Deoxyribonucleic Acid Relatedness Among Erwiniae and Other *Enterobacteriaceae*: the Soft-Rot Organisms (Genus *Pectobacterium* Waldee)

DON J. BRENNER, A. G. STEIGERWALT, G. V. MIKLOS, and G. R. FANNING

Division of Biochemistry, Walter Reed Army Institute of Research, Washington, D.C. 20012

Relatedness among soft-rot-producing organisms of the genus *Pectobacterium* Waldee was assessed by means of interspecific deoxyribonucleic acid reassociation followed by chromatography on hydroxyapatite. Relatedness was also determined between pectobacteria and representatives from all other established genera of the family *Enterobacteriaceae*. The results indicate five distinct groups of pectobacteria: (i) *Pectobacterium carotovorum* (Jones) Waldee, including *Erwinia aroideae* (Townsend) Holland, *E. atroseptica* (van Hall) Jennison, *E. solanisapra* (Harrison) Holland, and *Bacillus oleraceae* Harrison; (ii) *P. carnegieana* (Lightle et al.) comb. nov.; (iii) cornstalk-rot bacterium and *P. chrysanthemi* (Burkholder et al.) comb. nov. (including *E. dieffenbachiae* McFadden, *E. cytolytica* Chester, and *P. carotovorum* f. sp. *parthenii*); (iv) *P. cypripedii* (Hori) comb. nov.; and (v) *P. rhapontici* (Millard) Patel and Kulkarni. Relatedness between groups of pectobacteria is 20 to 50% except between the *P. carotovorum* group and strains of *P. carnegieana*, which exhibit 60 to 70% relatedness. With few exceptions, the pectobacteria are 20 to 50% related to other members of the family *Enterobacteriaceae*. The data presented support the inclusion of pectobacteria in the family *Enterobacteriaceae*.

According to the latest edition of *Bergey's Manual of Determinative Bacteriology* (2), the genus *Erwinia* Winslow et al., as typified by *E. amylovora*, contains phytopathogens that cause wilts and blights as well as those that cause soft-rot diseases, the latter exemplified by *E. carotovora*. In addition to differences in the type of infection produced, the above-mentioned species exhibit significantly different biochemical characteristics. For these reasons, it has been repeatedly suggested that they be placed in separate genera, *Erwinia* and *Pectobacterium* Waldee, respectively (13, 17).

Two additional types of organism are cited in *Bergey's Manual* (2) as belonging to *Erwinia*: yellow-pigmented organisms (*E. ananas*, *E. herbicola*, etc.) that are often isolated from clinical sources (16) but that do not appear to be phytopathogenic (8) (it was recently recommended [11] that these organisms be placed in the genus *Enterobacter* as *E. agglomerans*), and a group of atypical erwiniae thought to be related to *Klebsiella* and *Enterobacter* (9).

We became interested in erwiniae as part of a continuing study of relatedness among members of the family *Enterobacteriaceae* (6).

Preliminary results indicated that the soft-rot erwiniae are significantly related to the other erwiniae and to organisms in other genera of *Enterobacteriaceae*. Nonetheless, extensive divergence is evident among species of *Erwinia* and between erwiniae and other *Enterobacteriaceae*. Our preliminary results were recently confirmed and extended by Gardner and Kado (12). These data (6, 12) and reawakened interest in erwiniae on the part of taxonomists, clinical microbiologists, and plant pathologists stimulated a thorough investigation of relatedness in erwiniae.

This report deals with deoxyribonucleic acid (DNA) relatedness in the organisms that produce soft-rot diseases.

MATERIALS AND METHODS

Organisms and media. The organisms used in this study are listed in Table 1 by the name under which they were received. The organisms were maintained on meat extract agar or brain heart infusion agar. Routine cultivation was carried out on brain heart infusion broth or nutrient broth on a dry-air rotary shaker. The incubation temperature was 30 C for pectobacteria.

TABLE 1. *Bacterial strains used*

Organism received as	Source ^a	Organism received as	Source ^a
<i>Arizona</i> 1	CDC	<i>Erwinia</i> 5730-69	CDC
<i>Citropacter freundii</i> 460-61	CDC	<i>Erwinia chrysanthemi</i> EC16	ICPB
<i>C. freundii</i> 5118-60	CDC	fluidal SR31 (ICPB EC176)	Kelman, Univ. of Wisconsin
Cornstalk-rot bacterium SR56 (ICPB EC209)	Kelman, Univ. of Wisconsin	SR32 (ICPB EC16)	Kelman, Univ. of Wisconsin
SR80 (ICPB EC252)	Kelman, Univ. of Wisconsin	EC205	ICPB
SR84	Kelman, Univ. of Wisconsin	11662	ATCC
SR98 (ICPB EC253)	Kelman, Univ. of Wisconsin	11663	ATCC
EC210	ICPB	<i>Erwinia cypripedii</i> EC155	ICPB
EC107	ICPB	EC160	ICPB
<i>Edwardsiella tarda</i> 1126-64	CDC	<i>Erwinia cytolytica</i> EC207	ICPB
<i>Enterobacter aerogenes</i> 1627-66	CDC	<i>Erwinia dieffenbachiae</i> ED102	ICPB
banana strain 3151	ICPB	ED103	ICPB
<i>Enterobacter cloacae</i> 1347-71	CDC	<i>Erwinia dissolvens</i> ED105	ICPB
yellow 165	V. J. Brenner, NIH	ED114	ICPB
yellow 256-4	V. J. Brenner, NIH	23373	ATCC
<i>Enterobacter hafniae</i> 4360-67	CDC	<i>Erwinia herbicola</i> Y46 EH103	ICPB
<i>Erwinia amylovora</i> EA11	ICPB	3163	ICPB
EA169	ICPB	<i>Erwinia lathyri</i> EL102	ICPB
EA178	ICPB	<i>Erwinia nigrifluens</i> EN101	ICPB
15357	ATCC	EN104	ICPB
19382 (ICPB EA195)	ATCC	<i>Erwinia nimipressuralis</i> EN1	ATCC
7400 (ICPB EA196)	ATCC	9912	ATCC
<i>Erwinia ananas</i> EA181	ICPB	<i>Erwinia oleraceae</i> EO1	ICPB
<i>Erwinia aroideae</i> EA13	ICPB	<i>Erwinia carotovora</i> f. sp. <i>parthenii</i> EC115	ICPB
EA144	ICPB	<i>Erwinia quercina</i> EQ101	ICPB
12286 (ICPB EA189)	ATCC	<i>Erwinia rhapontici</i> ER106	ICPB
15390 (ICPB EA190)	ATCC	<i>Erwinia rubrifaciens</i> ER105	ICPB
25207 (ICPB EA191)	ATCC	<i>Erwinia salicis</i> ES102	ICPB
<i>Erwinia atroseptica</i> EA112	ICPB	<i>Erwinia solanisapra</i> ES101	ATCC
494 (ICPB EA192)	ATCC	8061	ATCC
4446 (ICPB EA193)	ATCC	<i>Erwinia tracheiphila</i> 27004	ATCC
7403 (ICPB EA194)	ATCC	<i>Escherichia coli</i> K-12	Univ. of Washington
7404	ATCC	<i>Klebsiella ozaenae</i>	CDC
<i>Erwinia carnegieana</i> EC186	ICPB	<i>Klebsiella pneumoniae</i> 2	CDC
EC223	ICPB	<i>Proteus mirabilis</i> 1	Falkow, Univ. of Washington
13452 (ICPB EC251)	ATCC	<i>Salmonella typhimurium</i> LT2	Theodore, NIH
<i>Erwinia carotovora</i> EC105	ICPB	<i>Serratia marcescens</i> SM6 W2	WRAIR
EC153	ICPB	<i>Shigella flexneri</i> 2a 24570	Falkow, Univ. of Washington
EC208	ICPB	<i>Xanthomonas uedovorus</i> XU104	ICPB
495	ATCC		
15359 (ICPB EC250)	ATCC		
15713	ATCC		
25270 (ICPB EC248)	ATCC		
25272 (ICPB EC249)	ATCC		

^a CDC, Center for Disease Control, Atlanta, Ga, from W. H. Ewing and G. Hermann; ICPB, International Collection of Phytopathogenic Bacteria, Univ. of California, Davis, from M. P. Starr; NIH, National Institutes of Health, Bethesda, Md; ATCC, American Type Culture Collection, Rockville, Md; WRAIR, Walter Reed Army Institute of Research, Washington, D.C.

Erwinia amylovora and related organisms were grown at 23 C to 25 C, and atypical erwiniae, members of *Enterobacter agglomerans* (Herbicola-Lathyri group), and all other *Enterobacteriaceae* were grown at 37 C. The medium used for labeling cells with $^{32}\text{PO}_4$ has been described previously (3).

DNA preparation. Both unlabeled and labeled DNA were prepared by a modification (3) of the method of Berns and Thomas (1). Cells were normally lysed by 0.5% sodium dodecyl sulfate (SDS) at 37 C overnight. Certain erwiniae required lysozyme treatment for lysis. A few erwiniae apparently contain a potent deoxyribonuclease, as judged from rapid loss of DNA viscosity after cell lysis. In these strains, DNA was obtained by one of the following modifications in the lysing procedure: (i) shortening the SDS treatment to 1 h or less and immediately extracting the lysate with phenol; (ii) lysing the cells directly into phenol; or (iii) lysing cells as in (i) or (ii) at 75 C for 15 min and then holding them at 37 C.

DNA reassociation. The conditions used for DNA reassociation and for the separation of DNA duplexes from unreacted single-stranded DNA on hydroxyapatite have been presented in detail elsewhere (3, 4, 5). Essential reaction conditions are described below.

In all cases 0.1 μg of sheared, $^{32}\text{PO}_4$ -labeled DNA per ml was reacted with 150 μg of sheared, unlabeled DNA per ml in 0.28 M phosphate buffer (equimolar mixture of Na_2HPO_4 and NaH_2PO_4 , pH 6.8) for 16 h. The temperature of incubation was 60 or 75 C. At 60 C, interspecific DNA reassociation is maximized yet specific, and is restricted to sequences with more than statistical-base-sequence similarity. At 75 C, reassociation is restricted to related sequences showing a high degree of thermal stability.

After incubation, the samples were diluted with distilled water to 0.14 M phosphate buffer, and SDS was added to 0.4%. The samples were then passed through hydroxyapatite, and unreacted DNA was eluted. The thermal stability of reassociated DNA was determined by washing the hydroxyapatite with 0.14 M phosphate buffer at increasing temperature increments of 5.0 C. As the temperature exceeded the thermal stability of a class of DNA, the DNA was denatured and eluted from hydroxyapatite. When thermal stability was not of interest, the bound DNA was eluted from hydroxyapatite with 0.4 M phosphate buffer.

The specific activity of the labeled DNA was between 8×10^4 and 1.5×10^5 counts per min per μg . Ten samples were run simultaneously by using a batch procedure for thermal elution of DNA from hydroxyapatite (4). One homologous DNA reaction was included with each 10 samples. Observed homologous binding was arbitrarily designated 100%. Reassociation of heterologous DNA reactions was determined relative to that of homologous DNA. Heterologous reactions were carried out three to four times at 60 C and twice at 75 C.

RESULTS AND DISCUSSION

Bacterial relationships determined from DNA-DNA reassociation reactions are dependent upon the specificity and completeness of DNA reassociation and upon the ability to

distinguish DNA duplexes from single-stranded DNA. In this and previous studies we have chosen 60 C and 75 C as incubation temperatures for DNA reassociation. At 60 C, we hope to detect all reasonably stable complementary sequences shared by the test organisms. At 75 C, only interspecies DNA duplexes with closely related or highly complementary polynucleotide sequences will be stable.

Assuming a common ancestor, relative reassociation at 60 C indicates the amount of DNA from two organisms that is now related and how much DNA has diverged to a point where it can no longer form stable duplexes. The difference in thermal stability between homologous and heterologous DNA duplexes allows an approximation of divergence within related DNAs. We equate each 1 C decrease in duplex thermal stability with approximately 1% unpaired bases within the duplex (14).

We use the name *Pectobacterium* or its vernacular equivalent, pectobacteria, to refer to the five species of soft-rot-producing *Enterobacteriaceae* recognized herein and to related organisms. To our knowledge, formal transfers to the genus *Pectobacterium* of three of the five species recognized have not been made. Therefore, on the basis of the morphological and biochemical characteristics previously reported for these three organisms (7, 13) and on the basis of the DNA similarities of these three species to each other and to other pectobacteria, as discussed below, the following new combinations are proposed: *Pectobacterium carnegieana* (Lightle et al.) comb. nov., *P. chrysanthemi* (Burkholder et al.) comb. nov., and *P. cypripedii* (Hori) comb. nov.

P. carotovorum (Jones) Waldee ATCC 495, *P. cypripedii* ICPB EC155, and *P. carnegieana* ICPB EC186 were the reference strains used to determine DNA relatedness among pectobacteria and relatedness between these organisms and the remainder of *Enterobacteriaceae* studied. Reactions using $^{32}\text{PO}_4$ -labeled reference DNA from *P. carotovorum* ATCC 495 are shown in Table 2.

The six *P. carotovorum* strains tested at 60 C show an average of 91% relatedness to ATCC strain 495. The average $\Delta T_{m(e)}$ is 3.3 C, and relatedness remains high (85% average) when reassociation is carried out at 75 C. ATCC 15359 is least related to ATCC 495 (80%) and exhibits a $\Delta T_{m(e)}$ of almost 7 C, yet 68% of the genomes of these strains form stable duplexes at 75 C.

E. atroseptica and *E. aroideae* strains exhibit an average 85% relatedness to ATCC 495. There is evidence of some 4% unpaired bases in the related nucleotide sequences, and relatedness at 75 C averages 84%. The relatedness values for

individual strains, with the exception of *E. aroideae* ATCC 12286 (72%) and *Erwinia* sp. 5730-69 (74%), are well within the range of relatedness observed between *P. carotovorum* strains. *Erwinia* sp. 5730-69, based on biochemical reactions, is classified as a typical *Pectobacterium* (W. H. Ewing, personal communication). Two strains of *E. solanisa* and *E. oleraceae* ICPB EO 1 are also highly related to ATCC 495. Reassociation reactions among *P. carotovorum* strains and between ATCC 495 and strains of *E. atroseptica*, *E. aroideae*, *E. solanisa*, and *E. oleraceae* form gaussian-type distributions with the mode between 90 and 99% relatedness (Fig. 1). These distributions of relatedness are indicative of patterns seen previously only with strains of one species or of very closely related species (5).

Two of three strains of *P. carnegieana* are 60% related to ATCC 495. Approximately one-half of the related sequences are unstable in 75 C incubations. The duplex stability indicates some 13% unpaired bases within related sequences. The third strain, *P. carnegieana* ATCC 13452, exhibits about 40% relatedness to ATCC 495 with some 12% unpaired bases. About 25% relatedness is maintained at 75 C. *P. carnegieana* ATCC 13452 is not closely related to the other two *P. carnegieana* strains (Table 3). The distribution of relatedness of *P. carnegieana* strains to *P. carotovorum* ATCC 495 is midway between the relatedness shown by the *P. carotovorum* group of strains and that shown by all other pectobacteria that are considered below (Fig. 1).

DNA relatedness studies to be reported later (Brenner and Kelman, manuscript in preparation) show that constalk-rot bacteria and strains of *P. chrysanthemi* (as well as of *E. dieffenbachiae*, *E. cytolytica*, and *P. carotovorum* f. sp. *parthenii*) form a closely related group. These organisms are 40% related to *P. carotovorum* ATCC 495. Substantial divergence is present in related DNA sequences as shown both by the $\Delta T_{m(e)}$ of 17 C and the fact that only 10% relative reassociation occurs at the more stringent 75 C incubation temperature. Other pectobacteria tested for relatedness to ATCC 495 are *P. cyprripedii* (29% relatedness, 19% unpaired bases) and *P. rhapontici* (Millard, Patel and Kulkarni) (46% relatedness, 16% unpaired bases).

Relatedness of *P. carotovorum* to species of *Erwinia* (Table 2) is from 24 to 56%. Substantial divergence is present in all cases as judged by unpaired bases (11.5-19%) and by the marked decrease in binding at 75 C. It appears that *P. carotovorum* is less diverged from *E. nigri*, *E. salicis*, *E. rubrifaciens*, and

perhaps *E. quercina* than from strains of *E. amylovora* and *E. tracheiphila*. In fact relatedness between *P. carotovorum* and *E. nigri* is higher than that observed between *P. carotovorum* and all other pectobacteria except *P. carnegieana*.

Strains of the four yellow-pigmented organisms tested (*E. ananas*, *E. lathyri*, *E. herbicola*, and *Xanthomonas uredo*) are presumed, based on pigmentation and preliminary biochemical characterization, to be *Enterobacter agglomerans*. These organisms show an average of 32% relatedness to *P. carotovorum* with about 18% unpaired bases in the duplexed DNA. A similar degree of relatedness exists between *P. carotovorum* and *E. dissolvens* and *E. nimipressuralis*, two atypical erwiniae.

P. carotovorum ATCC 495 shares one-fourth to one-third of its genome with species of *Serratia*, *Citrobacter*, *Salmonella*, *Arizona*, *Escherichia*, *Enterobacter*, and *Shigella*. This relatedness falls to an average of 6% at 75 C. *P. carotovorum* is least related to *Edwardsiella tarda* (18%) and *P. mirabilis* (11%).

Reassociation reactions at 60 C established that *P. cyprripedii* ICPB EC 155 and *P. cyprripedii* ICPB EC160 are virtually identical based on relative DNA binding and duplex thermal stability (Table 4). Relatedness of *P. cyprripedii* to other pectobacteria, species of *Erwinia*, and other *Enterobacteriaceae* is 21 to 42% with some 14 to 20% unpaired bases within the related nucleotide sequences. Reactions of ICPB EC155 are highest with DNA from the strains of *Enterobacter agglomerans* (*E. herbicola* and *X. uredo*) and perhaps lowest with strains of other species of *Pectobacterium*. In the main, ICPB EC155 exhibits a 25 to 33% core relatedness to strains belonging to other species of *Enterobacteriaceae*.

FIG. 1. Frequency distribution of relatedness between *Pectobacterium carotovorum* ATCC 495 and other pectobacteria. Values shown here were obtained from reactions at 60 C. The value for the reference strain, *P. carotovorum* ATCC 495, was inadvertently included. Therefore, the number of *P. carotovorum* strains that show 100 to 109% relative binding is one, not two.

TABLE 2. Reassociation of DNA from *Pectobacterium carotovorum* 495

Source of unlabeled DNA	Relative binding at 60 C (%)	$\Delta T_{m(e)}^a$ at 60 C	Relative binding at 75 C (%)
<i>Pectobacterium carotovorum</i> ATCC 495	100		100
ICPB EC208	104	0.4	102
ICPB EC105	94	2.1	92
ATCC 25270	91	4.0	84
ATCC 15713	90	3.0	84
ATCC 25272	84	3.5	79
ATCC 15359	80	6.7	68
Average	91	3.3	84
<i>Erwinia atroseptica</i> ATCC 7403	96	1.0	94
ATCC 7404	92	1.1	84
ATCC 494	90	1.7	90
ATCC 4446	90	1.5	85
ICPB EA112	83	7.5	71
<i>E. aroideae</i> ATCC 25207	87	3.6	84
ICPB EA144	87	4.8	81
ATCC 15390	86	3.5	82
ICPB EA13	83	6.4	72
ATCC 12286	72	6.6	72
<i>Pectobacterium</i> 5730-69	74	4.7	68
Average	85	3.9	80
<i>Erwinia solanisapra</i> ICPB ES101	99	0.3	104
ATCC 8061	71	8.0	60
<i>Bacillus oleraceae</i> ICPB EO1	94	1.0	93
Average	88	3.1	86
<i>Erwinia amylovora</i> ICPB EA169	37	17.5	10
ATCC 15357	30	18.0	4
ATCC 19382	28	18.1	5
ICPB EA11	24	17.2	6
<i>E. tracheiphila</i> ATCC 27004	25	18.8	3
Average	29	17.9	6
<i>E. nigrifluens</i> ICPB EN104	56	11.5	32
<i>E. salicis</i> ICPB ES102	49	16.5	17
<i>E. rubrifaciens</i> ICPB ER105	48	15.4	17
<i>E. quercina</i> ICPB EQ101	42	12.3	24
Average	49	13.9	23
<i>E. ananas</i> ECPB EA181	37	16.5	7
<i>E. herbicola</i> ICPB 3163	33	17.8	5
<i>E. lathyri</i> ICPB EL102	32	17.3	6
<i>Xanthomonas uredovorus</i> ICPB XU104	26	18.2	5
Average	32	17.5	6
<i>Erwinia nimipressuralis</i> ICPB EN1	34	15.4	11
<i>E. dissolvens</i> ICPB ED105	32	16.8	7
Average	33	16.1	9

Table 2 continued on next page

TABLE 2—Continued

Source of unlabeled DNA	Relative Binding at 60 C (%)	$\Delta T_{m(e)}^a$ at 60 C	Relative Binding at 75 C (%)
<i>Serratia marcescens</i> SM6 W2	36	15.3	10
<i>Citrobacter freundii</i> CDC 5118-60	30	15.2	5
<i>Salmonella typhimurium</i> LT2	30	17.3	5
Arizona 1	30	17.9	5
<i>Escherichia coli</i> K-12	28	17.2	4
<i>Pectobacterium carnegieana</i> ICPB EC223	62	13.2	30
ICPB EC186	69	12.6	31
ATCC 13452	41	12.3	24
Average	54	12.7	28
Cornstalk-rot bacterium SR98	41	17.5	10
SR84	40	17.8	9
ICPB EC210	40	18.0	7
SR56	39	17.2	8
SR80	39	17.4	9
ICPB EM107	39	18.0	9
<i>Pectobacterium chrysanthemi</i> ATCC 11663	43	14.8	13
ICPB EC205	42	16.3	10
SR32	41	16.9	8
ATCC 11662	41	17.0	8
ICPB EC16	40	17.3	8
SR31	38	15.7	11
<i>Erwinia dieffenbachiae</i> ICPB ED103	47	16.9	13
ICPB ED102	44	18.6	11
<i>E. cytolytica</i> ICPB EC207	42	18.2	10
<i>Pectobacterium carotovorum</i> f. sp. <i>parthenii</i> ICPB EC115	38	17.6	9
Average	41	17.2	10
<i>Pectobacterium rhapontici</i> ICPB ER106	46	15.9	18
<i>P. cypripedii</i> ICPB EC155	29	19.2	5
ICPB EC160	28	18.4	5
Average	34	17.8	9
<i>Enterobacter aerogenes</i> banana ICPB 3151	28	17.4	5
CDC 1627-66	27	17.6	5
<i>Shigella flexneri</i> 2a 24570	28	17.3	5
Average	28	17.4	5
<i>Edwardsiella tarda</i> CDC 1126-64	18	17.9	2
<i>Proteus mirabilis</i> 1	11	18.4	—
Average	15	18.2	2

^a $T_{m(e)}$, Thermal elution midpoint; the temperature at which 50% of the DNA bound to hydroxyapatite (at the 60 or 75 C incubation temperature) is eluted. $\Delta T_{m(e)}$ is the decrease in $T_{m(e)}$ between heterologous DNA reactions and the homologous *P. carotovorum* ATCC 495 reaction. The average extent of reassociation of *P. carotovorum* ATCC 495 DNA was 83% at both the 60 and 75 C incubation temperatures. *P. carotovorum* DNA reassociated at 60 C had an average $T_{m(e)}$ of 91.9 C.

Table 3 shows relatedness data obtained by using reference DNA from *P. carnegieana* ICPB EC186. *P. carnegieana* strains ICPB EC186 and ICPB EC223 are essentially identical. DNA from *P. carnegieana* ATCC 13452 is only one-third related to DNA from ICPB EC186, and the related sequences contain 15% unpaired bases. At 75 C, relatedness between ICPB

EC186 and ATCC 13452 falls to 12%. To rule out errors such as mislabeling or contamination in our handling of the culture, a new culture of *P. carnegieana* ATCC 13452 was obtained from the ATCC, and its DNA was tested. Results obtained with both cultures of ATCC 13452 are similar.

Relatedness between ICPB EC186 and mem-

TABLE 3. Reassociation of DNA from *Pectobacterium carnegieana* ICPB EC186

Source of unlabeled DNA	Relative binding at 60 C (%)	$\Delta T_{m(e)}^a$ at 60 C	Relative binding at 75 C (%)
<i>Pectobacterium carnegieana</i> ICPB EC186	100		100
ICPB EC223	99	0.2	99
ATCC 13452 (old)	34	15.3	12
ATCC 13452 (new)	32	17.0	
<i>Erwinia aroideae</i> ICPB EA144	64	12.1	37
ATCC 12286	64	11.0	41
<i>E. atroseptica</i> ICPB EA112	64	11.0	41
ATCC 7403	65	11.2	38
<i>Pectobacterium carotovorum</i> ICPB EC153	62	11.2	38
ATCC 495	62	12.1	35
ATCC 25272	71	11.1	43
<i>Bacillus oleraceae</i> ICPB EO1	63	12.8	40
<i>Erwinia solanisaera</i> ICPB ES101	63	12.4	39
ATCC 8061	57	12.2	32
<i>Pectobacterium cyripedii</i> ICPB EC155	28	17.1	7
ICPB EC160	27	18.9	
Cornstalk-rot SR80	37	18.0	10
<i>Pectobacterium rhapontici</i> ICPB ER106	45	17.5	14
<i>Erwinia nigrifluens</i> ICPB EN101	49	15.0	17
<i>E. salicis</i> ICPB ES102	47	17.2	14
<i>E. rubrifaciens</i> ICPB ER105	43	16.8	13
<i>E. quercina</i> ICPB EQ101	33	16.5	
<i>E. amylovora</i> ICPB EA169	31	18.0	
ICPB 178	26	15.5	
ATCC 7400	24	18.0	9
<i>E. tracheiphila</i> ATCC 27004	21	18.3	
<i>E. herbicola</i> ICPB EH103	30	18.9	9
<i>Xanthomonas uedovorus</i> ICPB XU104	28	19.7	
<i>Erwinia dissolvens</i> ICPB ED114	38	13.8	
ICPB ED105	31	18.8	
ATCC 23373	31	19.1	
<i>E. nimipressuralis</i> ICPB EN1	33	18.9	
ATCC 9912	33	19.6	
<i>Serratia marcescens</i> SM6 W2	35	17.7	11
<i>Klebsiella pneumoniae</i> 2	34	17.6	9
<i>K. ozaenae</i>	31	18.2	
<i>Enterobacter cloacae</i> 165	32	18.2	
256-4	31	17.9	
CDC 1347-71	31	18.0	
<i>Salmonella typhimurium</i> LT2	30	17.3	10
<i>Escherichia coli</i> K-12	29	18.7	
<i>E. aerogenes</i> banana strain ICPB 3151	29	18.2	
CDC 1627-66	28	18.3	9
<i>E. hafniae</i> CDC 4360-67	28	19.2	
<i>Citrobacter freundii</i> CDC 460-61	26	18.4	

^a Same as Table 2. The average extent of reassociation of *P. carnegieana* ICPB EC186 DNA was 86% at both 60 and 75 C incubation temperatures. *P. carnegieana* ICPB EC186 DNA reassociated at 60 C had an average $T_{m(e)}$ of 92.0 C.

bers of the *P. carotovorum* group is 57 to 71%, with 11 to 12% divergence in related sequences. Most of the related sequences remain stable at 75 C (32-43% relatedness). As expected, these results are similar to those obtained in reciprocal reactions (Table 2) where *P. carotovorum* ATCC 495 was the reference organism.

Relative relatedness of *P. carnegieana* ICPB EC186 to other *Enterobacteriaceae* is significantly lower. In all cases, 14 to 20% unpaired bases are present, and only 7 to 17% relative binding occurs at 75 C. Among other pectobacteria, relatedness of ICPB EC186 is 45% to *P. rhapontici*, 37% to cornstalk-rot bacterium, and 28% to *P. cypripedii*. Relatedness of ICPB EC186 to erwiniae is 21 to 49%. *E. nigrifluens*, *E. salicis*, and *E. rubrifaciens* are the erwiniae most closely related to EC186 (43-49%). ICPB EC186 shows 30% reaction with strains of *Enterobacter agglomerans* and 25 to 38% relatedness to other *Enterobacteriaceae*.

Surprisingly, except for two organisms in the *E. agglomerans* group (*E. uredoovora* and *E. cassavae*), there are no type or neotype strains of *Erwinia* in the ATCC. To our knowledge, only *P. cypripedii* ICPB EC155 has been designated as a type strain (7). Therefore, it is possible that some of our data are based on strains that may be misidentified. We feel that this possibility is unlikely because the nature of these experiments is such that misidentified strains will be detected by lack of relatedness to validly identified strains. Furthermore, strains of *P. carotovorum* and of other designated species thought to be closely related to *P. carotovorum* (a total of 21 strains from three culture collections) exhibit a high level of DNA relatedness. Similar findings were obtained with the *P. chrysanthemi* group and cornstalk-rot organisms (Brenner and Kelman, manuscript in preparation). We are therefore confident that most of the strains used are correctly identified.

By using *P. carotovorum* ATCC 15713 as the reference strain in a previous study from this laboratory (6), relatedness of *P. carotovorum* to erwiniae and to other *Enterobacteriaceae* was significantly lower at 60 C than that observed in this study. The magnitude of the difference was mainly 10 to 15%, although with certain organisms the differences were larger and quite striking. For example, *P. carotovorum* ATCC 15713 exhibited 15% relatedness to *E. coli*, 13% relatedness to *S. typhimurium*, and 18% relatedness to both *S. marcescens* and *P. carnegieana* ATCC 13452, whereas in the present study *P. carotovorum* ATCC 495 exhibited relatedness values to these organisms of 28, 30, 36, and 41%, respectively. Despite differences in relative binding, thermal stability

of interspecies DNA duplexes was similar in both studies. *P. carotovorum* ATCC 495 is 90% related to *P. carotovorum* ATCC 15713. The 10% divergence in these strains may account for some of the quantitative binding differences. A difference in genome size could also explain these differences. For this to be true, the genome in ATCC 15713 would have to be substantially larger than that of ATCC 495.

Gardner and Kado (12) used direct DNA-DNA binding and competition experiments on nitrocellulose filters to determine relationships among erwiniae. They used formamide to decrease the T_m of DNA and to allow DNA binding to occur at lower temperatures. In many cases, their data show quantitative differences in relatedness as determined by direct binding and by competition. Where similar strains are used, their data are in good agreement with the data presented here.

All pectobacteria are significantly related to other enteric bacteria. This core reaction, where tested, is 25% or greater except with *Edwardsiella tarda* and *P. morganii* (about 20%), and *P. mirabilis* (about 10%). *E. tarda* and *P. mirabilis* exhibit the same low level of relatedness with all genera of *Enterobacteriaceae*. There is no doubt that pectobacteria should remain in the family *Enterobacteriaceae*.

Five groups of pectobacteria are evident from this study. The *P. carotovorum* group and the *P. carnegieana* group are about 65% related. All other groups show less than 50% relatedness. The *P. carotovorum* group includes *P. carotovorum*, *E. atroseptica*, *E. aroideae*, *E. solanisaepara*, and *E. oleraceae*. We cannot distinguish between these organisms. Strains of *P. carnegieana* form a second group. One strain, *P. carnegieana* ATCC 13452, is not closely related to the other *P. carnegieana* strains. A third group of pectobacteria contains cornstalk-rot bacteria, *P. chrysanthemi*, *E. dieffenbachiae*, *E. cytolytica*, and *P. carotovorum* f. sp. *parthenii*. These organisms will be discussed in detail later (Brenner and Kelman, manuscript in preparation). Strains of *P. cypripedii* are a fourth group. Finally, *P. rhapontici*, which is not closely related to any other group, is placed in a fifth group.

Table 5 presents five classifications of the soft-rot erwiniae, or pectobacteria, along with guanine plus cytosine contents of their DNAs. *Bergey's Manual of Determinative Bacteriology* (2) lists nine species of soft-rot-producing erwiniae. *E. ananas* is not a soft-rot-producing species according to Waldee (17). Dye (8) has placed *E. ananas* in *E. herbicola*, which Ewing and Fife (11) have now classified as *Enterobacter agglomerans*. Graham reported *E. citro-*

TABLE 4. Reassociation of DNA from *Pectobacterium cypripedii* ICPB EC155

Source of unlabeled DNA	Relative binding at 60 C (%)	$\Delta T_{m(e)}^a$ at 60 C
<i>Pectobacterium cypripedii</i> ICPB EC155	100	
ICPB EC160	98	+0.2
<i>Erwinia aroideae</i> ICPB EA144	24	18.9
ATCC 12286	21	18.6
<i>E. atroseptica</i> ICPB EA112	24	18.2
ATCC 7403	24	18.0
<i>Pectobacterium carnegieana</i> ICPB EC186	22	19.0
ICPB EC223	22	19.6
ATCC 13452	29	17.3
<i>P. carotovorum</i> ICPB EC153	25	18.0
ATCC 495	24	17.6
ATCC 15359	25	19.1
ATCC 25272	23	19.0
Cornstalk-rot bacterium SR80	30	18.9
<i>Bacillus oleraceae</i> ICPB EO1	24	19.0
<i>Pectobacterium rhapontici</i> ICPB ER106	24	19.5
<i>Erwinia solanisapra</i> ICPB ES101	27	18.4
ATCC 8061	21	18.1
<i>E. amylovora</i> ICPB EA169	33	17.5
ICPB EA178	31	16.2
ATCC 7400	30	17.8
<i>E. nigrifluens</i> ICPB EN101	28	17.9
<i>E. quercina</i> ICPB EQ101	23	18.2
<i>E. rubrifaciens</i> ICPB ER105	21	19.3
<i>E. salicis</i> ICPB ES102	25	17.9
<i>E. tracheiphila</i> ATCC 27004	26	17.9
<i>E. herbicola</i> ICPB EH103	42	14.3
<i>Xanthomonas uredoovorus</i> ICPB XU104	37	15.6
<i>Erwinia dissolvens</i> ICPB ED105	33	16.9
ICPB ED114	33	17.3
ATCC 23373	34	17.4
<i>E. nimipressuralis</i> ICPB EN1	29	18.2
ATCC 9912	31	17.0
<i>Citrobacter freundii</i> CDC 460-61	25	18.5
<i>Enterobacter aerogenes</i> banana strain ICPB 3151	29	18.5
<i>E. aerogenes</i> CDC 1627-66	31	18.3
<i>E. cloacae</i> yellow 165	31	17.5
CDC 1347-71	32	17.8
yellow 256-4	32	17.3
<i>E. hafniae</i> CDC 4360-67	21	19.4
<i>Escherichia coli</i> K-12	25	16.6
<i>Klebsiella ozaenae</i>	33	17.1
<i>K. pneumoniae</i> 2	33	17.8
<i>Serratia marcescens</i> SM6 W2	31	17.2
<i>Salmonella typhimurium</i> LT2	24	17.2

maculans to be identical to *E. ananas* (13). Two other organisms, *E. dissolvens* and *E. nimipressuralis*, are not considered to be *Erwinia* species by Dye (9), Graham (13), or Waldee (17). Waldee described *E. dissolvens* as a new species of *Enterobacter* (17), and Graham described *E. nimipressuralis* as resembling *Enterobacter cloacae* (13). Our data support these conclusions; both *E. dissolvens* and *E. nimipressuralis* are far more closely related to strains of *Enterobacter cloacae* (50-70%) than to any other *Enterobacteriaceae* (Brenner, unpublished data).

P. carnegieana, according to Graham (13), is not a soft-rot-producing organism, and it resembles *Enterobacter cloacae*. Our data, from two of three strains, indicate substantial relatedness between *P. carnegieana* and *P. carotovorum*. The third strain we tested is not closely related to the other strains of *P. carnegieana* or to other pectobacteria. Ewing (personal communication) showed that *P. carnegieana* strains ICPB EC186 and ICPB EC223 are pectolytic and biochemically similar to other pectobacteria. In contrast, ATCC 13452 is not pectolytic and has biochemical characteristics typical of *K. pneumoniae*. In all probability, the confusion surrounding the status of *P. carnegieana* is due to a series of strains called *P. carnegieana* which are in reality *K. pneumoniae*. Before receiving the two pectolytic strains we sent to Ewing, he found all strains labeled *P. carnegieana* to be *K. pneumoniae*. Dye (personal communication) has also encountered two types of strains labeled *P. carnegieana*.

The remaining *Erwinia* species cited in *Bergey's Manual* are less controversial. *P. carotovorum*, *E. atroseptica*, and *E. aroideae* all produce soft rot. The question is whether they merit species status. Waldee (17) lists them as separate species, but Graham (13) and Dye (7) list them as varieties of *P. carotovorum*. We cannot distinguish between these organisms and feel that they and also *E. solanisapra* and *E. oleraceae* should be included in one species. *P. chrysanthemi* is listed as a variety of *P. carotovorum* by Dye (7) and by Graham (13), although the latter states that it may merit separate species status. *P. chrysanthemi* is certainly distinct from *P. carotovorum* based on DNA relatedness.

We find *P. chrysanthemi* to be indistinguishable from *E. cytolytica*, *P. carotovorum* f. sp. *parthenii*, and *E. dieffenbachiae*, and closely related to cornstalk-rot bacteria (Brenner and

^a Same as Table 2. The average extent of reassociation of *P. cypripedii* ICPB EC155 DNA was 90%. The $T_{m(e)}$ of reassociated *P. cypripedii* ICPB EC155 DNA was 92.9 C.

TABLE 5. Comparison of taxonomic groupings for soft-rot *Erwinia* species

Species	Guanine + cytosine ^a	Grouping in					Present study
		Bergey's Manual (2)	Waldee (17)	Graham (13)	Dye (7-9)		
<i>Erwinia ananas</i> <i>E. aroideae</i>	53.1-54.1	<i>E. ananas</i>	<i>Pectobacterium aroideae</i> <i>P. atroseptica</i>	Not a soft-rot species <i>E. carotovora</i> var. <i>aroideae</i>	<i>Erwinia herbicola</i> <i>E. carotovora</i> var. <i>carotovora</i>	<i>E. agglomerans?</i>	
	51.0-53.2	<i>E. aroideae</i>				<i>P. carotovorum</i> group	
<i>E. atroseptica</i> <i>E. carnegiana</i>	51.3-53.1	<i>E. atroseptica</i>	<i>P. carotovorum</i>	<i>E. carotovora</i> var. <i>atroseptica</i> Not a soft-rot, resembles <i>E. cloacae</i>	<i>E. carotovora</i> var. <i>atroseptica</i>	<i>P. carotovorum</i> group	
	50.8-51.7	<i>E. carnegiana</i>				<i>P. carnegiana</i> group	
<i>E. carotovora</i> <i>E. chrysanthemi</i>	51.0-53.9	<i>E. carotovora</i>	<i>P. carotovorum</i>	<i>E. carotovora</i> var. <i>carotovora</i> <i>E. carotovora</i> var. <i>chrysanthemi</i> (separate species?)	<i>E. carotovora</i> var. <i>carotovora</i> <i>E. carotovora</i> var. <i>chrysanthemi</i>	<i>P. carotovorum</i> group	
	55.1-57.1	<i>E. chrysanthemi</i>				<i>P. chrysanthemi</i> group	
<i>E. citromaculans</i>		<i>E. citromaculans</i>		<i>E. ananas</i> , not a soft-rot species			
Cornstalk-rot bacterium <i>E. cypripedii</i>	53.1-54.1		Possibly <i>P. carotovorum</i> <i>P. aroideae</i>	<i>E. carotovora</i> var.	<i>E. carotovora</i> var. <i>chrysanthemi</i>	<i>P. chrysanthemi</i> or cornstalk-rot group	
	54.1-54.6					<i>E. carotovora</i> var. <i>cypripedii</i>	<i>P. cypripedii</i> group
<i>E. cytolytica</i> <i>E. dieffenbachiae</i>	56.1		<i>Enterobacter dissolvens</i>	<i>E. carotovora</i> var.	<i>E. carotovora</i> var. <i>chrysanthemi</i>	<i>P. chrysanthemi</i> group	
	55.1-56.1					Not an <i>Erwinia</i> species	<i>P. chrysanthemi</i> group
<i>E. dissolvens</i>	53.5-57.6	<i>E. dissolvens</i>				<i>Enterobacter (cloacae?)</i>	
<i>E. nimipressuralis</i>	55.1-55.3	<i>E. nimipressuralis</i>		Not a soft-rot, resembles <i>E. cloacae</i>	Not an <i>Erwinia</i> species	<i>Enterobacter (cloacae?)</i>	
<i>Bacillus oleraceae</i> <i>E. carotovora</i> f. sp. <i>parthenii</i>	50.5		<i>P. carotovorum</i>	<i>E. carotovora</i> var.	<i>E. carotovora</i> var. <i>carotovora</i> <i>E. carotovora</i> var. <i>chrysanthemi</i>	<i>P. carotovorum</i> group	
	52.6					<i>E. carotovora</i> var. <i>chrysanthemi</i>	<i>P. chrysanthemi</i> group
<i>E. rhapontici</i> <i>E. solanisapra</i>	51.0-53.2		<i>P. carotovorum</i>		<i>E. carotovora</i> var. <i>rhapontici</i> <i>E. carotovora</i> var. <i>carotovora</i>	<i>P. rhapontici</i> group	
	51.0					<i>E. carotovora</i> var. <i>carotovora</i>	<i>P. carotovorum</i> group

^a All determinations are according to Starr and Mandel (15). In addition, determinations of Gardner and Kado (12) were used for *Erwinia aroideae*, *E. carotovora*, *E. dissolvens*, *E. nimipressuralis*, and *E. rhapontici*.

Kelman, manuscript in preparation). Graham (13) and Dye (7) agree that *E. dieffenbachiae* and *P. carotovorum* f. sp. *parthenii* are synonyms for *P. chrysanthemi*. *E. cytolytica* is considered as *P. carotovorum* by Waldee (17), Dye (7), and Graham (13). Relatedness of *E. cytolytica* to *P. chrysanthemi* is supported by the higher guanine plus cytosine content present in their DNA as compared with DNA from *P. carotovorum* (15, Table 5). Dye has placed the cornstalk-rot organisms with *P. chrysanthemi* (7). *P. cypripedii* and *P. rhapontici* are not included in the current *Bergey's Manual* (2). Waldee (17) states that *P. cypripedii* is possibly similar to *P. carotovorum*. Dye (7) treats both organisms as separate varieties of *P. carotovorum*. Our data show that these organisms are distinct from each other and from other pectobacteria.

Neither *P. cypripedii* nor *P. rhapontici* produces pectinase on pectate media, and these organisms do not produce soft-rot diseases. Furthermore, neither of these organisms is significantly more related to pectobacteria than to other *Enterobacteriaceae*. Despite these facts, we recommend their inclusion in pectobacteria because they are most biochemically similar to pectobacteria (7, 17). Failure to produce pectinase and failure to cause soft-rot disease are not unique to these species. At 22 to 25 C, 25% of 90 pectobacteria (mainly *P. carotovorum*) did not produce pectinase within 48 h, and at 37 C only 42% of these strains produced pectinase within 48 h (10).

Even if one accepts the five groups of pectobacteria as shown by DNA relatedness, classification is complicated by additional considerations. One of these concerns phytopathogenicity and host range. Phytopathogenicity can be lost by simple mutation (for example, a single base mutation that affects pectinase activity). Since phytopathogenicity and host range are of critical interest to the plant pathologist, he requires a means of differentiation based on these parameters. DNA relatedness of pectobacteria to erwiniae and other *Enterobacteriaceae* is sometimes greater than relatedness between groups of pectobacteria. For instance, *P. carotovorum* shows significantly higher relatedness to *E. nigrifluens*, *E. salicis*, and *E. rubrifaciens* than to *P. cypripedii* (see Tables 2, 3 and 4 for further examples) despite the fact that the pectobacteria are biochemically much closer to each other than they are to erwiniae.

In the absence of one perfect system of taxonomy that satisfies the bacteriologist, the plant pathologist, and the evolutionist, one can

either have separate taxonomies or make certain compromises. Waldee's (17) system of two genera in the tribe *Erwinia* separates organisms by biochemistry and phytopathogenicity. Both Graham (13) and Dye (7) attack the problems by severely restricting the number of species. They recognize *E. carotovora* as the only soft-rot species. Phytopathogenicity is dealt with at the level of variety, for example, *E. carotovora* var. *carotovora*, *E. carotovora* var. *chrysanthemi*, etc.

We feel that a combination of these approaches provides a means for obtaining one taxonomy for *Erwiniae* that can be embraced by and be meaningful to all concerned. Our proposals are as follows: (i) maintain the tribe *Erwiniae* as it now stands in *Bergey's Manual* (2); (ii) reserve the genus *Erwinia* for species that produce wilts or blight diseases and whose biochemical characteristics are similar to those of *E. amylovora*, the type species; (iii) reserve the genus *Pectobacterium* for soft-rot-producing organisms and other organisms whose biochemical characteristics are similar to those of *P. carotovorum*, the type species; (iv) on the basis of morphological and biochemical characteristics and similarities in DNA, transfer *Erwinia carnegieana*, *E. chrysanthemi*, and *E. cypripedii* to the genus *Pectobacterium*; (v) place yellow-pigmented "erwiniae" in *Enterobacter agglomerans* unless DNA relatedness data taken together with biochemical characteristics show them to belong more properly in another group; (vi) require host range patterns before admitting additional organisms to either *Erwiniae* or *Pectobacterium*; (vii) use infrasubspecific terms such as "forma specialis" or "variety" to designate host range or other important traits; and (viii) remove *E. nimipresuralis* and *E. dissolvens* from *Erwinia*. These organisms are more properly classified in the genus *Enterobacter* as *E. cloacae* or in one or two additional species (9, 13, 17; Brenner, unpublished data).

Following these proposals, the genus *Pectobacterium* would presently consist of five species: *P. carotovorum*, *P. chrysanthemi*, *P. carnegieana*, *P. cypripedii*, and *P. rhapontici*. Infrasubspecific forms would be recognized as warranted.

REPRINT REQUESTS

Address requests for reprints to: Dr. Don J. Brenner, Division of Biochemistry, Walter Reed Army Institute of Research, Washington, D.C. 20012.

LITERATURE CITED

1. Berns, K. I., and C. A. Thomas. 1965. Isolation of high molecular weight DNA from *Hemophilus influenzae*. J. Mol. Biol. 11:476-490.
2. Breck, R. S., E. G. D. Murray, and N. R. Smith. 1957. Bergey's manual of determinative bacteriology, 7th Ed. The Williams & Wilkins Co., Baltimore.
3. Brenner, D. J., G. R. Fanning, K. E. Johnson, R. V. Citarella, and S. Falkow. 1969. Polynucleotide sequence relationships among members of the *Enterobacteriaceae*. J. Bacteriol. 98:637-650.
4. Brenner, D. J., G. R. Fanning, A. Rake, and K. E. Johnson. 1969. A batch procedure for thermal elution of DNA from hydroxyapatite. Anal. Biochem. 28:447-460.
5. Brenner, D. J., G. R. Fanning, F. J. Skerman, and S. Falkow. 1972. Polynucleotide sequence divergence among strains of *Escherichia coli* and closely related organisms. J. Bacteriol. 109:953-965.
6. Brenner, D. J., G. R. Fanning, and A. G. Steigerwalt. 1972. Deoxyribonucleic acid relatedness among species of *Erwinia* and between *Erwinia* species and other bacteria. J. Bacteriol. 110:12-17.
7. Dye, D. 1969. A taxonomic study of the genus *Erwinia*. II. The "Carotovora" group. N.Z.J. Sci. 12:81-97.
8. Dye, D. 1969. A taxonomic study of the genus *Erwinia*. III. The "Herbicola" group. N.Z.J. Sci. 12:223-236.
9. Dye, D. 1969. A taxonomic study of the genus *Erwinia*. IV. "Atypical" erwinias. N.Z.J. Sci. 12:833-839.
10. Edwards, P. R., and W. H. Ewing. 1972. Identification of *Enterobacteriaceae*, 3rd ed. Burgess Publishing Co., Minneapolis, Minn.
11. Ewing, W. H., and M. A. Fife. 1971. *Enterobacter agglomerans*. Publication of the Center for Disease Control, Atlanta, Ga.
12. Gardner, J. M., and C. I. Kado. 1972. Comparative base sequence homologies of the deoxyribonucleic acids of *Erwinia* species and other enterobacteria. Int. J. Syst. Bacteriol. 22:201-209.
13. Graham, D. C. 1964. Taxonomy of the soft rot coliform bacteria. Annu. Rev. Phytopathol. 2:13-43.
14. Laird, C. D., B. L. McConaughy, and B. J. McCarthy. 1969. On the rate of fixation of nucleotide substitutions in evolution. Nature (London) 224:149-154.
15. Starr, M. P., and M. Mandel. 1969. DNA base composition and taxonomy of phytopathogenic and other enterobacteria. J. Gen. Microbiol. 56:113-123.
16. Von Graevenitz, A. 1970. *Erwinia* species isolates. Ann. N.Y. Acad. Sci. 174:436-443.
17. Waldee, E. L. 1945. Comparative studies of some peritrichous phytopathogenic bacteria. Iowa State J. Sci. 19:435-484.