Comparison of Adansonian Analysis and Deoxyribonucleic Acid Hybridization Results in the Taxonomy of *Yersinia enterocolitica*

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A total of 190 strains of *Yersinia enterocolitica* from environmental sources, humans, and other animals were scored on biochemical, nutritional, and resistance tests. These were run in duplicate at 30 and 35°C, and the results were analyzed by numerical taxonomy. The computer-generated phenograms derived from simple matching coefficients and unweighted average linkage analysis delineated seven phenons from the 30°C data and five phenons from the 35°C data at a similarity value of 85%. A centrostrain was derived from each cluster in both matrixes, and these centrostrains were used as reference strains for subsequent deoxyribonucleic acid hybridizations. The relative binding ratios of the centrostrains to one another, as well as to other organisms of their own phenons, were determined. The genetic relationships as denoted by relative binding ratios were compared with the phenetic relationships developed by numerical taxonomy. Our results demonstrate that taxonomic relationships generated by numerical taxonomy do not necessarily correlate highly with those derived by deoxyribonucleic acid hybridizations in either a linear or a numerical sense (correlation coefficients, 0.48 and 0.40; mean correlation ratio, 0.60). In addition, it was found that numerical taxonomies may show considerable fluctuations in phenomic composition, which are dependent on the temperature of incubation. Deoxyribonucleic acid hybridizations among the various strains of this study divided the species *Y. enterocolitica* into at least four deoxyribonucleic acid relatedness groups. The level of intergroup homology was high enough (relative binding ratio, >20%) to justify the inclusion of all groups as species in the genus *Yersinia*.

The majority of bacterial taxonomists presently base their classification schemes on one of two techniques: numerical taxonomy and deoxyribonucleic acid (DNA) hybridizations. Each procedure has its ardent adherents. Only rarely have both procedures been used together, and, to our knowledge, there has never been a comparative evaluation of the relative correlation of the two techniques. Since the taxonomy of *Yersinia enterocolitica* had not been resolved and since we had isolated many environmental strains (10) whose relationship to this species was unclear, the opportunity to do several inter-related taxonomic studies presented itself.

Thus, a tripartite study was initiated, using a heterogeneous collection of organisms, all of which had been identified as members of *Y. enterocolitica*. The primary objective was to determine what correlation, if any, exists between phenetic analysis and genetic analysis when each is applied to the same group of organisms for the purpose of developing taxonomic relationships. A necessary prerequisite to this project (and a discrete study in itself) was the development of two numerical taxonomies, one

with tests incubated at 35°C and one with tests incubated at 30°C. This 5°C difference in incubation temperature served two purposes: (i) to provide a lower (and thus perhaps a more nearly optimum) temperature of incubation for *Y. enterocolitica* than 35°C (reports in the literature [14, 21] suggest that temperatures from 22 to 30°C are more conducive to growth of *Y. enterocolitica* than are higher temperatures), and (ii) to assess the intrinsic error in a taxonomic system which relies on the expression of phenotypic characteristics which may be affected by even a slight change in incubation temperature. The third objective was to determine the genetic relationships of the environmental strains of *Y. enterocolitica* to each other and to strains from humans and from other animals in order to render an opinion as to their species status.

MATERIALS AND METHODS

Numerical taxonomy. (i) Bacterial strains. A total of 190 strains of *Y. enterocolitica* and *Y. enterocolitica*-like organisms and 5 non-*Yersinia* control organisms was used in the study. In addition to our own 66 isolates identified as strains of *Y. enterocolitica*

(62 from environmental sources, 3 from rodents, and 1 from a human), 124 strains were contributed by a variety of individuals. This collection encompassed isolates from humans, other animals, and environmental sources and included all presently classified biovars. The five non-*Yersinia* control organisms were *Pseudomonas fluorescens* Ps 19, *Enterobacter agglomerans* Ea 42, *Citrobacter diversus* Y-U, and two strains of unidentified oxidase-negative water organisms. In addition to these non-*Yersinia* control organisms, three pairs of *Y. enterocolitica* strains were included as intrinsic controls. One pair was a duplicate subculture of a single colony, whereas one member of each of the other pairs was a lactose-positive variant of an original lactose-negative strain. The designations and sources of all of the strains studied are listed in Table 1. To promote objectivity during scoring of the results, each strain was handled unnamed under a serial number (throughout this paper strain numbers refer to these serial numbers).

During the study, all stock strains were maintained on Kligler iron agar (Difco Laboratories). The stock organisms were subcultured every 4 to 5 months and were stored at 4°C.

For all test medium inoculations with the exception of single-carbon-source replica plates, working cultures were 18- to 24-h-old heart infusion broth (Difco) cultures incubated at the appropriate test temperature. One standard loopful of this broth culture was used as inoculum. Replica plate inoculation was done with three 72-grid templates prepared on heart infusion agar (Difco) incubated for 18 to 24 h at the appropriate test temperature.

(ii) **Media.** All media were used within 5 days of preparation. Every batch of medium was made in a volume sufficient for testing the 195 organisms. The standard procedure was to inoculate all 195 organisms onto two to four sets of different test media within 1 day. Because of the uniform inoculation of the whole group of organisms onto a single batch of medium, any error inherent in doing the tests piecemeal was avoided.

(iii) **Growth tests.** The ability of the organisms to utilize single carbon sources for growth was tested on a basal medium containing 1.0 g of NH_4Cl , 2.14 g of $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$, 1.09 g of KH_2PO_4 , 0.2 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 10 ml of trace salts solution, 15.0 g of agar, and 1 liter of distilled water. The trace salts solution contained 300 mg of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 180 mg of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 130 mg of $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$, 1,000 mg of CaCl_2 , and 1 liter of 0.1 N HCl. The trace salts solution was added to the base before autoclaving, the medium was cooled, and a presterilized carbon source (final concentration, 0.1%, wt/vol) was added. Growth was recorded at 24 h and 7 days. Plates without carbon source served as controls for background growth. Heart infusion agar plates were included to check bacterial viability after the last plate in a series was stamped. No more than four test plates plus controls were included in any run. The carbon sources tested were acetate, benzoate, butyrate, glycerate, lactate, malate, malonate, nicotinate, phenylacetate, propionate, pyruvate, succinate, tartrate, valerate, adonitol, amygdalin, arabinose, *d*-arabitol, *l*-arabitol, cellobiose, erythritol, galactose, *myo*-inositol, lactose, maltose, man-

nose, melezitose, melibiose, raffinose, rhamnose, salicin, sorbose, sorbitol, trehalose, xylose, alanine, β -alanine, glutamate, glycine, histidine, hydroxy-L-proline, ornithine, serine, benzylamine, glucosamine, hippurate, histamine, putrescine, sarcosine, betaine, 1,3-butanediol, creatine, ethanol, galacturonate, geraniol, glycerol, pectin, and trigonelline.

The ability of the organisms to grow in heart infusion broth at pH 4.0, 4.5, 5.0, 6.0, 8.0, and 9.0 was recorded at 24 h and 7 days.

Growth in the presence of NaCl incorporated in nutrient broth (Difco) at concentrations of 3, 4, 5, 6, 6.5, 7, and 7.5% (wt/vol) was tested. Bacteria were also inoculated into nutrient broth alone as a control. Growth was recorded at 24 h and 7 days.

Growth of the organisms on various selective media was tested. The following dehydrated selective media were prepared according to the directions of the manufacturers: MacConkey agar (Difco), deoxycholate citrate agar (Oxoid), Hektoen agar (BBL Microbiology Systems), xylose lysine Desoxycholate agar (BBL), salmonella-shigella agar (BBL), brilliant green agar (Difco), mannitol salt agar (Difco), and cetrimide agar (BBL). Triphenyl tetrazolium chloride at 0.001 and 0.01% (wt/vol) was incorporated in heart infusion agar before autoclaving, as were 0.05% (wt/vol) sodium azide and 1.0 and 1.5% (wt/vol) sodium deoxycholate. Growth was recorded at 24 h and 7 days.

The ability of the organisms to grow at 42°C in heart infusion broth was determined visually after incubation in a water bath. Readings were made every 24 h for 7 days.

(iv) **Production of acid from carbohydrates.** A basal medium composed of 1.0% (wt/vol) Casitone (Difco), 0.001% (wt/vol) bromocresol purple, and distilled water was dispensed in 3-ml amounts into test tubes (13 by 100 mm) containing Durham vials. After autoclaving, the medium was cooled, and 0.15 ml of the appropriate prefiltered carbohydrate (20%, wt/vol) was aseptically added to each tube. Tubes were scored for acid and gas formation at 24 h and 4 days. The carbohydrates tested were adonitol, amygdalin, *d*-arabinose, *l*-arabinose, arbutin, cellobiose, dulcitol, galactose, glucose, fructose, inositol, inulin, lactose, maltose, mannitol, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose.

(v) **Miscellaneous tests.** Urease activity was detected in Christensen medium (4). Alkalinization of the medium was noted at 24-h intervals for 4 days. Hydrolysis of esculin was observed as a blackening of bile esculin medium (Difco). The medium was prepared and inoculated as described in the directions of the manufacturer, except that serum was omitted.

The tartrate medium of Jordan and Harmon (13) was used for the detection of acid production from tartrate. Any definite yellow color in the medium was scored as a positive result.

Heart infusion agar supplemented after autoclaving with 5% sterile human or sheep blood served as the substrate for the observations of hemolytic activity. The presence or absence of hemolysis was noted only around single colonies.

For the detection of deoxyribonuclease activity, dehydrated medium (Difco) was prepared according to the directions of the manufacturer. Development of a

TABLE 1. *List of strains used in this study*

Serial no.	Received as:		Biovar ^a		Source ^b	Habitat
	Species	Strain designation	Rhamnose	Sucrose		
1	<i>Y. enterocolitica</i>	Ye 1	+	+	1	Environmental
2	<i>Y. enterocolitica</i>	Ye 2	+	—	1	Environmental
3	<i>Y. enterocolitica</i>	Ye 3	—	—	1	Environmental
4	<i>Y. enterocolitica</i>	Ye 4	+	+	1	Environmental
5	<i>Y. enterocolitica</i>	Ye 5	+	+	1	Environmental
6	<i>Y. enterocolitica</i>	Ye 6	+	+	1	Environmental
7	<i>Y. enterocolitica</i>	Ye 7	+	+	1	Environmental
8	<i>Y. enterocolitica</i>	Ye 8	+	+	1	Environmental
9	<i>Y. enterocolitica</i>	Ye 9	+	+	1	Environmental
10	<i>Y. enterocolitica</i>	Ye 10	+	+	1	Environmental
11	<i>Y. enterocolitica</i>	Ye 11	—	+	1	Environmental
12	<i>Y. enterocolitica</i>	Ye 12	+	—	1	Environmental
13	<i>Y. enterocolitica</i>	Ye 13	+	+	1	Environmental
14	<i>Y. enterocolitica</i>	Ye 14	+	+	1	Non-environmental
15	<i>Y. enterocolitica</i>	Ye 15	+	—	1	Environmental
16	<i>Y. enterocolitica</i>	Ye 16	+	+	1	Non-environmental
17	<i>Y. enterocolitica</i>	Ye 17	+	+	1	Environmental
18	<i>Y. enterocolitica</i>	Ye 18	+	+	1	Environmental
19	<i>Y. enterocolitica</i>	Ye 19	+	+	1	Environmental
20	<i>Y. enterocolitica</i>	Ye 20	+	+	1	Environmental
21	<i>Y. enterocolitica</i>	Ye 21	—	—	1	Environmental
22	<i>Y. enterocolitica</i>	Ye 22	+	+	1	Environmental
23	<i>Y. enterocolitica</i>	Ye 23	+	+	1	Environmental
24	<i>Y. enterocolitica</i>	Ye 24	—	+	1	Environmental
25	<i>Y. enterocolitica</i>	Ye 25	+	+	1	Environmental
26	<i>Y. enterocolitica</i>	Ye 26	+	+	1	Environmental
27	<i>Y. enterocolitica</i>	Ye 27	+	+	1	Environmental
28	<i>Y. enterocolitica</i>	Ye 28	+	+	1	Environmental
29	<i>Y. enterocolitica</i>	Ye 29	+	—	1	Environmental
30	<i>Y. enterocolitica</i>	Ye 30	+	—	1	Environmental
31	<i>Y. enterocolitica</i>	Ye 31	+	+	1	Environmental
32	<i>Y. enterocolitica</i>	Ye 32	—	+	1	Environmental
33	<i>Y. enterocolitica</i>	Ye 33	+	—	1	Environmental
34	<i>Y. enterocolitica</i>	Ye 34	+	+	1	Environmental
35	<i>Y. enterocolitica</i>	Ye 35	—	+	1	Environmental
36	<i>Y. enterocolitica</i>	Ye 36	+	+	1	Environmental
37	<i>Y. enterocolitica</i>	Ye 37	+	+	1	Environmental
38	<i>Y. enterocolitica</i>	Ye 38	+	+	1	Environmental
39	<i>Y. enterocolitica</i>	Ye 39	+	+	1	Environmental
40	<i>Y. enterocolitica</i>	Ye 40	+	+	1	Environmental
41	<i>Y. enterocolitica</i>	Ye 41	+	+	1	Environmental
42	<i>E. agglomerans</i>	Ye 42	—	—	1	Environmental
43	<i>Y. enterocolitica</i>	Ye 43	+	+	1	Environmental
44	<i>Y. enterocolitica</i>	Ye 44	+	+	1	Environmental
45	<i>Y. enterocolitica</i>	Ye 45	+	+	1	Environmental
46	<i>Y. enterocolitica</i>	Ye 46	+	—	1	Environmental
47	<i>Y. enterocolitica</i>	Ye 20	+	+	1	Environmental
48	<i>Y. enterocolitica</i>	Ye 48	+	—	1	Environmental
49	<i>Y. enterocolitica</i>	Ye 49	+	+	1	Environmental
50	?(Water isolate)	H-3	—	+	1	Environmental
51	?(Water isolate)	35-2	+	+	1	Environmental
52	<i>P. fluorescens</i>	Ps 19	—	—	1	Environmental
53	<i>Y. enterocolitica</i>	74-457	—	+	2	Non-environmental
54	<i>Y. enterocolitica</i>	74-458	—	+	2	Non-environmental

^a Rhamnose and sucrose reactions, 4 days, 30°C.^b Sources: 1, Original isolate, this laboratory; 2, Analytab Products, Inc., Plainview, N.Y.; 3, M. Bissett, Department of Health, Berkeley, Calif.; 4, B. Chester, Veterans Administration Hospital, Miami, Fla.; 5, M. J. Pickett, Microbiology Department, University of California, Los Angeles; 6, S. Hsu, Department of Public Health, Lansing, Mich.

TABLE 1—continued

Serial no.	Received as:		Biovar ^a		Source ^b	Habitat
	Species	Strain designation	Rhamnose	Sucrose		
55	<i>Y. enterocolitica</i>	74-459	—	+	2	Non-environmental
56	<i>Y. enterocolitica</i>	74-461	—	—	2	Non-environmental
57	<i>Y. enterocolitica</i>	74-463	+	+	2	Non-environmental
58	<i>Y. enterocolitica</i>	74-593	—	+	2	Non-environmental
59	<i>Y. enterocolitica</i>	74-702	—	+	2	Non-environmental
60	<i>Y. enterocolitica</i>	74-780	—	+	2	Non-environmental
61	<i>Y. enterocolitica</i>	74-939	—	+	2	Non-environmental
62	<i>Y. enterocolitica</i>	74-940	—	+	2	Non-environmental
63	<i>Y. enterocolitica</i>	74-941	—	+	2	Non-environmental
64	<i>Y. enterocolitica</i>	74-942	—	+	2	Non-environmental
65	<i>Y. enterocolitica</i>	74-1875	—	+	2	Non-environmental
66	<i>Y. enterocolitica</i>	74-984	—	+	2	Non-environmental
67	<i>Y. enterocolitica</i>	74-983	—	+	2	Non-environmental
68	<i>Y. enterocolitica</i>	74-982	—	+	2	Non-environmental
69	<i>Y. enterocolitica</i>	74-981	—	+	2	Non-environmental
70	<i>Y. enterocolitica</i>	74-979	—	+	2	Non-environmental
71	<i>Y. enterocolitica</i>	74-978	—	+	2	Non-environmental
72	<i>Y. enterocolitica</i>	74-975	—	+	2	Non-environmental
73	<i>Y. enterocolitica</i>	74-974	—	+	2	Non-environmental
74	<i>Y. enterocolitica</i>	74-973	—	+	2	Non-environmental
75	<i>Y. enterocolitica</i>	74-972	—	+	2	Non-environmental
76	<i>Y. enterocolitica</i>	74-971	—	+	2	Non-environmental
77	<i>Y. enterocolitica</i>	74-970	—	+	2	Non-environmental
78	<i>Y. enterocolitica</i>	74-969	—	+	2	Non-environmental
79	<i>Y. enterocolitica</i>	74-968	—	+	2	Non-environmental
80	<i>Y. enterocolitica</i>	74-967	—	+	2	Non-environmental
81	<i>Y. enterocolitica</i>	74-966	—	+	2	Non-environmental
82	<i>Y. enterocolitica</i>	74-965	—	+	2	Non-environmental
83	<i>Y. enterocolitica</i>	74-964	—	+	2	Non-environmental
84	<i>Y. enterocolitica</i>	74-962	—	+	2	Non-environmental
85	<i>Y. enterocolitica</i>	74-960	—	+	2	Non-environmental
86	<i>Y. enterocolitica</i>	74-959	—	+	2	Non-environmental
87	<i>Y. enterocolitica</i>	74-957	—	+	2	Non-environmental
88	<i>Y. enterocolitica</i>	74-956	—	+	2	Non-environmental
89	<i>Y. enterocolitica</i>	74-955	—	+	2	Non-environmental
90	<i>Y. enterocolitica</i>	74-954	—	+	2	Non-environmental
91	<i>Y. enterocolitica</i>	74-953	—	+	2	Non-environmental
92	<i>Y. enterocolitica</i>	74-952	—	+	2	Non-environmental
93	<i>Y. enterocolitica</i>	74-951	—	+	2	Non-environmental
94	<i>Y. enterocolitica</i>	74-950	—	+	2	Non-environmental
95	<i>Y. enterocolitica</i>	74-949	—	+	2	Non-environmental
96	<i>Y. enterocolitica</i>	74-947	—	+	2	Non-environmental
97	<i>Y. enterocolitica</i>	74-946	—	+	2	Non-environmental
98	<i>Y. enterocolitica</i>	74-945	—	+	2	Non-environmental
99	<i>Y. enterocolitica</i>	74-944	—	+	2	Non-environmental
100	<i>Y. enterocolitica</i>	74-943	—	+	2	Non-environmental
101	<i>Y. enterocolitica</i>	74-1892	—	+	2	Non-environmental
102	<i>Y. enterocolitica</i>	75-169	—	+	2	Non-environmental
103	<i>Y. enterocolitica</i>	75-1199	—	+	2	Non-environmental
104	<i>Y. enterocolitica</i>	75-1200	—	+	2	Non-environmental
105	<i>Y. enterocolitica</i>	75-1201	—	+	2	Non-environmental
106	<i>Y. enterocolitica</i>	75-1202	—	+	2	Non-environmental
107	<i>Y. enterocolitica</i>	75-1203	—	+	2	Non-environmental
108	<i>Y. enterocolitica</i>	75-1205	—	+	2	Non-environmental
109	<i>Y. enterocolitica</i>	75-1206	—	+	2	Non-environmental
110	<i>Y. enterocolitica</i>	75-1207	—	+	2	Non-environmental
111	<i>Y. enterocolitica</i>	75-1208	—	+	2	Non-environmental
112	<i>Y. enterocolitica</i>	75-1210	—	+	2	Non-environmental
113	<i>Y. enterocolitica</i>	75-1211	—	+	2	Non-environmental

TABLE 1—continued

Serial no.	Received as:		Biovar ^a		Source ^b	Habitat
	Species	Strain designation	Rhamnose	Sucrose		
114	<i>Y. enterocolitica</i>	75-1212	—	+	2	Non-environmental
115	<i>Y. enterocolitica</i>	75-1213	—	+	2	Non-environmental
116	<i>Y. enterocolitica</i>	75-1214	—	+	2	Non-environmental
117	<i>Y. enterocolitica</i>	75-1215	—	—	2	Non-environmental
118	<i>Y. enterocolitica</i>	75-1216	—	—	2	Non-environmental
119	<i>Y. enterocolitica</i>	75-1217	—	—	2	Non-environmental
120	<i>Y. enterocolitica</i>	75-1218	—	—	2	Non-environmental
121	<i>Y. enterocolitica</i>	75-1219	—	—	2	Non-environmental
122	<i>Y. enterocolitica</i>	75-1220	—	+	2	Non-environmental
123	<i>Y. enterocolitica</i>	75-1221	—	+	2	Non-environmental
124	<i>Y. enterocolitica</i>	75-1222	—	+	2	Non-environmental
125	<i>Y. enterocolitica</i>	75-1223	—	—	2	Non-environmental
126	<i>Y. enterocolitica</i>	75-1224	+	+	2	Environmental
127	<i>Y. enterocolitica</i>	75-1225	+	+	2	Environmental
128	<i>Y. enterocolitica</i>	75-1226	—	+	2	Non-environmental
129	<i>Y. enterocolitica</i>	75-1227	—	+	2	Non-environmental
130	<i>Y. enterocolitica</i>	75-1228	—	+	2	Non-environmental
131	<i>Y. enterocolitica</i>	75-1229	—	+	2	Environmental
132	<i>Y. enterocolitica</i>	75-1231	—	+	2	Non-environmental
133	<i>Y. enterocolitica</i>	75-1335	—	+	2	Non-environmental
134	<i>Y. enterocolitica</i>	75-1531	—	+	2	Non-environmental
135	<i>Y. enterocolitica</i>	75-1538	—	+	2	Non-environmental
136	<i>Y. enterocolitica</i>	75-1763	—	+	2	Non-environmental
137	<i>Y. enterocolitica</i>	Ye 137	+	+	1	Environmental
138	<i>Y. enterocolitica</i>	Ye 138	—	—	1	Environmental
139	<i>Y. enterocolitica</i>	75-1806	—	+	2	Non-environmental
140	<i>Y. enterocolitica</i>	75-1896	—	+	2	Non-environmental
141	<i>Y. enterocolitica</i>	75-2360	—	+	2	Non-environmental
142	<i>Y. enterocolitica</i>	75-2361	—	+	2	Non-environmental
143	<i>Y. enterocolitica</i>	107 A	—	+	3	Non-environmental
144	<i>Y. enterocolitica</i>	107 B	—	+	3	Non-environmental
145	<i>Y. enterocolitica</i>	107 C	—	+	3	Non-environmental
146	<i>Y. enterocolitica</i>	107 D	—	+	3	Non-environmental
147	<i>Y. enterocolitica</i>	107 E	—	+	3	Non-environmental
148	<i>Y. enterocolitica</i>	107 F	—	+	3	Non-environmental
149	<i>Y. enterocolitica</i>	107 G	—	+	3	Non-environmental
150	<i>Y. enterocolitica</i>	107 H	—	+	3	Non-environmental
151	<i>Y. enterocolitica</i>	107 I	—	+	3	Non-environmental
152	<i>Y. enterocolitica</i>	107 J	—	+	3	Non-environmental
153	<i>Y. enterocolitica</i>	107 K	—	—	3	Non-environmental
154	<i>Y. enterocolitica</i>	107 L	—	—	3	Non-environmental
155	<i>Y. enterocolitica</i>	107 M	—	+	3	Non-environmental
156	<i>Y. enterocolitica</i>	107 N	—	+	3	Non-environmental
157	<i>Y. enterocolitica</i>	107 O	—	+	3	Non-environmental
158	<i>Y. enterocolitica</i>	107 P	+	+	3	Non-environmental
159	<i>Y. enterocolitica</i>	107 Q	+	+	3	Environmental
160	<i>Y. enterocolitica</i>	107 R	—	+	3	Non-environmental
161	<i>Y. enterocolitica</i>	107 S	—	+	3	Non-environmental
162	<i>Y. enterocolitica</i>	107 T	—	+	3	Non-environmental
163	<i>Y. enterocolitica</i>	107 U	—	—	3	Environmental
164	<i>Y. enterocolitica</i>	Ye 164	—	+	1	Non-environmental
165	<i>Y. enterocolitica</i>	Ye 165	—	+	1	Non-environmental
166	<i>Y. enterocolitica</i>	Ye 166	+	+	1	Environmental
167	<i>Y. enterocolitica</i>	Ye 167	+	+	1	Environmental
168	<i>Y. enterocolitica</i>	Ye 168	—	—	1	Environmental
169	<i>Y. enterocolitica</i>	Ye 169	+	+	1	Environmental
170	<i>Y. enterocolitica</i>	Ye 170	+	+	1	Environmental
171	<i>Y. enterocolitica</i>	Ye 171	+	+	1	Environmental

TABLE 1—continued

Serial no.	Received as:		Biovar ^a		Source ^b	Habitat
	Species	Strain designation	Rhamnose	Sucrose		
172	<i>Y. enterocolitica</i>	Ye 172	+	+	1	Environmental
173	<i>Y. enterocolitica</i>	Ye 173	—	—	1	Environmental
174	<i>Y. enterocolitica</i>	Ye 174	—	—	1	Environmental
175	<i>Y. enterocolitica</i>	Ye 175	+	+	1	Environmental
176	<i>Y. enterocolitica</i>	Ye 176	—	—	1	Environmental
177	<i>Y. enterocolitica</i>	Ye 1-L	+	+	1	Environmental
178	<i>Y. enterocolitica</i>	Ye 167-L	+	+	1	Environmental
179	<i>Y. enterocolitica</i>	Ye-49	+	+	4	Non-environmental
180	<i>Y. enterocolitica</i>	Y-48	+	+	4	Non-environmental
181	<i>Y. enterocolitica</i>	Y-50	+	+	4	Non-environmental
182	<i>Y. enterocolitica</i>	Y-51	+	+	4	Non-environmental
183	<i>Y. enterocolitica</i>	Y-53	+	+	4	Non-environmental
184	<i>Y. enterocolitica</i>	Y-54	+	+	4	Non-environmental
185	<i>Y. enterocolitica</i>	Y-55	+	+	4	Non-environmental
186	<i>Y. enterocolitica</i>	Y-56	+	+	4	Non-environmental
187	<i>Y. enterocolitica</i>	Y-57	+	+	4	Non-environmental
188	<i>Y. enterocolitica</i>	Y-58	+	+	4	Non-environmental
189	<i>Y. enterocolitica</i>	Y-59	+	+	4	Non-environmental
190	<i>Y. enterocolitica</i>	Y-60	+	+	4	Non-environmental
191	<i>Y. enterocolitica</i>	Y-61	+	+	4	Non-environmental
192	<i>C. diversus</i>	Y-U	+	+	5	Non-environmental
193	<i>Y. enterocolitica</i>	Ye A	+	+	6	Environmental
194	<i>Y. enterocolitica</i>	Ye E	+	+	6	Environmental
195	<i>Y. enterocolitica</i>	Ye H	—	—	6	Environmental

pale pink to rose color around the inoculum was recorded as a positive result.

Alkalinization and/or growth on Simmons citrate agar at 24 h or 7 days was scored as a positive result.

Catalase and oxidase tests were performed with 24-h-old growth from heart infusion agar plates. The oxidase test was performed by rubbing a loopful of cell paste onto a moistened strip of filter paper previously saturated with a solution of 1% tetramethyl-*p*-phenylenediamine dihydrochloride in 0.2% ascorbic acid. Development of a purple color within 10 s was read as positive for cytochrome *c* oxidase. For detection of catalase activity, the plates were flooded with 3% H₂O₂, and the appearance of bubbles was recorded as a positive result.

Motility and indole production were both scored from motility indole ornithine medium (Difco). A tube of medium was stabbed to the bottom, and motility was read as diffusion of growth from the initial stab line. Indole was detected at 24 h with Kovacs reagent. Ornithine decarboxylation was also recorded in this medium, but the results were not used in the numerical analysis.

Moeller medium (19) supplemented with ornithine, lysine, or arginine (1%, wt/vol), along with an unsupplemented control tube, was used to score decarboxylase activity. Tubes were inoculated and covered with a layer of mineral oil.

To detect hydrolysis of Tweens 20, 40, 60, and 80 (Sigma Chemical Co.), a 1% (vol/vol, final concentration) solution of the appropriate Tween was added to a medium composed of 10 g of peptone (Difco), 5 g of

NaCl, 0.1 g of CaCl₂·H₂O, 15 g of agar, and 1 liter of distilled water (pH 7.4). Zones of precipitation were recorded for 5 days.

Double-strength heart infusion agar was autoclaved, cooled, and then added to an equal volume of an autoclaved skim milk solution (15%, wt/vol). After mixing, this medium was poured into petri dishes, inoculated, and observed for clearing as evidence of casein hydrolysis. The test was terminated at 1 week.

Tributyrin hydrolysis was detected on a medium composed of heart infusion agar to which tributyrin (1%, vol/vol) was added and emulsified after autoclaving.

Phenylalanine agar (Difco) was inoculated and incubated for 2 days. Each slant was then flooded with an aqueous ferric chloride solution (13%, wt/vol); development of a green color was read as a positive result.

Two methods were used for the detection of gelatin hydrolysis. (i) Nutrient gelatin (5 g of peptone [Difco], 3 g of beef extract [Difco], 120 g of gelatin [Difco], 1 liter of distilled water) was dispensed in 3-ml amounts into tubes (13 by 100 mm), autoclaved, inoculated, and incubated; liquefaction was recorded at 7 days and 4 weeks. (ii) Sterile heart infusion agar containing gelatin (12%, vol/vol; [Difco]) was poured into petri plates and inoculated; zones of hydrolysis were detected by flooding with aqueous mercuric chloride (10%, wt/vol) after incubation for 7 days.

The production of hydrogen sulfide from cystine was tested in a medium containing 10 g of Trypticase (BBL), 0.2 g of L-cystine, 5 g of sodium chloride, 0.2 g

of ferric ammonium citrate, 2 g of agar, and 1 liter of distilled water. The medium was dispensed in 4-ml amounts into tubes (13 by 100 mm) and inoculated by stabbing to the bottom.

Lipase and lecithinase activities were recorded from inoculations on egg yolk agar (Difco).

Starch hydrolysis was detected on heart infusion agar supplemented with cornstarch (1%, wt/vol). After 7 days of incubation, zones of hydrolysis were enhanced by flooding the plates with Gram iodine.

The methyl red and Voges-Proskauer tests were performed by incubating inoculated tubes of buffered glucose-peptone broth for 48 h and then dividing the broth for use in the two tests. The medium and reagents for these tests were as outlined in the *Laboratory Manual for Medical Bacteriology*, 5th ed. (16).

Nitrate reduction was tested in a broth composed of 10 g of a pancreatic digest of casein (Difco), 3 g of yeast extract, 2 g of potassium nitrate, and 1 liter of distilled water. After 24 h of incubation of the inoculated medium, nitrite formation was detected by using the method described in the *Laboratory Manual for Medical Bacteriology*.

The test for beta-galactosidase activity was done with tableted medium from Key Scientific Products Co., Los Angeles, Calif. Inoculum for the test was obtained by harvesting 18-h-old growth from a Kligler iron agar slant. The test was performed according to the recommendations of the manufacturer.

Kligler iron agar (Difco) supplemented with esculin (0.002%, wt/vol; Difco), lysine iron agar (Difco), Selenite-F broth (BBL Microbiology Systems), Rogosa agar (Difco), malonate broth (Difco), and tetrathionate broth (Difco) were also used. None of the results from the tests in which these media were employed was scored for numerical analysis, however, because of equivocal or duplicate results. Unless otherwise noted, all tests were read at 24 h and 4 days.

(vi) Coding of characters. All tests performed resulted potentially in mutually exclusive character states. Consequently, characters were scored 1 (positive) or 0 (negative). Weakly positive results were scored 1. Gas production from carbohydrates was not scored for numerical taxonomy.

Computation. All computations were computer directed by programs included in the "Numerical Taxonomy System of Multivariate Statistical Programs." These programs were developed at the University of Kansas and refined at the State University of New York at Stony Brook by F. James Rohlf, John Kishpaugh, and David Kirk. From the coefficients offered by this program, the simple matching coefficient was chosen on the basis of a recent comparative study (1) which showed this coefficient to be one of the most useful for differentiating clusters. Unweighted pair group method with averages was chosen from the several types of sequential agglomerative hierarchical cluster analyses offered by the program Taxon. This method of determining and graphically depicting the relationships among strains is recommended by the "Numerical Taxonomy System of Multivariate Statistical Programs" as the option of choice.

DNA hybridizations. (i) **Bacterial strains.** The centrostrains of the phenons from both the 30 and 35°C matrixes were used as reference strains in the subsequent DNA-DNA hybridization experiments.

Centrostrain is defined here as the bacterial strain within each cluster which has the highest mean relationship to every other organism in that cluster. In addition, two other organisms from each phenon were selected as sources of cold DNA for hybridizations. These organisms were chosen on the basis of their phenetic relationships to the centrostrains. One organism of each pair was highly related (similarity coefficient, ≥ 0.90) to the centrostrain, whereas the other had the lowest similarity coefficient in comparison with the centrostrain for any organism of the cluster. The DNAs from additional organisms were labeled and/or extracted as deemed necessary for the adjunct genetic study of the environmental strains.

(ii) Media. Bacteria to be labeled were grown in a glucose salts medium containing 10 ml of 10 $\times$ salts, 5 ml of 10% (wt/vol) Casamino Acids (Difco), 0.1 ml of 0.1 M $MgSO_4$, 1 ml of 20% (wt/vol) glucose, and distilled water to 100 ml. The 10 $\times$ salts solution was composed of 4.0 g of KH_2PO_4 , 10.5 g of K_2HPO_4 , 0.47 g of sodium citrate $\cdot 5H_2O$, 1.0 g of $(NH_4)_2SO_4$, and distilled water to 100 ml. Other bacteria were grown in Brucella broth (Difco).

(iii) DNA isolation. An overnight Brucella broth (Difco) culture (10 ml) was added to 500 ml of fresh medium and incubated with shaking at 30°C for 10 to 12 h. Cells were harvested by centrifugation and then resuspended in 50 ml of saline-ethylenediaminetetraacetate (0.15 M NaCl plus 0.1 M ethylenediaminetetraacetate, pH 8.0). A 1-ml amount of 25% (wt/vol) sodium dodecyl sulfate and 2.5 mg of pronase (B grade; nuclease free; Calbiochem) were added, and the mixture was incubated overnight at 35°C. The subsequent isolation and purification technique was essentially that described by Marmur (17). The only modifications were the addition of several more purification steps. After completion of the Marmur technique, isolated DNA was subjected to an additional ribonuclease step, which was followed by repeated (three or four) deproteinizations before a final reprecipitation in isopropyl alcohol. The purified DNA was stored in dilute saline citrate (0.1 $\times$ SSC; 1 $\times$ SSC is 0.15 M NaCl plus 0.015 M trisodium citrate, pH 7.0) over chloroform at 4°C.

(iv) Preparation of radioactive DNA. Radioactive DNA was produced by adding 10 ml of an overnight glucose salts broth culture to 490 ml of fresh glucose salts broth containing 72 μg of [*methyl*- 3H]-thymidine (6.7 Ci/mmol; New England Nuclear Corp., Boston, Mass.). The flask cultures were incubated with shaking at 30°C for 16 to 18 h. Cells were harvested, and the DNA was extracted and purified as described above. All DNA concentrations were determined by using a Beckman model 22 spectrophotometer (Beckman Instruments, Inc., Scientific Instruments Div., Irvine, Calif.) at 260 nm and a standard curve.

DNA-DNA hybridizations. Numbered microfilters (diameter, 6 mm) were punched from filter membranes (type HAWP; Millipore Corp., Bedford, Mass.) and soaked in 6 $\times$ SSC for 3 to 4 h. Solutions of DNA in 0.1 $\times$ SSC were denatured for 10 min in a boiling water bath, chilled quickly on ice, and made up to 6 $\times$ SSC. Microfilters were individually placed in a specially designed filter apparatus with a working diameter of 5 mm. After the filters were washed with 6 $\times$

FIG. 2. 30°C phenogram.

enterocolitica are represented in phenons 2 and 3, with the typical biogroups predominating. The 56 organisms in phenon 2 are rhamnose negative with only two exceptions, both nonenvironmental isolates. In contrast, 11 of the environmental strains of cluster 3 belong to the atypical rhamnose-positive biogroup. Phenon 5 is composed of seven biochemically atypical strains of *Y. enterocolitica*, three of which are ornithine decarboxylase negative.

The phenogram in Fig. 2 represents an analysis of the results of test incubations at a temperature only 5°C lower than the temperature on which the phenogram in Fig. 1 was based, yet numerical analysis produced a different taxonomic ordering. Instead of five clusters at the 85% similarity level, there are seven. The relative arrangement of the 56 strains in cluster 1 changed considerably, but the overall composition remained fairly stable. Phenon 1 lost one of its rhamnose-negative organisms to phenon 2 and gained three of the atypical (rhamnose-positive) organisms which were in other clusters of the 35°C phenogram. This shift gives cluster 1 of the 30°C phenogram a composition of 95% atypical strains. Cluster 2 expanded to 70 organisms, gaining many of the typical *Y. enterocolitica* strains from cluster 3 of the 35°C phenogram and losing all but two of its environmental organisms. Phenon 3 of the 30°C phenogram is composed entirely of environmental organisms, both rhamnose positive and rhamnose negative. Phenon 4 is unremarkable except that two of the strains are ornithine decarboxylase negative. Phenons 5 and 7 are composed primarily of typical *Y. enterocolitica* strains from clusters 2 and 3 of the 35°C phenogram, although five sucrose-negative strains are in phenon 5. Phenon 6 has all but one of the atypical environmental strains of cluster 4 of the 35°C phenogram. Three organisms (an atypical environmental strain and two nonenvironmental strains) are not associated with any of the clusters.

DNA hybridizations. Tables 2 and 3 give the results of the DNA hybridization studies within each cluster. Percent relatedness is expressed as the RBR of heterologous DNA to homologous DNA times 100. Also included in these tables are the phenetically generated relatedness values. In each case, this is the similarity coefficient expressed as percent relatedness.

If one examines the data presented for clusters 1 through 3 in Table 2, it becomes apparent that although there is high correlation between numerical analysis and DNA hybridization data when an organism has a high degree of phenetic relatedness to the centrostrain, the converse is true with a less highly related organism. In each of these cases, the organism which showed only

TABLE 2. *Percent relatedness of centrostrains to test strains*

35°C phenon no.	Centrostrain	Test strain	% Similarity ^a	% DNA binding
1	167	178	93	83
		109	83	24
2	112	161	95	100
		16	84	27
3	68	114	95	99
		2	87	39
4	166	175	92	82
		126	86	86
5	78	79	92	94
		72	85	92

^a Simple matching coefficient.TABLE 3. *Percent relatedness of centrostrains to test strains*

30°C phenon no.	Centrostrain	Test strain	% Similarity ^a	% DNA binding
1	191	189	97	96
		178	90	84
2	89	95	92	99
		161	87	80
3	29	30	99	73
		3	86	25
4	72	73	95	93
		65	86	92
5	53	142	93	94
		35	82	20
6	166	175	93	82
		171	88	23
7	63	61	95	63
		79	87	74

^a Simple matching coefficient.

a low degree of DNA relatedness was in a different phenon than the other two organisms in the 30°C matrix. Unfortunately, the same is true of clusters 4 and 5. Although all three organisms show a high degree of DNA relatedness, the one less related by numerical analysis in both clusters was placed in a different phenon than the other two in the 30°C matrix.

Clusters 1, 2, 4, and 7 of the 30°C matrix in Table 3 show a high degree of correlation between numerical analysis and DNA hybridization data. With only two exceptions, however, the percent DNA relatedness is less than the corresponding numerical analysis relationship. Clusters 3, 5, and 6 exhibit the same phenomenon seen in the 35°C matrix. Organisms which have a high degree of relationship to the centrostrain by numerical analysis have good correlations with DNA relatedness values, whereas the less highly related organisms do not.

The results of DNA hybridization studies using the centrostrains from the 35 and 30°C matrices are shown in Tables 4 and 5. The centrostrains of clusters 1 and 4 and clusters 3 and 5 from Table 4 are obviously highly related by genetic data, as are those of clusters 1 and 6 and clusters 2, 4, 5, and 7 from Table 5.

Table 6 presents the summation of all DNA hybridization studies along with the concomi-

TABLE 4. *Percent relatedness of 35°C centrostrains, as determined by DNA hybridizations*

Centrostrain of phenon no.	% Binding of centrostrain of phenon no.:				
	1	2	3	4	5
1	100	25	23	93	33
2		100	57	27	57
3			100	21	98
4				100	29
5					100

TABLE 5. *Percent relatedness of 30°C centrostrains, as determined by DNA hybridizations*

Centrostrain of phenon no.	% Binding of centrostrain of phenon no.:						
	1	2	3	4	5	6	7
1	100	29	27	21	17	70	20
2		100	32	76	70	25	65
3			100	27	24	26	19
4				100	83	30	89
5					100	27	73
6						100	17
7							100

tant numerical analysis data. For comparative purposes, correlation ratios and the overall correlation coefficients have been computed for the genetic and phenetic percent relatedness figures.

TABLE 6. *Comparison of DNA hybridization and numerical taxonomy data*

Source of labeled DNA (strain)	Source of unlabeled DNA (strain)	% Binding	Standard error of mean	% Similarity when culture incubated at:°	
				30°C	35°C
29	29	100	7.3	100	100
	2	102	1.7	93	97
	3	25	4.1	86	88
	30	73	3.0	99	98
	35	21	1.5	80	89
	63	19	0.4	74	87
	89	32	1.5	81	85
53	53	100	3.8	100	100
	3	44	2.7	85	81
	29	24	0.6	85	89
	35	20	1.9	82	87
	63	73	6.7	86	94
	72	83	1.8	84	84
	89	70	0.4	86	85
	142	94	4.4	93	90
	191	17	2.4	74	72
63	63	100	3.8	100	100
	61	63	2.0	95	94
	79	74	9.3	87	85
68	68	100	6.6	100	100
	2	39	3.0	85	87
	3	48	1.4	84	83
	35	36	1.8	85	94
	78	98	2.9	78	85
	114	99	1.6	82	95
	166	21	3.5	79	89
72	72	100	6.6	100	100
	29	27	3.5	85	83
	63	89	6.1	72	83
	65	92	9.2	86	76
	73	93	3.7	95	96
	89	76	3.0	82	83
78	78	100	0.9	100	100
	72	92	2.0	76	85
	79	94	2.1	97	92
89	89	100	2.4	100	100
	63	65	3.2	80	87
	95	99	3.2	92	90
	161	80	7.8	87	85
112	112	100	3.9	100	100
	3	51	3.0	83	80
	16	27	0.8	82	84
	35	39	6.1	84	80
	68	57	7.1	85	82
	78	57	3.3	72	69
	161	100	2.6	89	95
	166	27	0.8	80	85
	167	25	0.9	89	85
166	166	100	5.2	100	100
	2	25	1.9	81	85
	16	74	1.9	85	85
	29	26	5.5	85	85
	35	22	1.8	77	88
	53	27	2.0	82	87
	63	17	0.6	80	87
	72	30	2.0	79	79
	78	29	1.5	79	80

° The coefficients of correlation were 0.48 and 0.40 for the 30 and 35°C data, respectively. The mean correlation ratios were 0.60 and 0.60 for the 30 and 35°C data, respectively.

TABLE 6—continued

Source of labeled DNA (strain)	Source of unlabeled DNA (strain)	% Binding	Standard error of mean	% Similarity when culture incubated at:°	
				30°C	35°C
167	89	25	1.0	84	85
	126	86	5.4	83	86
	167	92	7.6	89	87
	171	23	2.2	88	94
	175	82	2.6	93	92
	191	70	4.4	85	81
	167	100	7.8	100	100
	68	23	1.8	85	82
	78	33	1.9	72	74
	109	24	1.7	86	83
	166	94	5.6	89	87
	178	83	6.0	95	93
	191	100	7.2	100	100
	3	37	2.2	76	76
191	29	27	0.6	83	75
	63	20	1.0	66	72
	72	21	1.2	81	71
	89	29	0.9	82	78
	178	84	7.5	90	84
	189	96	5.0	97	94
	195	100	7.6	100	100
	2	44	4.2	89	86
	3	89	6.6	88	86
	29	42	1.8	87	85
195	35	60	4.4	83	84
	191	43	1.8	80	85

In addition, the standard error of the mean was calculated for all DNA hybridization results to illustrate concisely the amount of fluctuation in the raw data.

DISCUSSION

Numerical taxonomy involves counting the number of similar and dissimilar features between bacterial strains and resolving these by any of several formulas into similarity coefficients. With the aid of an electronic computer and an additional formula, strains were sorted into groups, based upon these similarity coefficients. The result is a graphic depiction of the degree of relatedness (based on observable features) among the strains tested. The chief criticism of this method is that it tests indirectly a relatively small portion of a bacterial genome. The most favorable estimates gauge the amount of a genome tested by numerical methods to be in the range of 10 to 20% (6). In contrast, DNA hybridization techniques theoretically offer a direct method of comparing the molecular structures of entire genomes.

Proponents of the numerical taxonomy approach have long argued that there should be (and in fact is) a high correlation between the evaluation of phenetic similarities and DNA ho-

mologies. Their contention is that both reflect the similarity of base sequences and thus can be equated as techniques for determining relationships among organisms (5, 6, 12). Unfortunately, this philosophy overlooks certain basic facts. Even though enzymes may perform the same function and thus be ranked as similarities in phenetic comparisons, the coding sequences of enzymes may differ, and thus the molecular structure of the DNA would differ. The choice and coding of features is also a problem in numerical taxonomy. Some taxonomies rely heavily on morphological features which, in addition to requiring qualitative judgements for coding, may also be the end results of the interaction of multiple enzymes. In addition, unless the individuals undertaking the study are very knowledgeable in bacterial physiology, a single feature can be weighted multiply. For example, in some genera of bacteria, a single enzyme is responsible for the oxidation of at least 10 carbohydrates to the corresponding sugar acids (2). If several or all of these sugars are included as test substrates for carbohydrate utilization by certain bacteria, the erroneous conclusion may be drawn that the ability to produce acid from each sugar attacked should be scored as a separate character. Observations such as these can result in a very biased

picture of similarity relationships within a group. The mathematical approach of numerical taxonomy is sound, but the experimental data available for mathematical conversion are often the result of subjective decisions and genomic intrarelationships. The purity of the end can be obscured by the means.

This view was formed after the completion of the first phase of this study (the duplicate numerical taxonomies differing only in test incubation temperatures). Even a cursory comparison of the 35°C phenogram (Fig. 1) with the 30°C phenogram (Fig. 2) discloses a basic flaw of the method. The two phenograms differ considerably. If the organisms were accurately clustered by this method, a 5°C difference in incubation temperature should affect highly related strains in a homogeneous manner so that, although the relative order of the phenons might change, the individual cluster composition should remain basically stable. Experimentally, this was not the case. Statistical analysis of the 35°C data produced a phenogram of five clusters, whereas the 30°C data generated a phenogram with seven clusters plus three ungrouped organisms. Only cluster 1 remained relatively stable for the two phenograms. As the examples shown in Table 7 demonstrate, the members of this cluster typically exhibit higher intracluster similarity values at 30°C than at 35°C. This, in combination with the knowledge that all *Y. enterocolitica* biovars are metabolically more active at the lower temperature, leads us to believe that the 30°C phenogram is the more accurate representation of taxonomic divisions within the groups. Unfortunately, however, the genetic evidence disputes this. The DNA hybridization study of the centrostrains from the 30°C phenogram (Table 5) justifies the existence of only three taxonomic divisions since the centrostrains of clusters 1 and 6 are highly related (RBR, $\geq 70\%$), as are those of clusters 2, 4, 5, and 7. The 35°C phenogram does not fare much better by the same type of genetic comparison. As Table 4 shows, only a maximum of three of the five clusters of this matrix is confirmed by DNA hybridization evidence. In our opinion, all that has been elicited by the numerical taxonomies are subdivisions of these organisms into temperature-dependent biovars, with no discernment of the real genetic relationships among the various groups. The computer-aided statistical analysis has not increased our understanding of the true taxonomic interrelationships of this group of organisms past the biovar stage of Niléhn (21), which was achieved manually 10 years ago.

The methods of numerical taxonomy may separate organisms accurately into discrete taxa

TABLE 7. Comparative computer-generated similarity values of centrostrain of 30°C phenon 1 versus 30°C phenon 1 strains at 30 and 35°C

Comparison (strains)	% Similarity at:	
	30°C	35°C
191 × 1	92	87
191 × 172	93	92
191 × 4	92	86
191 × 5	94	88
191 × 8	92	90
191 × 36	93	95
191 × 9	95	94
191 × 10	94	90
191 × 17	94	87
191 × 167	93	90
191 × 23	92	85
191 × 26	93	86
191 × 28	94	90
191 × 31	91	83
191 × 38	91	84
191 × 18	93	90
191 × 43	92	86
191 × 49	91	85
191 × 34	93	85
191 × 44	92	83
191 × 45	91	83
191 × 137	91	89
191 × 20	95	91
191 × 47	95	92
191 × 24	94	87
191 × 41	95	84
191 × 127	97	93
191 × 189	97	94
191 × 188	96	92
191 × 159	95	95
191 × 6	91	82
191 × 14	91	80
191 × 7	92	90
191 × 25	95	87
191 × 11	93	87
191 × 107	93	87
191 × 22	93	89
191 × 169	94	87
191 × 170	90	92
191 × 186	95	95
191 × 187	93	92
191 × 190	93	97
191 × 194	95	93
191 × 193	93	92
191 × 16	93	85
191 × 179	92	89
191 × 182	92	92
191 × 184	93	92
191 × 180	92	92
191 × 181	93	90
191 × 183	92	94
191 × 185	93	93
191 × 57	91	92
191 × 27	91	85
191 × 37	90	84
191 × 177	92	86
191 × 178	89	84
191 × 178	89	84

when those organisms diverge radically when compared by the available testing procedures, but with organisms whose testable metabolic pathways are extremely similar, no valid conclusions can be drawn. The five control organisms, all obviously not *Y. enterocolitica* strains by superficial testing, separated out nicely on both phenograms. None showed a relationship to any of the *Y. enterocolitica* clusters at a similarity level higher than 78%, and most linked at levels well below the 80 to 85% range considered analogous to species level by most advocates of numerical taxonomy (22). The *Y. enterocolitica* paired controls (strains 20 and 47, 1 and 177, 167 and 178) did not diverge radically at either temperature.

The criticism leveled here at the validity of establishing taxonomic divisions on the basis of phenetic clustering is based not only on the intrinsic errors of the testing procedures for numerical taxonomy but also on the results of comparisons with DNA hybridization data. DNA hybridization is theoretically much more valid for determining taxonomic relationships than is numerical taxonomy. Conceivably, however, the experimental error could offset this theoretical advantage. Unfortunately, few publications on DNA hybridization studies contain data on experimental error or mention whether replicate tests were used. Two studies which included replicate testing and standard error of the mean for the results are those of Garvie (8) and Gibbins and Gregory (9). Both studies used the membrane filter technique, but the incubation medium of Garvie was 2× SSC-30% dimethyl sulfoxide instead of the 6× SSC-50% formamide used in this study and in that of Gibbins and Gregory. The average standard error of the mean of Garvie was 4.8 (range, 0.01 to 27.4), whereas that of Gibbins and Gregory was 0.4 (range, 0.06 to 1.26). The average standard error of the mean of this study was 3.4 (range, 0.4 to 9.3); the fluctuation among replicate tests was greater than that found by Gibbins and Gregory but not as much as that noted by Garvie. The deviation in the replicate counts of Gibbins and Gregory, as evidenced by an overall standard error of the mean of 0.4, is extraordinarily low. Even though the average standard error of the mean calculated for the *Y. enterocolitica* study was higher and thus the reproducibility was lower than that of the Gibbins and Gregory study, data from reciprocal hybridizations (Table 8) give considerable support to the validity of the method.

Additional support for the validity of the method derives from the fact that DNA hybridization measures the relationship of the molecular structure of the entire genome of one bac-

TABLE 8. Reciprocal DNA hybridizations

Group	Source of labeled DNA (strain)	Source of unlabeled DNA (strain)	% Binding
1	112	78	57
	112	68	57
	68	78	98
2	112	166	27
	112	167	25
	166	167	92
3	195	2	44
	195	29	42
	29	2	102
4	166	2	25
	166	29	26
	29	2	102
5	166	72	30
	166	78	29
	78	72	92
6	167	78	33
	166	78	29
	167	166	94
7	166	167	92
	167	166	94

terium to that of another, whereas numerical taxonomy measures, at best, only 20% of the genome through phenotypic capabilities. When one evaluates this indirect measurement of 20% of the genome in terms of the error in test reproducibility plus the unknown error in inadvertent testing duplication, the disparity between the values of the two procedures for taxonomic purposes becomes even more obvious. Although Colwell and colleagues (6) derived correlation coefficients ranging from 0.5 to 0.9 in a comparison between a numerical taxonomy done in the laboratory of Colwell and DNA hybridizations done with some of the same strains in the laboratory of D. Brenner, these correlation coefficients are misleading. Omitted from the calculations were all matches where the percent similarity was 60% or less. This omitted many sets of aberrant data from the computations (for example, RBR of 71%, percent similarity of 48%; RBR of 70%, percent similarity of 48%; RBR of 70%, percent similarity of 58%; RBR of 73%, percent similarity of 57%). These data were taken from Colwell et al. (6), who compared *Escherichia coli* with *Shigella* species. All of the DNA hybridizations gave values which showed a species relationship, whereas numerical taxonomy indicated that these organisms are not significantly related.

In addition, the correlation coefficient r is simply a measure of the linear relationship between two variables. It gives no indication of how closely related numerically these two variables are. In a comparison of numerical taxonomy similarity values to DNA hybridization ho-

mologies, some indication of how closely related these values are numerically is desirable. As an indication of the misleading impression given by an r value of 0.9, consider the following data set taken from the study by Colwell et al. (6), in which *Salmonella typhimurium* was used as the reference organism against *Citrobacter* species, RBR of 58%, percent similarity of 84%; RBR of 58%, percent similarity of 83%; RBR of 50%, percent similarity of 82%; RBR of 49%, percent similarity of 84%; RBR of 49%, percent similarity of 77%; RBR of 46%, percent similarity of 79%; RBR of 46%, percent similarity of 79%; RBR of 42%, percent similarity of 80%. The data give a good linear correlation, as evidenced by the correlation coefficient of 0.9 for the set as a whole. However, in the examples shown, all of the percent similarity values denote a high phenetic relationship, whereas the RBRs indicate low DNA base sequence homology. If one assumes a significant taxonomic relationship at the 80% similarity level for numerical taxonomy (22) and at an RBR of 70% for DNA hybridization (3) and also assumes that phenetic similarity is a measure of genetic similarity, then one should expect both a high numerical correlation and a high linear correlation between the data sets. As can be seen by the comparative data generated in this study (Tables 2, 3, and 6) and by the examples just cited, this does not occur consistently. The average correlation ratio of 0.60 for both the 30 and 35°C data sets, as well as the overall correlation coefficients of 0.48 and 0.40, respectively, are indicative of low numerical and linear correlations between numerical taxonomy and DNA hybridization.

Consideration of the final part of the study (determination of the intra- and interrelationships of the organisms from environmental sources) must depend on the results of DNA hybridizations. No coherent separations of this group of strains can be made on the basis of the numerical taxonomy results.

The one previous attempt at a comprehensive study of the species *Y. enterocolitica* was by Brenner et al. (3). The conclusions drawn from their study, which used an RBR of 70% or greater at stringent temperature as indicative of species relationship, were as follows: (i) the strains in their study formed three, possibly four, DNA relatedness groups; (ii) one DNA relatedness group biochemically is "typical" *Y. enterocolitica*, the second and third are rhamnose positive, the third is additionally melibiose, α -methyl glucoside, and raffinose positive, and the possible fourth group is sucrose negative and rhamnose negative; (iii) all of the observed DNA relatedness groups should remain in the genus *Yersinia*, but only those organisms which are

biochemically typical should be called *Y. enterocolitica*. The tenuousness of the fourth DNA relatedness group derived from the omission of hybridization of these strains to one of the rhamnose-positive groups plus the intermediate RBRs (67, 59, and 55%) of the group 4 strains when hybridized to the group of typical *Y. enterocolitica* strains.

Except for data on two strains, our data agree with those in the above-mentioned study with respect to the DNA relatedness of typical *Y. enterocolitica* strains. The two aberrant strains (63 and 112) gave RBRs of less than 70% with some typical *Y. enterocolitica* strains and greater than 70% with others. Strain 112 had an RBR of 57% with both strain 68 and strain 78. These strains in turn had RBRs of greater than 70% with each other and with other typical strains. In contrast, strain 112 had an RBR of 100% with another typical *Y. enterocolitica* strain (strain 161), which subsequently hybridized with other strains of the biochemically typical group at RBR levels of greater than 70%. Similar hybridization values occurred with strain 63. Hybridizing this strain with typical strains 61 and 69 resulted in RBR values of 63 and 65%, respectively, whereas hybridization of all of these strains with other typical *Y. enterocolitica* strains resulted in RBR values of greater than 70%. Unfortunately, strains 63 and 112 were not tested by hybridization to each other or to a common strain. Since ascertaining the DNA relatedness of typical *Y. enterocolitica* strains was not a goal of this study, the enigma was only fortuitously discovered by the inter- and intraphenon hybridizations. Johnson (11) proposed a subspecies designation for organisms that have 60 to 70% homology with organisms of the high homology group, and it may be that typical *Y. enterocolitica* strains 63 and 112 belong to a subspecies of *Y. enterocolitica*.

The results of hybridizations among the environmental organisms and between environmental and nonenvironmental strains of this study indicated the existence of three additional DNA relatedness groups. All of the groups can be characterized on the basis of their 30°C rhamnose and sucrose fermentation patterns: (i) rhamnose negative, sucrose positive; (ii) rhamnose negative, sucrose negative; (iii) rhamnose positive, sucrose positive; (iv) rhamnose positive, sucrose negative. Additional 30°C differential features of these groups are given in Table 9. This table also gives the total number of strains which, when cross-hybridized, comprised the respective relatedness groups. No attempt was made to categorize all of the strains in the study into the four groups.

The biochemical reactions of the four DNA

TABLE 9. Differential features of *Y. enterocolitica* DNA relatedness groups

Characteristic	Group ^a			
	1 (n = 15)	2 (n = 2)	3 (n = 8)	4 (n = 3)
Utilization of:				
Rhamnose	—	—	+	+
Sucrose	+	—	+	—
Salicin	d	—	+	—
Lactose	d	—	d	—
Arbutin	d	—	+	—
Cellobiose	+	+	+	—
Raffinose	—	—	d	—
Citrate (Simmons)	—	—	+	+
Esculin hydrolysis	d	—	+	—
Indole production	d	—	+	—

^a +, All strains positive; —, all strains negative; d, different biochemical types.

relatedness groups of this study do not entirely conform to the four DNA relatedness groups of *Y. enterocolitica* identified by Brenner et al. (3). Based on comparative biochemical reactions, group 3 of this study is identical to their group 3. Their other rhamnose-positive group (group 2) appears to have little biochemical relationship, other than the rhamnose reaction, to our group 4. Their potential group 4 (rhamnose and sucrose negative) may include both groups 2 and 4 of this study. The majority of biochemical reactions recorded by Brenner et al. were at 35°C. At a temperature of 35°C, group 4 of this study is rhamnose negative as well as sucrose negative. Their group 4 is comprised of organisms with different reactions in a variety of biochemical tests; these reactions conform to those of our groups 2 and 4 at 35°C. Group 1 of both studies encompasses the biochemically typical *Y. enterocolitica* strains.

One environmental organism (strain 35) gave low RBR values with members of all DNA relatedness groups except group 2. With group 2, the relative homology value for this strain was 60%. Again, it is difficult to interpret these intermediate values. Biochemically, this organism differs slightly from the members of group 2, giving reactions more compatible with those of members of group 1, the typical *Y. enterocolitica* strains. Cross-hybridizations with DNA from three members of this group, however, yielded RBR values of 20, 36, and 39%. These ratios are indicative of a relationship no closer than genus level. If one adheres to the philosophy of Johnson (11), this is another example of subspecies relationship, this time within group 2. Another possible explanation, of course, is experimental error. The time involved in the several phases of DNA hybridization experiments precludes hybridizations between every pair of organisms in a study. Only further work by other investigators

with different strains will result in a cohesive and complete picture of the taxonomic relationships within this group of organisms.

Based on this study and the study of Brenner et al. (3), it can be stated, however, that the strains presently identified as members of *Y. enterocolitica* comprise at least five DNA relatedness groups. One group contains the biochemically typical (rhamnose-negative, sucrose-positive) *Y. enterocolitica* strains. Two groups are rhamnose positive and sucrose positive. Brenner et al. differentiated these groups on the basis of their melibiose, methyl glucoside, and raffinose reactions; this study found only one DNA relatedness group of rhamnose-positive, sucrose-positive organisms. The fourth and fifth DNA relatedness groups contain sucrose-negative organisms. One group is rhamnose negative as well as sucrose negative, and the other is rhamnose positive. The level of intergroup homology is high enough (RBR, >20%) to justify the inclusion of all groups as species in the genus *Yersinia* unless subsequent work demonstrates greater homology between members of these new groups and another genus.

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