Numerical and DNA : DNA Reassociation Analyses of *Erwinia rubrifaciens* and Other Members of the *Enterobacteriaceae*

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(Received 29 January 1980; revised 20 March 1980)

Phenetic data on 75 strains of *Erwinia* and other representative genera of the *Enterobacteriaceae* were collected and analysed using two numerical taxonomic methods. In both methods the same subclusters were recovered. The subclusters, however, were defined at different similarity levels and were classified into clusters of different composition. *Erwinia rubrifaciens* strains formed a very tight, homogeneous subcluster, completely distinct and readily distinguishable from other *Erwinia* and enterobacteria species studied. DNA : DNA hybridizations between *E. rubrifaciens* and *Erwinia* and *Shigella* species were analysed to corroborate the numerical classifications. A good correlation between the numerical and DNA : DNA hybridization analyses was found and provided sufficient evidence for not supporting the previously proposed subspecific taxonomic position of *E. rubrifaciens*. The data clearly showed that *E. rubrifaciens* is a separate species in its own right. Based on the existence of very high genetic relatedness and high similarities in phenetic characters among *E. rubrifaciens* strains and the confinement of the pathogen to the state of California, the hypothesis is offered that *E. rubrifaciens* originated from a single source.

INTRODUCTION

A bacterium that causes deep bark canker disease of Persian walnuts (*Juglans regia* L.) was first described as *Erwinia rubrifaciens* by Wilson *et al.* (1967). Thorough biological and epidemiological studies of the disease have been reported (Schaad & Wilson, 1970*a, b*, 1971*a, b*; Schaad *et al.*, 1973; Gardner & Kado, 1973; Kado & Gardner, 1977; Kado *et al.*, 1977) but the taxonomic position of *E. rubrifaciens* remains unclear. *Erwinia rubrifaciens* was thought to share phenotypic characters with *E. amylovora* and thus was ranked as a variety of *E. amylovora* (Dye, 1968). DNA : DNA hybridization experiments did not support these findings because of the low (13 to 15 %) DNA homologies between *E. rubrifaciens* and *E. amylovora* (Gardner & Kado, 1972; Brenner *et al.*, 1972; Murata & Starr, 1974). Recently, Young *et al.* (1978*a, b*) have included *E. rubrifaciens* as a pathovar of *E. quercina* on the basis of a few physiological and biochemical tests.

The taxonomic status of *E. rubrifaciens* therefore seemed to remain in a state of flux and could only be resolved by detailed comparative studies. We have examined *E. rubrifaciens* strains from all areas where they occur naturally and in this paper we report comparative analyses of these strains with other enterobacteria.

METHODS

Bacterial strains. Some *E. rubrifaciens* strains were obtained from the laboratory culture collection; others were isolated during the summers of 1976 and 1977 from infected walnut trees located in various counties of California (Table 1) using methods previously described (Wilson *et al.*, 1967; Schaad & Wilson, 1971*b*; Kado *et al.*, 1977). The remaining strains studied are listed in Table 2.

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Table 1. *Erwinia rubrifaciens* strains studied

Strain	California county of isolation	Source*
6D365	Tehama	This paper
6D366, 6D373	Glenn	This paper
6D374, 6D375, 6D376, 6D379	Butte	This paper
6D345	Colusa	N. Schaad, UC Davis
6D369, 6D381	Yuba	This paper
6D372, 6D368	Sutter	This paper
6D360, 6D356	Sutter	N. Schaad, UC Davis
6D3, 6D34	Yolo	E. E. Wilson, UC Davis
6D330	Yolo	Lee Jackson, UC Davis
6D370	Yolo	This paper
6D364, 6D380	Solano	This paper
6D377	Contra Costa	This paper
6D361	San Joaquin	N. Schaad, UC Davis
6D327	Stanislaus	J. Gardner, UC Davis
6D331, 6D332, 6D337,		
6D339, 6D348, 6D349	Stanislaus	N. Schaad, UC Davis
6D343, 6D344	Merced	N. Schaad, UC Davis
6D363	Madera	W. Moller, UC Davis
6D367	Fresno	This paper
6D371	Tulare	This paper
6D378	Kings	This paper

* UC, University of California, U.S.A.

Culture media. All strains were cultured on medium YDC [yeast extract (Difco), 10.0 g; glucose, 20.0 g; CaCO_3 , 20.0 g; Bacto-agar (Difco), 15.0 g; distilled water, 1 l] at 30 °C for 48 h and then stored at 4 °C. Medium 925E (Langley & Kado, 1972) was used as a basal medium and medium 523 (Kado *et al.*, 1972) for mass culture. The differential media employed were medium D3 (Kado & Heskett, 1970), MacConkey agar (Difco), EMB agar (Difco) and modified EMB agar [glycerol, 10.0 ml; lactose, 10.0 g; casein enzymatic hydrolysate (Calbiochem), 10.0 g; K_2HPO_4 , 2.0 g; eosin Y, 0.4 g; methylene blue, 0.065 g; Bacto-agar, 15.0 g; distilled water, 1 l].

Test for motility. Motility was observed in hanging drops prepared from exponential phase cultures in medium 523 broth. Air-dried films of the broth cultures were stained for the presence of flagella after Blendén & Goldberg (1965).

Biochemical and physiological tests. Cytochrome oxidase (Kovacs, 1956) and catalase activity (Rhodes, 1959) were examined on cultures taken from medium 523 agar plates incubated for 24 h at 30 °C, and indole production was tested in medium 523 broth cultures after 48 h at 30 °C (Happold & Hoyle, 1934). Methyl red reaction was recorded according to the method of Barry *et al.* (1970) after 3 d growth in shake flasks of MR broth (peptone, 5.0 g; glucose, 5.0 g; K_2HPO_4 , 5.0 g; distilled water, 1 l) and glucose broth ($\text{NH}_4\text{H}_2\text{PO}_4$, 0.5 g; K_2HPO_4 , 0.5 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g; NaCl, 0.6 g; yeast extract, 1.0 g; glucose, 10.0 g; distilled water, 1 l; pH 6.8), formulated by Dye (1968). The Voges-Proskauer test was recorded after 3 d (Coblentz, 1943). Lipase production was tested after 12 d growth by incorporating Tween 80 into medium 523 agar modified by replacing K_2HPO_4 with 2.0 g $\text{CaCl}_2 \cdot \text{l}^{-1}$; opacity around the areas of growth, due to the precipitation of calcium oleate, was recorded as a positive result (Sierra, 1957). Nitrate reductase activity was detected in nitrate agar slants (glucose, 10.0 g; KNO_3 , 5.0 g; yeast extract, 1.0 g; K_2HPO_4 , 2.0 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g; Bacto-agar, 15.0 g; distilled water, 1 l) using the reagents described by Bailey & Scott (1974). Urease activity was detected after 24 and 48 h incubation (Christensen, 1946) and amino acid decarboxylases were detected in Bacto-decarboxylase broth base (Difco) and Moeller's medium B containing either 0.8 % (w/v) L-arginine, L-lysine, L-ornithine or L-glutamic acid (Moeller, 1955). The deamination of phenylalanine and tryptophan was measured after 24 h according to Ewing *et al.* (1957) and Singer & Volcani (1955) and β -galactosidase activity according to Holding & Collee (1971). Protopectinase activity was observed on washed, peeled, alcohol-flamed, potato slices (5 to 7 mm thick) that were one-quarter immersed in sterile water and their surfaces streaked with bacteria; after 2 to 4 d, complete collapse of the tissue designated a positive reaction. 2-Ketogluconate production in medium 925E salts containing 3 % (w/v) potassium gluconate was scored according to Lelliott *et al.* (1966). The presence of arginine dihydrolase was determined in Thornley's (1960) medium after 7 d incubation at 30 °C while the degradation of starch (Bailey & Scott, 1974), hippurate (Brooks & Hucker, 1944), aesculin (Dye, 1968) and gelatin (Smith *et al.*, 1952) were observed after 9, 14, 24

Table 2. *Other bacteria studied*

Species and strain	Source*
<i>Klebsiella pneumoniae</i> 3759, 3739 (<i>oxytoca</i>), 3744	ICPB
<i>Serratia marcescens</i> 2875	ICPB
<i>Serratia plymuthica</i> 3992	ICPB
<i>Serratia liquefaciens</i> 3989	ICPB
<i>Shigella dysenteriae</i> 2872	ICPB
<i>Shigella alkalescens</i> 2026	ICPB
<i>Shigella flexneri</i> 3538	ICPB
<i>Enterobacter cloacae</i> 4021	ICPB
<i>Enterobacter agglomerans</i> 3424	ICPB
<i>Enterobacter aerogenes</i> 4022	ICPB
<i>Enterobacter aerogenes</i> A-170-40	D. G. Gilchrist, UC Davis
<i>Salmonella typhimurium</i> LT-2	J. L. Ingraham, UC Davis
<i>Salmonella typhimurium</i> TA-1534, TA-1536, TA-1537, TA-1538	B. N. Ames, UC Berkeley
<i>Salmonella typhimurium</i> JL-1002	A. Warren, UC Davis
<i>Erwinia carotovora</i> B-374	P. F. Lurquin, Nuclear Centre, Mol, Belgium
<i>E. carotovora</i> 3D31	R. H. Segall, USDA, Orlando, Fla, U.S.A.
<i>E. carotovora</i> EC-201	M. N. Schroth, UC Berkeley
<i>Erwinia herbicola</i> Y-46	L. N. Gibbins, University of Guelph, Ont., Canada
<i>E. herbicola</i> Y-74	L. N. Gibbins, University of Guelph, Ont., Canada
<i>E. herbicola</i> 25D36	E. E. Wilson, UC Davis
<i>E. herbicola</i> 25D38	W. Wescott, UC Davis
<i>Erwinia amylovora</i> FB-1	M. N. Schroth, UC Berkeley
<i>E. amylovora</i> 27-3	S. V. Beer, Cornell University, N.Y., U.S.A.
<i>E. amylovora</i> EA-167, EA-178	ICPB
<i>E. amylovora</i> (NC)NYGA	H. L. Keil, USDA, Beltsville, Md, U.S.A.
<i>Erwinia salicis</i> ES-102	ICPB
<i>Erwinia nigrifluens</i> 5D34, 5D36, 5D38	E. E. Wilson, UC Davis
<i>E. nigrifluens</i> 5D312, 5D313	J. M. Gardner, UC Davis
<i>Erwinia quercina</i> UCBPP389, UCBPP400	M. N. Schroth, UC Berkeley
<i>Erwinia rhapontici</i> 8D31	E. E. Wilson, UC Davis

* ICPB, International Collection of Phytopathogenic Bacteria (M. P. Starr, curator), Davis, California, U.S.A.; UC, University of California, U.S.A.

and 31 d incubation, respectively. The formation of reducing substance from sucrose was determined after 48 h incubation at 30 °C using the medium and method described by Dye (1968).

Bacteria were stabbed into and streaked over slants of triple sugar iron agar (TSI) and then incubated at 30 °C. The reactions were interpreted 18 to 24 h later based on the scheme presented by Hajna (1945).

Reactions in litmus milk were observed daily for 28 d (Bailey & Scott, 1974) and levan production was identified by the method of Lelliott *et al.* (1966) from cultures grown at 30 °C for 5 d on nutrient agar (Difco) or on medium 523 agar containing 5 % (w/v) sucrose. The ability of strains to grow in the presence of 1 to 10 % (w/v) NaCl (at 1 % increments) and 0.1 % (w/v) KCN was recorded in medium 523 broth over 14 d. Cultures showing turbidity were re-inoculated into medium containing the same NaCl or KCN concentration as a further check on growth.

Four media were selected to demonstrate the production of H₂S: 925E plus cysteine hydrochloride (0.8 g l⁻¹), 925E plus sodium thiosulphate (0.8 g l⁻¹), 925E plus Bacto-peptone (0.8 g l⁻¹) and TSI. Cultures were incubated at 30 °C for 9 d and the H₂S produced was detected by the filter paper strip method (Bailey & Scott, 1974) or by the darkening of TSI medium (Hajna, 1945). Only the presence or absence of H₂S was scored but not more than once.

Sensitivity to antibiotics. Antibiotic-impregnated filter paper discs (7 mm diam., Difco) were placed in the centre of medium 523 agar plates that had been overlaid with 3 ml 0.7 % molten agar containing 0.5 ml portions of 24 h broth cultures. The antibiotics (Difco) used were chloramphenicol, neomycin, kanamycin, novobiocin and tetracycline (each at 5, 10 and 30 µg ml⁻¹), erythromycin (2 and 15 µg ml⁻¹), streptomycin (2 and 10 µg ml⁻¹) and penicillin G (2 and 10 units). The plates were incubated at 30 °C for 24 h before measuring the diameter of the inhibition zone around the discs.

Sensitivity to colicins. *Escherichia coli* strains W3110 (ColE1), W3110 (ColE2) and W3110 (ColE3) (from D. R. Helinski, University of California, San Diego, U.S.A.) were each inoculated in the centre of medium 925 agar plates and incubated for 48 h at 30 °C. Each culture was then exposed to short-wave u.v. radiation from a UVII lamp (Ultraviolet Products, San Gabriel, Calif., U.S.A.) held 10 to 15 cm over the culture for

20 to 25 s and incubated for 36 to 48 h at 30 °C to induce synthesis of colicin. Then 0.5 ml portions of the test bacteria were thoroughly mixed with 3 ml 0.7 % molten Bacto-agar and overlaid on the *E. coli* colicin producer. The plates were checked for a zone of growth inhibition around the *E. coli* colicin producer 24 h later.

Nutritional tests. Growth factor requirements were tested in minimal medium 925 and in medium 925 supplemented with 1.0 g yeast extract l⁻¹. Growth in the presence of yeast extract (but not in its absence) within 14 d of incubation on a rotary shaker at room temperature indicated that growth factors were necessary.

Utilization of organic acids as sources of carbon and energy was tested in a medium containing: organic acid (see below), 10.0 g; casein enzymatic hydrolysate, 2.0 g; NaCl, 5.0 g; K₂HPO₄, 0.3 g; 1 % (w/v) bromothymol blue, 3.0 ml; Bacto-agar, 10.0 g; distilled water, 1 l; adjusted to pH 6.8 before autoclaving the mixture. The organic acids used were: acetic, adipic, anthranilic, ascorbic, azelaic, benzoic, butyric, capric, caproic, caprylic, citric, deoxycholic, formic, fumaric, galacturonic, gluconic, glucuronic, glutaric, glycollic, heptanoic, itaconic, lactic, maleic, malic, malonic, mandelic, *m*-hydroxybenzoic, nicotinic, oxalic, polygalacturonic, propionic, protocatechuic, pyruvic, quinic, saccharic, salicylic, sebacic, suberic, succinic, tannic, tartaric and thioglycollic. All organic acids were used as sodium salts except gluconic acid which was used as the potassium salt. Cultures were checked daily for 3 d and a dark blue colour around the bacterial growth was considered positive. Uninoculated tubes and inoculated tubes lacking the organic acid but containing the other ingredients were used as controls. Subcultures from the positive tests to a second set of the test media were made to provide a check on false positive results attributable to possible carry-over of compounds in the initial inoculum.

Utilization of organic acids was also tested in the absence of dye, to avoid possible dye-mediated inhibition, by the following method. A medium was prepared as above but without bromothymol blue and its pH was adjusted to 6.8 after autoclaving. Inoculated plates were incubated at 30 °C and checked for growth after 7 d. Plates with all ingredients except the organic acid served as controls.

For utilization of rutin, Casamino acids and β -alanine as sources of carbon and energy, medium 925E agar with 2.0 g β -alanine l⁻¹ or 5.0 g Casamino acids or rutin l⁻¹ was used. β -Alanine and Casamino acids were filter-sterilized and added to autoclaved basal medium. Rutin was first dissolved in half the total volume of 0.1 M-NaOH, the pH was adjusted to 8.0 and an equal volume of 925 basal medium was added before autoclaving. Plates were streaked with the bacterial cultures and checked for growth 7 d after incubation at 30 °C.

To test for utilization of carbohydrates and related compounds as sources of carbon and energy, medium 925 supplemented with yeast extract (0.1 %), bromocresol purple (0.7 ml of a 1.5 % solution l⁻¹) and 0.5 % (w/v) of the following compounds was used: amygdalin, D-cellobiose, D-fructose, D-fucose, D-galactose, D-glucose, D-lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, D-melezitose, D-raffinose, D-ribose, D-sorbitol, D-sucrose, D-trehalose, D-xylose, dulcitol, aesculin, glycerol, *i*-inositol, inulin, L-arabinose, L-rhamnose, L-sorbose, salicin and starch. The medium was inoculated with the test strains and incubated at 30 °C. All tests were performed at least twice and cultures were examined for growth and acid production after 3, 7, 14 and 21 d. A second medium containing Bacto-peptone (1 %), bromocresol purple (0.7 ml of a 1.5 % solution l⁻¹) and 0.75 % (w/v) carbon source was prepared and adjusted to pH 6.8 before autoclaving. Durham tubes were also used in glucose medium to detect gas formation. Growth and acid production were recorded after 3, 7, 14 and 21 d incubation at 30 °C. In both methods, the controls consisted of uninoculated media and inoculated media without the carbon sources. At the end of all of the above experiments, culture purity was checked by streaking on YDC or medium 523 plates.

The ability of cultures to grow with amino acids as their sole carbon and nitrogen source was tested in the broth medium described by Dye (1968). Concentrated solutions of glycine and the L-amino acids alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, histidine, isoleucine, leucine, lysine, methionine, ornithine, phenylalanine, proline, serine, threonine, tryptophan and valine were filter-sterilized and added aseptically to give 0.4 % (w/v). Tubes were inoculated with a loopful of a 24 h bacterial suspension and examined for growth after 3, 7, 14 and 24 d incubation with shaking at 30 °C. Growth was further checked by transferring a loopful of 24 d culture to fresh tubes of the amino acids media. Control tubes without added amino acids were also inoculated.

Numerical analysis. To code characters, most data collected from the 140 tests performed on 75 bacterial strains (Tables 1 and 2) that existed in one of two mutually exclusive states were assigned a code of 1 for a positive character and 0 for a negative character. The remaining multistate characters, such as pigmentation or colony morphology, were divided into several mutually exclusive states and strains were coded 1 for the particular character state exhibited and 0 for the others. The binary data were entered on standard IBM punched cards. The similarity coefficient (Sneath, 1957), defined as $S_j = NS \times 100 / (NS + ND)$, where NS is the number of positive characters common to a pair of organisms and ND is the number of unshared characters, and the matching coefficient (Sokal & Michener, 1958), defined as $S_{SM} = (NS + NT)100 / (NS + ND + NT)$, where NS and ND are the same as in S_j and NT is the number of negative characters common to

both organisms, were calculated using the computer programs outlined by Quadling (1970). Sorted similarity matrices were obtained using single linkage clustering assignments (Sneath & Sokal, 1973). Data from S_r , S_{ss} and single linkage clustering were then used to prepare a sorted dendrogram. The homogeneity of the defined clusters and subclusters were checked by determining their mean intragroup and intergroup similarity values (Sneath, 1957).

DNA: DNA hybridization. Bacteria were grown in 4 l Fernbach flasks containing 200 ml medium 523 broth aerated by shaking at 225 rev. min⁻¹ for 4 d at 28 °C, harvested by centrifugation, and washed twice with glass-distilled water. For labelled DNA, cells were grown in basal medium 925 salts containing glucose (0.5 %), deoxyadenosine (200 µg ml⁻¹) and [methyl-³H]thymidine [50 µCi ml⁻¹; 60 Ci mmol⁻¹ (2.22 TBq mmol⁻¹); New England Nuclear]. Yeast extract (0.1 mg) was also added to the culture medium of *Shigella flexneri* because of its inability to grow in the absence of growth factors. Radioactively labelled cells were harvested by centrifugation during the late exponential phase (as judged by absorbance at 590 nm) and washed with 0.05 M-sodium borate/0.01 M-EDTA buffer, pH 9.0. The packed cells from both media were stored in a frozen state overnight. DNA was prepared as outlined by Gardner & Kado (1972). The final preparation of DNA was dissolved in 0.1 × SSC (SSC buffer is 0.15 M-NaCl, 0.015 M-sodium citrate, pH 7.0), dialysed exhaustively against 0.1 × SSC and diluted to approximately 50 µg ml⁻¹ with the same buffer. The purity of each DNA preparation was then checked by its absorbance spectrum, absorbance ratios at 260/280 nm and 260/230 nm and by measuring the helix to coil transition. Each preparation met the criteria standards for highly purified DNA (Gardner & Kado, 1972; Flavell *et al.*, 1974). Labelled DNA was dialysed against 0.01 M-sodium phosphate buffer, pH 6.8, collected on hydroxylapatite and washed with 0.12 M-sodium phosphate buffer. The DNA was eluted with 0.6 M-sodium phosphate buffer and the eluted DNA was dialysed exhaustively against 0.1 × SSC.

Heat-denatured, non-labelled DNA was fixed to nitrocellulose filters (Schleicher & Schüll Type B-6) as described by Drlica & Kado (1974). The amount of DNA retained on 6 mm diam. filter discs cut from 47 mm diam. filters was determined by a diphenylamine method (Burton, 1968) but modified by increasing the acetaldehyde concentration three-fold to enhance the colour development.

Filters were placed individually in small vials and pretreated with the preincubation mixture of Denhardt (1966) for 8 h at 46 °C. The filters were then resuspended in 0.4 ml 50 % formamide/5 × SSC. Labelled DNA was sheared ultrasonically at 0 °C using 30 s pulses with a Biosonik IV sonicator (Bronwill Co., Rochester, N.Y., U.S.A.) to fragments 500 to 600 nucleotides long (Studier, 1965). Sheared, heat-denatured, labelled DNA (1 to 2.5 µg) was then added to the above solution containing a filter and the mixture was incubated at 46 °C with reciprocal shaking as described elsewhere (McConaughy *et al.*, 1969). Each sample was in duplicate. Incubation, washing and isotope counting of the filters were done according to the procedures of Gardner & Kado (1972). After counting, the filters were removed from the vials, placed in Kahn tubes to dry and the amount of DNA on each filter was determined by the diphenylamine method (Burton, 1968). Counts were then normalized per µg DNA and percentage DNA sequence homologies were calculated. For competitive hybridization, the reaction volume was increased to 0.6 ml, and 0.15 ml of sheared, heat-denatured, unlabelled competitor DNA (total 150 µg) was added to the reaction mixture. Percentage competition was calculated by the equation of Gardner & Kado (1972).

The melting curve of DNA was determined spectrophotometrically using a Beckman 5230 recording spectrophotometer equipped with specially designed thermal cuvette holders regulated by a temperature programmer (Gardner & Kado, 1972). The absorbance and temperature were simultaneously recorded on a Honeywell X-Y recorder. The mol % guanine plus cytosine (GC) of the DNA was calculated from T_m values using the equation of Marmur & Doty (1962).

RESULTS

In the similarity coefficient (S_r) analysis, the 75 test strains were classified into three clusters – A, B and C – defined at the 46 to 55 % similarity level (S-level, Fig. 1). Cluster A, which accommodated all *erwiniae* except *Erwinia herbicola* and *E. carotovora* strains, was further divided at the 58 to 63 % S-level into subclusters a, b, c, and e and d. Cluster B was somewhat more heterogeneous than cluster A and contained subclusters f, g, h, i and j. *Shigella dysenteriae* and *Shigella flexneri*, which align at the 46 % S-level, comprised cluster C. The mean intragroup similarity of each cluster and subcluster and the corresponding intergroup similarities are shown in Table 3. The three clusters were well separated and the mean intragroup similarities were high.

Fig. 1. Dendrogram showing the relationship between clusters and subclusters based on the S_7 coefficient and single linkage clustering.

Table 3. Mean inter- and intragroup similarities (%) for the defined clusters and subclusters in the similarity coefficient (S_J) analysis

					Cluster A	91.58																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																																								</
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Table 4. Mean inter- and intragroup similarities (%) for the defined clusters and subclusters in the matching coefficient (S_{SM}) analysis

		Cluster A		97.20																	
		B		65.93		82.15															
		C		59.20		67.70		88.15													
		D		71.73		59.90		64.06		100.00											
				A		B		C		D											
Subcluster	a	98.27																			
	b	83.38	91.43																		
	c	79.81	86.43	100.00																	
	d	82.29	80.72	82.62		94.76															
	e	81.57	82.57	74.71		76.19		92.11													
	f	79.07	78.57	73.22		74.40		77.64		83.57											
	g	63.85	60.00	54.86		66.00		60.26		58.93		94.51									
	h	55.30	51.67	50.12		58.93		51.21		47.62		76.52		90.11							
	i	62.19	58.00	56.29		65.38		58.20		55.65		76.54		79.36		78.97					
			a	b	c	d	e	f	g	h	i										

Thirty-five strains of *E. rubrifaciens*, sharing very high similarity with one another, were recovered at the 95 % S-level in subcluster a (Fig. 1). Subcluster b, defined at the 83 to 91 % S-level, was composed of *E. nigrifluens* strains and *E. salicis*. Subcluster c, containing strains of *E. quercina*, shared relatively high similarity with subcluster d (Table 3) which accommodated the *E. rhapontici* strain. *Erwinia amylovora* strains were recovered in subcluster e, defined at a 65 to 77 % S-level. *Erwinia herbicola* strains and *Enterobacter agglomerans* comprised a relatively tight subcluster, f, defined at the 90 to 93 % S-level. *Klebsiella pneumoniae* 3759 and 3739, *Serratia plymuthica*, *Serratia liquefaciens* and *Enterobacter aerogenes* were classified in subcluster g, while *Klebsiella pneumoniae* 3744, *Serratia marcescens*, *Enterobacter cloacae* and *E. carotovora* formed subcluster h which was defined at the 72 to 81 % S-level. High intergroup similarities were obtained for subclusters f, g and h (Table 3). The strains in subcluster i, defined at the 67 to 84 % S-level, formed a relatively loose group which included *Shigella alkalescens* 2026 and all *Salmonella typhimurium* strains. *Erwinia carotovora* EC-201, sharing little similarity with the other two *E. carotovora* strains in subcluster h, comprised subcluster j.

Examination of the data by the matching coefficient (S_{SM}) produced the same subclusters defined at higher S-levels (Fig. 2). The grouping of the subclusters into clusters, however, was different from that found in the S_J analysis. In this instance, the strains fell into four clusters – A, B, C and D – at the 77 to 80 % S-level. *Shigella dysenteriae* 2872 and *Shigella*

Fig. 2. Dendrogram showing the relationship between clusters and subclusters based on the S_{SM} coefficient and single linkage clustering.

Table 5. Percentage GC values for DNA preparations and DNA homologies (%) between *Erwinia rubrifaciens* strains 6D34, 6D332, 6D337, 6D371 and 6D379 and some members of the family Enterobacteriaceae

Source of unlabelled DNA	Mol % GC	6D34		6D332		6D337		6D371		6D379	
		D*	C*	D	C	D	C	D	C	D	C
<i>Erwinia rubrifaciens</i> 6D34	52.2	100.0	100.0	98.3	92.8	108.0	110.2	101.4	96.8	106.7	97.1
<i>E. rubrifaciens</i> 6D332	51.6	94.8	92.8	100.0	100.0	102.0	92.8	107.5	100.3	96.7	88.2
<i>E. rubrifaciens</i> 6D337	52.1	118.9	110.3	106.3	95.2	100.0	100.0	94.0	106.0	97.6	89.5
<i>E. rubrifaciens</i> 6D371	51.1	104.1	91.4	112.8	89.2	93.7	110.3	100.0	100.0	107.1	98.3
<i>E. rubrifaciens</i> 6D379	52.6	116.0	90.7	94.1	93.5	93.3	100.3	100.6	92.8	100.0	100.0
<i>Erwinia nigrifluens</i> 5D36	56.4	31.9	38.6	30.3	29.2	48.7	43.8	27.9	33.5	35.7	30.7
<i>E. nigrifluens</i> 5D38	56.2	31.1	36.8	37.9	32.1	50.5	44.2	30.9	33.5	39.8	36.5
<i>E. nigrifluens</i> 5D312	56.7	33.1	29.4	42.5	35.7	45.9	37.1	33.2	28.9	35.0	30.9
<i>Erwinia salicis</i> ES-102	51.7	44.9	46.5	50.0	53.6	48.3	53.0	37.7	47.5	39.3	50.0
<i>Erwinia quercina</i> UCBPP389	55.3	20.6	18.7	24.4	16.9	35.5	22.1	27.8	19.4	33.4	20.6
<i>E. quercina</i> UCBPP400	54.8	23.4	19.5	21.2	18.6	22.5	21.2	20.4	15.2	25.5	17.6
<i>Erwinia amylovora</i> FB-1	55.1	13.3	19.7	18.2	13.2	15.6	12.0	15.0	9.7	14.2	11.5
<i>E. amylovora</i> 27-3	54.9	12.9	16.0	13.5	11.7	13.3	11.3	13.6	9.3	14.9	9.4
<i>E. amylovora</i> EA-167	54.3	13.5	10.6	14.2	8.5	14.5	9.6	12.4	7.5	13.5	9.0
<i>Shigella dysenteriae</i> 2872	53.2	7.8	10.2	10.8	10.6	11.2	13.3	8.5	7.1	9.1	12.6
<i>Shigella alkalescens</i> 2026	54.5	10.4	10.0	10.5	8.6	10.4	9.6	10.7	8.1	10.4	10.0
<i>Shigella flexneri</i> 3538	53.3	8.5	11.2	11.6	8.3	10.3	12.0	11.0	7.9	8.8	7.4

Results are the average of two replicates.

* D, Direct hybridization; C, competitive hybridization.

flexneri 3538 were included in cluster A because of their relatively high similarities with the members of that cluster, while *Salmonella typhimurium* strains and *Shigella alkalescens* 2026 were recovered together distinctly enough to warrant the rank of a cluster (cluster B). *Erwinia carotovora* EC-201 was assigned to a new group, cluster D.

The mean inter- and intragroup similarities of the clusters and subclusters in the matching coefficient (S_{SM}) analysis are presented in Table 4.

DNA: DNA hybridization

The mol % GC values of the DNA (Table 5) indicated that a fairly wide range of values (from 51 to 57 %) exists within the *Erwinia* strains tested.

The direct hybridization data (Table 5) showed that the five randomly selected strains of *E. rubrifaciens* were extremely closely related. The relative homologies between *E. rubrifaciens* strains and other test organisms was under 50 % in all cases. *Erwinia rubrifaciens* was most closely related to *E. salicis*. These organisms exhibited 44 % average relatedness. *Erwinia nigrifluens* was somewhat less related to *E. rubrifaciens* (37 % average binding) and *E. quercina* ranked third in relatedness to *E. rubrifaciens* (26 % average binding). The average degree of relatedness between *E. rubrifaciens* and *E. amylovora*, *Shigella dysenteriae* 2872, *Shigella alkalescens* 2026 and *Shigella flexneri* 3538 was about 11 %.

Relatedness patterns similar to that of *E. rubrifaciens* were obtained in cross hybridization using reference DNA from *E. nigrifluens* 5D38 and *Shigella flexneri* 3538 (Table 6). The three *E. nigrifluens* strains used in this study were indistinguishable. *Shigella flexneri* 3538 and *Shigella dysenteriae* 2872 were highly interrelated (85 %); the relatedness between *Shigella flexneri* 3538 and *Shigella alkalescens* 2026 was lower (about 55 %).

Results of competitive hybridization experiments agreed with those of direct hybridization and percentage homologies fell in the same range as those of direct hybridization (Tables 5 and 6). Percentage homologies between *E. rubrifaciens* and *E. nigrifluens* strains were almost the same as those from direct hybridization. Percentage homologies between *E.*

Table 6. DNA homologies (%) between *Erwinia nigrifluens* 5D38 and *Shigella flexneri* 3538 and some members of the family Enterobacteriaceae

Source of unlabelled DNA	<i>E. nigrifluens</i> 5D38		<i>S. flexneri</i> 3538	
	D*	C*	D	C
<i>Erwinia rubrifaciens</i> 6D34	33.3	35.0	10.5	9.2
<i>E. rubrifaciens</i> 6D332	33.8	34.6	9.2	11.2
<i>E. rubrifaciens</i> 6D337	47.9	37.7	11.0	11.4
<i>E. rubrifaciens</i> 6D371	27.2	27.3	9.3	9.4
<i>E. rubrifaciens</i> 6D379	41.3	40.2	6.5	12.9
<i>Erwinia nigrifluens</i> 5D36	92.0	108.1	6.8	14.8
<i>E. nigrifluens</i> 5D38	100.0	100.0	6.9	12.9
<i>E. nigrifluens</i> 5D312	103.3	92.7	6.9	12.7
<i>Erwinia salicis</i> ES-102	27.0	38.5	5.8	11.1
<i>Erwinia quercina</i> UCBPP389	18.1	25.2	7.6	10.4
<i>E. quercina</i> UCBPP400	13.1	22.2	4.9	12.9
<i>Erwinia amylovora</i> FB-1	14.9	14.5	13.3	10.2
<i>E. amylovora</i> 27-3	15.9	13.4	7.6	13.5
<i>E. amylovora</i> EA-167	16.0	10.2	6.9	14.6
<i>Shigella dysenteriae</i> 2872	7.0	10.5	84.9	72.9
<i>Shigella alkalescens</i> 2026	5.7	9.8	54.4	53.8
<i>Shigella flexneri</i> 3538	7.5	10.1	100.0	100.0

Results are the average of two replicates.

* D, Direct hybridization; C, competitive hybridization.

rubrifaciens strains and *E. salicis* ES-102 increased, whereas those between *E. rubrifaciens* strains and *E. quercina* strains decreased in competitive hybridization (Tables 5 and 6).

DISCUSSION

Erwinia rubrifaciens strains formed a very tight, distinct subcluster (Figs 1 and 2) with a mean intragroup similarity of 94.5 and 98.3 % in similarity coefficient and matching coefficient analyses, respectively (Tables 3 and 4). The data do not support an earlier proposal to reclassify erwiniae within existing taxa in the Enterobacteriaceae because the erwiniae separated into subclusters quite distinct from those containing other enterobacteria (Moustardier *et al.*, 1961). All erwiniae have been placed into three arbitrary groups 'amylovora', 'carotovora' and 'herbicola' based primarily on their pathogenic traits (Dye, 1968, 1969*a*, *b*, *c*; Lelliott, 1974). Numerical taxonomic studies (Focht & Lockhart, 1965; Krieg & Lockhart, 1966; Komagata *et al.*, 1968; Goodfellow *et al.*, 1976) and DNA pairing experiments (Brenner *et al.*, 1972; Gardner & Kado, 1972; Brenner, 1973; Brenner *et al.*, 1973, 1974) have shown that these groups are distinct from one another and from other enteric taxa, but the rationale behind further grouping many erwiniae under arbitrarily selected species is not supported by these studies, which, in fact, show that erwiniae comprise a very heterogeneous group easily separable by present analytical systems. Thus, the earlier proposal that *E. rubrifaciens*, *E. nigrifluens*, *E. salicis* and *E. quercina* should be considered as varieties of *E. amylovora* (Dye, 1968) is not supported by more recent DNA:DNA hybridization studies (Gardner & Kado, 1972; Brenner *et al.*, 1972; Murata & Starr, 1974) and the present numerical taximetric analyses (Tables 3 and 4). The recovery of these species as separate subclusters is also at variance with the recent proposal of Young *et al.* (1978*b*), who view *E. rubrifaciens* as a pathovar of *E. quercina*. In fact, the mean intergroup similarities (Tables 3 and 4) show that *E. quercina*, *E. nigrifluens* and *E. salicis* are almost equally related to *E. rubrifaciens*. If, for example, bacteria in subclusters b, f, g, h and i (Fig. 1) with much higher intragroup similarities are considered separate species, then it is clear that *E. quercina* and *E. rubrifaciens* with much lower similarities each deserve species status.

It is well-known that test errors can distort similarity coefficients (Sneath & Johnson, 1972). The degree of confidence that can be placed in a classification is a reflection of the congruence found between the different types of information (Goodfellow & Alderson, 1977; Goodfellow *et al.*, 1978). The recovery of well-defined and homogeneous clusters and subclusters in the present study indicates that the test error has been within acceptable limits.

DNA:DNA reassociation analyses clearly demonstrate excellent agreement between nucleotide sequence homology and numerical phenetic data (e.g. Gross & Wayne, 1970; Sneath, 1972; Staley & Colwell, 1973; Colwell *et al.*, 1974; Mordarski *et al.*, 1976). The present results fully confirm this observation for plant pathogenic erwiniae. Representative strains of the species recovered in the cluster A (Fig. 2) were randomly selected and used in the DNA:DNA hybridization experiments. A fairly wide range of mol % GC values (from 51 to 57 %) exists among the bacteria tested. No distinct relationships between GC values and DNA homology data were found.

Erwinia rubrifaciens strains possess almost identical genomes as judged by extremely high DNA homologies (Table 5). This correlates well with the grouping of *E. rubrifaciens* strains in a very tight, distinct, subcluster in the phenetic numerical analyses (Figs 1 and 2).

Gardner & Kado (1972), Brenner *et al.* (1972) and Murata & Starr (1974) found that in the white, non-pectolytic phytopathogen group, *E. rubrifaciens*, *E. salicis* and *E. nigrifluens* formed a rather loose cluster (the *rubrifaciens-salicis* cluster), whereas *E. quercina* and *E. amylovora* were apparently independent of the *rubrifaciens-salicis* cluster and of each other. In the present studies, the homologies shown by strains of *E. salicis* and *E. nigrifluens* with *E. rubrifaciens* and those shown by *E. rubrifaciens* and *E. salicis* with *E. nigrifluens* confirm and extend the earlier findings. As expected, higher DNA homologies were observed between *E. rubrifaciens* and *E. quercina* than between *E. rubrifaciens* and *E. amylovora* (Table 5). It should be stressed that the *E. amylovora* strains and the two *Shigella* species seem to share about the same level of core relatedness (around 10 %) with *E. rubrifaciens* in the present study as they do with the other members of the *Enterobacteriaceae* (Brenner *et al.*, 1972; Gardner & Kado, 1972; Murata & Starr, 1974).

The higher DNA homology between *E. rubrifaciens* and *E. salicis*, a pathogen of willow, than between *E. rubrifaciens* and *E. nigrifluens*, both pathogens of the English walnut, suggests no co-evolutionary selection between the pathogen and its host, otherwise *E. salicis* would also be a pathogen of walnut. Furthermore, *E. rubrifaciens* strains are invariant, irrespective of where they originated, indicating that they may have come from a single source rather than multiple sources long ago.

The authors thank James E. DeVay, W. Harley English and John M. Gardner for helpful discussions during the preparation of this manuscript. Special thanks are due to Frank Schick and Jeff Hall for technical assistance.

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