	21		
<i>Erwinia carotovora</i> subsp. <i>wasabiae</i> ATCC 43316 ^T	21		
<i>Pantoea terreus</i> ATCC 31628	21		
<i>Erwinia rubrifaciens</i> ICPB ER 105	21		
<i>Erwinia salicis</i> ATCC 15712 ^T	20		
<i>Erwinia quercina</i> ICPB EQ 102	19		
<i>Erwinia cacticida</i> ATCC 49481 ^T	18		
<i>Pantoea punctata</i> ATCC 31624	18		
<i>Pantoea citreus</i> ATCC 31623	16		

^a NCPBP, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom; ATCC, American Type Culture Collection, Rockville, Md.; ICPB, International Collection of Phytopathogenic Bacteria, University of California, Davis; CDC, Centers for Disease Control and Prevention, Atlanta, Ga.

^b The relative binding ratios and divergence values are averages from two or more experiments. Before normalization to 100%, the average levels of DNA bound to hydroxyapatite in homologous reactions were 70% in 60°C reactions and 72% in 75°C reactions. The average levels of labeled DNA that bound to hydroxyapatite in control reaction mixtures that did not contain unlabeled DNA were 3% in 60°C reactions and 1% in 75°C reactions. These values were subtracted from all reassociation reaction values before normalization.

percentage of divergence was calculated to the nearest 0.5% by assuming that each 1°C decrease in the thermal stability in a heterologous DNA duplex compared with the thermal stability of the homologous "*E. nulanidii*" DNA duplex was caused by approximately 1% unpaired nucleotide bases.

RESULTS AND DISCUSSION

Cells of "*E. nulanidii*" NCPBP 3375 are gram negative, oxidase negative, catalase positive, fermentative, and motile. They produce a pink pigment on glucose-yeast extract-calcium carbonate agar and other media. They give positive reactions in tests for H₂S production from cysteine, H₂S production from sodium thiosulfate, acetoin production (Voges-Proskauer test), phosphatase activity, reduction of nitrate to nitrite, esculin hydrolysis, growth in the presence of 5% NaCl, and growth in the presence of 0.0005% tetrazolium chloride. They give negative reactions in tests for growth at 42°C, urease

activity, gas production from D-glucose, gelatin hydrolysis, starch hydrolysis, production of reducing substances from sucrose, indole production, methyl red, phenylalanine deaminase activity, arginine dihydrolase activity, lysine decarboxylase activity, ornithine decarboxylase activity, lipolysis of Tween 80, tyrosinase activity, pectinase activity, and production of protopectinase on potato, carrot, onion, and pepper slices.

"*E. nulanidii*" NCPBP 3375 produces acid during fermentation of cellobiose (weakly), D-fucose, fructose, D-galactose, D-glucose, β-methyl-D-glucoside, *m*-inositol, lactose, maltose, D-mannitol, D-mannose, melibiose, L-rhamnose, ribose, D-salicin, D-sorbitol, sucrose, and trehalose, but does not ferment D-arabinose, D-arabitol, L-arabitol, adonitol, dulcitol, erythritol, ethanol, α-methyl-D-glucoside, glycerol, inulin, melezitose, palatinose, raffinose, starch, tagatose, D-xylose, or L-xylose.

"*E. nulanidii*" NCPBP 3375 utilizes sodium salts of the organic acids acetate, citrate, fumarate, gluconate, glutarate, hippurate, malonate, mesaconate, nicotinate, oxalate, D-tartrate, L-tartrate, *m*-tartrate, and succinate, but does not utilize benzoate, glycerophosphate, and taurocholate. It utilizes the amino acids DL-arginine, L-arginine, L-glutamine, DL-histidine, L-histidine, L-ornithine, L-proline (weakly), and L-serine, but does not utilize betaine, DL-phenylalanine, L-threonine, L-tryptophan, and L-valine.

The biochemical reactions of "*E. nulanidii*" NCPBP 3375 are very similar to the reactions reported previously for the pink-pigmented species *E. persicinus* (5). The differences observed included acid production from D-fucose in "*E. nulanidii*" but not in *E. persicinus*; acid production from D-melezitose and raffinose in *E. persicinus* but not in "*E. nulanidii*"; and acetate utilization in "*E. nulanidii*" but not in *E. persicinus*. Like *E. persicinus*, "*E. nulanidii*" is differentiated from the pink-pigmented species *E. rhapontici* by its negative methyl red test, by acetoin production, by a lack of acid production from dulcitol, α-methyl-D-glucoside, glycerol, inulin, starch, and D-xylose, and by acid production from *m*-inositol (5). "*E. nulanidii*" is differentiated from the pink-pigmented species *E. rubrifaciens* by its negative methyl red test, by acetoin production, by acid production from cellobiose, *m*-inositol, lactose, and melibiose, and by a lack of acid production from α-methyl-D-glucoside and glycerol (5).

E. persicinus ATCC 35998^T (T = type strain), HK 200, and HK 201 and "*E. nulanidii*" NCPBP 3375 were tested for pathogenicity by inoculating them into plants. None of these strains produced clear-cut rot on cucumbers, tomatoes, or bananas. All strains did produce necrotic symptoms on bean pods and discoloration of bean seeds.

The membrane protein patterns obtained after SDS-PAGE were extremely similar for "*E. nulanidii*" NCPBP 3375 and three strains of *E. persicinus* (Fig. 1). Densitometer tracings of the protein patterns confirmed the similarity of the membrane proteins of these strains (data not shown).

The levels of relatedness between labeled "*E. nulanidii*" DNA and unlabeled DNAs from *Erwinia* strains are shown in Table 1. The levels of relatedness between "*E. nulanidii*" and four *E. persicinus* strains ranged from 78 to 87% (average, 82%) with 0% divergence in 60°C reactions and from 81 to 89% (average, 86%) in 75°C reactions. The next closest relative of "*E. nulanidii*" was *E. rhapontici*; the average level of relatedness at 60°C was 63%, with an average level of divergence among related sequences of 11.0%. *E. rhapontici* is also the closest relative of *E. persicinus*, exhibiting an average level of relatedness at 60°C of 66% and a level of divergence among related sequences of 10.5% (5).

The recommended genetic definition of a bacterial species is a group of strains that exhibit levels of relatedness of 70% or

FIG. 1. Membrane protein profiles of *E. persicinus* and "*E. nulan-dii*." Lanes A through C, *E. persicinus* HK 200, ATCC 35998^T, and HK 201, respectively; lane D, "*E. nulan-dii*" NCPPB 3375; lane M, molecular weight marker Dalton Mark VII.

more at an optimal reassociation temperature (60°C in this study) and levels of divergence among related DNA sequences of less than 5% (16). When this definition and the 86% average level of relatedness at the stringent reassociation temperature were used, there was no doubt that the strains represented by the names "*E. nulan-dii*" and *E. persicinus* are members of a single species and that these names are synonyms. This conclusion was strengthened by the similar biochemical profiles, protein profiles, and plant pathogenicity characteristics of the strains which we studied.

"*E. nulan-dii*" differed from *E. persicinus* in D-fucose, D-melezitose, and raffinose fermentation reaction results and in acetate utilization reaction results (5). It is not known whether these differences resulted from differences in the tests used or reflected variability among the strains used in these tests. The fact that the "*E. nulan-dii*" and *E. persicinus* strains were pathogenic for beans provides the first evidence that *E. persicinus* is a plant pathogen. The description of *E. persicinus* (5) should be emended to reflect this finding.

Resolution of the nomenclatural question of which of the two synonymous names should be used for the single genospecies is simple. Unfortunately, the name "*E. nulan-dii*" was never validated in the International Journal of Systematic Bacteriology and has no standing in nomenclature. Therefore, despite the fact that "*E. nulan-dii*" was published 9 years earlier than *E. persicinus*, *E. persicinus* is the valid name for this species.

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