Comparative Immunology of Ribosomes and Disc Gel Electrophoresis of Ribosomal Proteins from *Erwiniae*, *Pectobacteria*, and Other Members of the Family *Enterobacteriaceae*

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Ribosomes from *erwiniae*, *pectobacteria*, and other *Enterobacteriaceae* were compared by two criteria: (i) disc gel electrophoresis of 50S subunit proteins, and (ii) Ouchterlony gel diffusion of intact ribosomes. There were no significant differences in the electrophoretic protein profiles of strains of *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae*, or *Enterobacter agglomerans* (synonym: *Erwinia herbicola*). The only significant profile differences occurred with the faster-migrating proteins of enterobacteria grouped according to plant or animal source. Several non-enterobacteria also had similar over-all profiles; however, specific differences were evident. Immuno-diffusion data were effective in establishing relatedness at the subspecies level. A single antigenic determinant was responsible for the specificity and was found to differ in its sensitivity to treatment with trypsin, ribonuclease, or heat.

Results of investigations on the structure and function of ribosomes suggest the possibility of using ribosomes to determine phylogenetic relationships of bacteria. All bacterial ribosomes studied have subunits with similar sedimentation coefficients, ribonucleic acid species, and amounts of protein (28, 43). Ribosomal proteins are heterogenous (13, 21, 46), direct products of structural cistrons (36, 37, 44) and exhibit sequence and charge similarities between closely related species (21, 45). On the other hand, distantly related species have ribosomal proteins of similar molecular weights (41). Thus far, however, no single method of analysis has proven completely satisfactory in expressing comparative similarities or differences.

Preliminary studies of subunit proteins by column chromatography (27) or total proteins by one-dimensional acrylamide gel electrophoresis (19) have shown that protein profiles from closely related bacteria exhibit more similarities than do profiles from distantly related bacteria. In fact, 60 strains of *Escherichia coli* have been classified into seven groups according to their subunit protein composition (26). These methods have several disadvantages: accurate, unbiased comparisons are difficult; slight variability can occur between analyses; single bands often consist of more than one protein; and it has been difficult to infer any relationship between proteins from two differ-

ent ribosomes except when they are identical. The single-band problem has been solved by the two-dimensional gel electrophoresis technique (20); however, the other problems have not. The problems associated with analysis of total or subunit proteins suggested investigating not only the individual components but also ribosomal topography. Several studies have been initiated recently to determine the topography and intimate structural arrangement of *E. coli* subunit ribosomes (5, 6, 9, 14, 22). Based on trypsin sensitivity and gel electrophoresis, these studies have indicated that proteins are more exposed than ribonucleic acid (RNA) in the 30S subunit, with the reverse being true for the 50S subunit. I have used a more direct method, that is, immunology, to study the topography of intact ribosomes. Immunological techniques are not new to ribosomal investigations; immunology has been used to substantiate the heterogeneity of subunit proteins (11, 39, 40) and the interrelatedness of ribosomal structure and function (14). In a preliminary study, Quash et al. (31) showed that intact ribosomes were immunologically species specific, and Friedman et al. (14) demonstrated that *E. coli* 50S split-protein antiserum reacted only with enterobacterial ribosomes. Subsequently, however, intact ribosomes (24) and subunit proteins (49) have been shown to cross-react with those of unrelated microorganisms.

The purpose of this work was to use two

independent approaches to study the interesting and important problem of relatedness between closely and distantly related bacterial ribosomes. I have analyzed intact ribosomes by Ouchterlony double-diffusion and 50S subunit proteins by disc gel electrophoresis from several isolates of five members of the family *Enterobacteriaceae* plus several other gram-negative rods. A gram-positive coccus was also used for Ouchterlony double-diffusion tests. The family *Enterobacteriaceae* was chosen because of the voluminous taxonomic and genetic information available for comparing results and because of the recent association of *Enterobacter agglomerans* Ewing and Fife (synonym: *Erwinia herbicola*) with human infections (12, 15, 21, 23, 47). Recent genetic studies have shown the

close relationship of *erwiniae* to other *Enterobacteriaceae* (4, 7).

MATERIALS AND METHODS

Bacterial strains and preparation of cells. The bacterial strains used in this study are listed in Table 1. Bacteria were maintained on yeast extract-dextrose-calcium carbonate agar and cultivated in medium 523 (19) at 36 C in 2.4-liter Fernback flasks on a rotary shaker. Early log-phase cells (Klett reading of 30 to 50) were harvested by centrifugation in a refrigerated Sorvall RC2-B centrifuge with a GSA rotor at 10,000 rpm for 10 min. The pellet was washed overnight in 0.85% NaCl, recentrifuged, and stored frozen.

Preparation of ribosomes. Ribosomes were obtained from 2 to 5 g (packed wet weight) of cells. The cells were suspended at 10 C in 10 volumes of TKM/3 buffer [0.01 M tris(hydroxymethyl)aminomethane

TABLE 1. *Bacterial strains*^a

Strain		Origin	Date	Source
<i>Escherichia coli</i>	01a (Antigen type)	Pig		Ewing, CDC
<i>E. coli</i>	01b (Antigen type)	Pig		Ewing, CDC
<i>E. coli</i>	02a (Antigen type)	Pig		Ewing, CDC
<i>E. coli</i>	02b (Antigen type)	Pig		Ewing, CDC
<i>E. coli</i>	04 (Antigen type)	Pig		Ewing, CDC
<i>E. coli</i>	06 (Antigen type)	Standard Strain		Ewing, CDC
<i>Salmonella typhimurium</i>	13311 (ATCC)	Human		Roach Diagnostic Lab
<i>S. typhimurium</i>	4066	Human		Ewing, CDC
<i>S. cholerae-suis</i>	1348	Pig		Ewing, CDC
<i>Klebsiella pneumoniae</i>	15	Human		Ewing, CDC
<i>K. pneumoniae</i>	20	Human		Ewing, CDC
<i>K. pneumoniae</i>	30	Human		Ewing, CDC
<i>Pectobacterium carotovora</i>	14	Calla	1946	Dickey, Cornell
<i>P. carotovora</i>	34	Iris	1946	Dickey, Cornell
<i>P. carotovora</i>	105 (ICPB)			Goodman, Missouri
<i>P. carotovora</i>	150 (ICPB)			Goodman, Missouri
<i>P. carotovora</i>	495	Carrot		American Type Culture Collection
<i>P. carotovora</i>	A-8	Radish	1971	Schaad, Orlando, Fla.
<i>Erwinia rubrifaciens</i>	533 (ICPB 103)	Persian walnut	1963	Wilson, California
<i>E. rubrifaciens</i>	540 (ICPB 104)	Persian walnut	1965	Wilson, California
<i>E. rubrifaciens</i>	602-C (ICPB 105)	Persian walnut	1966	Wilson, California
<i>E. rubrifaciens</i>	605-C	Persian walnut	1967	Schaad, California
<i>E. rubrifaciens</i>	613	Persian walnut	1968	Schaad, California
<i>E. rubrifaciens</i>	668	Persian walnut	1970	Schaad, California
<i>Enterobacter agglomerans</i> (<i>Erwinia herbicola</i>)	4524-71	Human		Ewing, CDC
<i>Xanthomonas campestris</i>	BBs	Brussels sprout		Grogan, California
<i>X. campestris</i>	B24	Broccoli	1972	Moore, Oregon
<i>X. vesicatoria</i>	B201	Tomato	1971	Schaad, Sarasota, Fla.
<i>Pseudomonas syringae</i>	B-3	Peach		English, California
<i>P. marginalis</i>	10844	Lettuce		American Type Culture Collection
<i>Staphylococcus epidermidis</i>	14990 (ATCC)	Human		Roach Diagnostic Lab

^a ATCC, American Type Culture Collection, Rockville, Md.; CDC, Center for Disease Control, Atlanta, Ga.; ICPB, International Collection of Phytopathogenic Bacteria, Univ. of California, Davis, Calif.

(Tris) (Sigma Chemical Co.), 0.05 M KCl, 0.005 M MgCl_2] at pH 7.4. Deoxyribonuclease was added to a final concentration of 2 mg/ml, and the cells were extracted three times at 2,000 lb/in.² under nitrogen in a Parr disruption bomb (Parr Instr. Co.). Cell debris was removed by centrifugation in a Sorvall SS34 rotor at 12,000 rpm for 20 min.

Intact ribosomes were obtained from the low-speed supernatant by centrifuging at 30,000 rpm in a Spinco 30 rotor for 2 h. The pellets were resuspended overnight at 2°C in 5 ml of TNM/3 buffer (0.01 M Tris, 0.5 M NH_4Cl , 0.005 M MgCl_2) at pH 7.4, pooled, and centrifuged at 12,000 rpm. Ribosomes in the supernatant were concentrated by centrifuging at 36,000 rpm in a Spinco 40 rotor for 4 h. The sedimented ribosomes were resuspended in TKM/3 buffer, pooled, and centrifuged at 12,000 rpm. The spectra at 235, 260, and 280 nm were measured, and the ribosomes were stored frozen in TKM/3 buffer.

Washed 70S ribosomes were obtained by placing 100 U (absorption at 260 nm) of intact ribosomes (freshly extracted) onto 10 to 30% linear sucrose TKM/3 gradients and centrifuging for 4 h at 21,000 rpm using a Spinco SW25.1 rotor. The 70S ribosomes were removed with an Isco gradient collector, pooled, diluted 1:1 with TKM/3 buffer, and centrifuged at 27,000 rpm in a Spinco 30 rotor for 4 h. The sedimented ribosomes were resuspended in TKM/3 and were stored frozen after the absorption spectra were measured.

Highly purified 50S subunits were obtained by dialyzing intact ribosomes against TKM/4 (3×10^{-4} M MgCl_2) buffer for 24 h. One hundred units of the dialysate were then layered onto 10 to 30% linear sucrose TKM/4 (10^{-4} M MgCl_2) gradients and centrifuged for 15.5 h at 19,500 rpm using a Spinco SW25.1 rotor. The fractions containing the 50S subunits were removed with a Isco gradient collector, pooled, diluted 1:1 with TKM/4 buffer, and centrifuged in a Spinco 30 rotor for 4 h at 27,000 rpm. The sedimented subunits were resuspended in TKM/4 buffer, the absorption spectra were measured, and the suspension stored frozen.

Crude ribosomes were extracted as above but from cells grown in 250-ml lots. The resuspended cells (0.1 to 0.2 g) were disrupted at 5 to 10°C for 2 min with a Bronwill Biosonik III at an intensity setting of 60. The suspension was then centrifuged at 12,000 rpm in a Sorvall RC2-B centrifuge with a SS34 rotor, the supernatant was removed, and the ribosomes were pelleted by centrifuging at 36,000 rpm in a Spinco 40 rotor for 2 h. The ribosomes were resuspended overnight in 2 ml of TKM/3 buffer, whereupon the suspension was clarified by a centrifugation at 12,000 rpm and stored frozen after the absorption spectra were measured. Crude ribosomes typically had a 260/235 ratio of 1.60, whereas the washed ribosomes had a ratio of 1.85.

Extraction of ribosomal protein. Ribosomal proteins were prepared from 50S subunits suspended in TKM/4 buffer by the chilled acetic acid method of Hardy et al. (17). The freeze-dried proteins were stored in a desiccator at -40°C.

Gel electrophoresis of subunit proteins. Disc gel electrophoresis of ribosomal proteins in 7.5% polyacrylamide gels at pH 4.3 was carried out as described

previously (19), but with a Buchler Polyanalyst. Five micrograms of protein was used in each electrophoretic run.

Analysis of protein bands. The positions of the stained protein bands in the gel were recorded by scanning the gel at 560 nm using a 0.05-mm slit width in a Gilford Linear Transport attached to a Beckman DU monochromator and a Gilford model 222 photometer.

Immunology. Washed ribosomes were emulsified 1:1 (vol/vol) with Freund incomplete adjuvant (Difco), and rabbits were injected intraperitoneally with 1 to 1.5 mg of ribosomes five times at 10-day intervals. The concentration of ribosomes was based on a specific extinction coefficient of 157 for a 1% solution at 260 nm (42). A booster injection was given 7 days after bleeding if the titer was less than 512. The total amount of antigen injected into the rabbit over the whole immunization period was 6 to 10.0 mg. Rabbits were bled 7 days after the last injection and, if needed, 7 days after the booster injection. Antisera were stored at -20°C.

Titration were performed in 0.5-ml test tubes. Eleven replicate twofold dilutions of each normal serum and of each antiserum were prepared in a final volume of 0.25 ml. Incubation was at 37°C and observation for agglutination was made after 4 and 18 h. The end point of each titration was defined as the highest dilution with a precipitation on the bottom of the tube.

For Ouchterlony double-diffusion experiments, 0.75 g of purified agar (Difco), 0.02 g of sodium azide, 0.85 g of NaCl, 2.0 g of MgCl_2 , and 1.0 g of Trypan blue were dissolved in 100 ml of distilled water, and the mixture was autoclaved. A 10-ml amount of the melted agar was added to each 88-mm diameter plastic petri plate. The distance from the edge of the central well to the surrounding wells was 11 mm. In all experiments unless stated otherwise, the center well contained 5 to 20 μl of undiluted normal serum or antiserum, and the outer wells contained 10 to 20 μl of antigen. Antigens consisted of intact or crude ribosomes and were adjusted to 3 mg/ml. The optimal antigen-antibody concentrations were determined by the diffusion method of Piazzi (29). The plates were incubated in a moist chamber at 8°C and observed after 72 h.

In tabulating double-immunodiffusion patterns, only the specific antigen precipitin line (see Table 4) was scored as complete identity (++). For the nonspecific precipitin line (see Table 3), no distinction was made between reactions of identity and partial identity; both are recorded as "+."

RESULTS

Disc gel electrophoresis. The *Enterobacteriaceae* strains studied had similar over-all profiles in that the protein bands were clustered into two major groups and a minor group (Fig. 1 and 2). The first group had four to five distinct bands, and the second group had three to five bands, separated from the first by a conspicuous space. A minor group of weakly stained diffuse bands occupied the lower third of the gel. The results showing that the strains of *E.*

FIG. 1. Comparative gel scans and corresponding acrylamide gels of 50S ribosomal proteins from strains of four enterobacteria genera: (A) *Salmonella typhimurium*, (B) *Klebsiella pneumoniae*, (C) *Escherichia coli* 01a, and (D) *Enterobacter agglomerans* (*Erwinia herbicola*). Arrows denote the three apparently common enterobacteria bands.

coli and *Salmonella typhimurium* had very similar 50S ribosomal protein profiles agreed with the results of others (25, 26).

Profiles from two strains each of *E. coli* (Fig. 2A, D), *Pectobacterium carotovora* (Fig. 2B, E), and *Erwinia rubrifaciens* (Fig. 2C, F) illustrate profile similarities and differences between the strains. For example, there were minor variations in the uppermost group of bands of *E. coli* strains 01a (A) and 04 (D). Even greater differences were present in the second group of bands of *P. carotovora* strains 105 (B) and 150 (E). These minor differences did not represent reproducible differences and therefore are not of any comparative value. Such minor variations also occurred from time to time in different electrophoretic runs of the same protein sample. For these reasons it was not possible to make accurate comparisons between different strains or species on the basis of individual bands within the two major band groups. On the other hand, the data suggested that similarities and differences exist between the enterobacteria with respect to the minor group of weakly stained, diffuse bands. *S. typhimurium* (Fig. 1A), *Klebsiella pneumoniae* (Fig. 1B), *E. coli* (Fig. 1C), and *Enterobacter agglomerans* (Fig. 1D) profiles were nearly identical in that each had three clearly resolved bands (arrows, Fig. 1A). Strains of plant origin, *P. carotovora* 150 (Fig. 2B, E) and *E. rubri-*

FIG. 2. Comparative gel scans and corresponding acrylamide gels of 50S ribosomal proteins from several enterobacteria strains: (A) *Escherichia coli* 01a, (D) *E. coli* 04, (B) *Pectobacterium carotovora* 105, (E) *P. carotovora* 150, (C) *Erwinia rubrifaciens* 540, (F) *E. rubrifaciens* 533. Note the similarities in the faster-migrating bands (to the right) of each isolate pair.

faciens 533 (Fig. 2C, F), also had nearly identical profiles. There were, however, clear differences between the two groups of organisms. Whereas the strains of animal origin had only three bands, those of plant origin had five clearly resolved bands.

To determine whether an organism unrelated to the *Enterobacteriaceae* might also have similar profiles, several strains belonging to the family *Pseudomonadaceae* were analyzed. The results showed that *Xanthomonas vesicatoria* (Fig. 3A), *Xanthomonas campestris* (Fig. 3C), *Pseudomonas marginalis* (Fig. 3B), and *Pseudomonas syringae* (Fig. 3D) indeed had over-all profiles which were similar to those of members of the family *Enterobacteriaceae*. In addition, the lower diffuse bands which appeared useful in differentiating the plant and animal enterobacteria were also somewhat similar. There was, however, a striking difference with respect to the area between the two major groups of bands. Whereas the enterobacteria profiles were void of any band between the two major band groups, the pseudomonad profiles contained one to two additional clearly resolved bands (Fig. 3).

Immunology. Ribosome preparations derived from the 25 enterobacteria and 4 non-enterobacteria strains were analyzed by Ouchterlony double diffusion against antisera to purified ribosomes of 7 enterobacteria and one non-enterobacterium (see Tables 3 and 4). Homologous systems produced one to three precipitin

FIG. 3. Gel scan and corresponding acrylamide gel of 50S ribosomal proteins of four members of the family *Pseudomonadaceae*: (A) *Xanthomonas vesicatoria*, (B) *Pseudomonas marginalis*, (C) *Xanthomonas campestris*, and (D) *Pseudomonas syringae*.

FIG. 4. Ouchterlony double-diffusion patterns of ribosomes from (1) *Escherichia coli* 01a, (2) *Salmonella typhimurium* 13311, (3) *Erwinia rubrifaciens* 533, (4) *Xanthomonas campestris* BBs, (5) *Pectobacterium carotovora* 150, and (6) *Klebsiella pneumoniae* 20. Center wells contained antiserum to ribosomes of (A) *E. coli* 01a, (B) *S. typhimurium* 13311, (C) *X. campestris*, and (D) *E. rubrifaciens*.

lines (Fig. 4). The precipitin which occurred as a single line near the antigen well, hereafter referred to as R-1 antigen, was specific at the subspecies level, whereas the precipitin which occurred as one to several lines midway between the two wells, hereafter referred to as the R-2 antigen, was common to most of the enterobacteria (Fig. 4). When antigens were crudely extracted, no additional lines occurred, and in no case did a line occur between antisera and concentrated high-speed supernatants.

To determine whether the R-1 and R-2 antigens were proteins or ribonucleic acid, 0.3 mg of ribosomes was treated separately with 10 μ g of trypsin and pancreatic ribonuclease for 48 h at 24 C. In addition, ribosomes were incubated at 90 C for 1 h. The results of testing treated ribosome preparations of *E. coli*, *S. typhimurium*, *K. pneumoniae*, *P. carotovora*, and *Erwinia rubrifaciens* are given in Table 2, and typical Ouchterlony gel-diffusion tests are shown in Fig. 5. It was apparent that the topography of these ribosomes was not universal but varied with the species. For example, with respect to the R-1 determinant, *E. coli* (Fig. 5A) was sensitive to trypsin and heat but not to ribonuclease; *P. carotovora* (Fig. 5C) was sensitive to all three treatments; and *S. typhimurium* (Fig. 5B) and *Erwinia rubrifaciens* were insensitive to all three treatments.

The R-2 determinant was responsible for

TABLE 2. Characterization of antigenic determinants R-1 and R-2^a

Antigen from	Digestion with trypsin		Digestion with ribonuclease		Heat sensitivity	
	R-1	R-2	R-1	R-2	R-1	R-2
<i>Escherichia coli</i>	+	+	—	+	+	—
<i>Salmonella typhimurium</i>	—	+	—	—	—	—
<i>Erwinia rubrifaciens</i>	±	+	—	+	—	+
<i>Pectobacterium carotovora</i>	+	+	+	+	+	—
<i>Klebsiella pneumoniae</i>		+		+		—

^a Symbols: +, precipitin line absent in homologous Ouchterlony double-diffusion tests; ±, weak line of precipitin; —, precipitin line present. Precipitin was scored as stated in Materials and Methods.

FIG. 5. Immunological reactions of R-1 and R-2 antigenic determinants after pretreatment of ribosomes with trypsin, ribonuclease, and heat. Center wells contained antiserum to ribosomes of (A) *Escherichia coli*, (B) *Salmonella typhimurium*, (C) *Pectobacterium carotovora*, and (D) *Erwinia rubrifaciens*. Outer wells in each pattern contained homologous ribosomes (1, 3, and 4) untreated, (2) trypsin treated, (5) ribonuclease treated, and (6) heat treated.

cross-reactions between most all enterobacteria strains tested. Whereas antisera to the seven enterobacteria isolates reacted against most enterobacteria ribosome preparations, no reaction occurred against ribosomes from three gram-negative rods and a gram-positive coccus (Table 3). However, due to the absence or weakness of the R-2 reaction between some enterobacteria systems, the usefulness of this antigen must await further investigations.

On the contrary, reactions of the R-1 determinant were highly consistent. A line of precipitin always occurred between homo-

gous systems, with the exception of *K. pneumoniae*. In no case did the R-1 reaction occur between antiserum to ribosomes of one genus or species and ribosomes of a different genus or species (Table 4 and Fig. 4). To determine the heterogeneity of the R-1 determinant at the subspecies level, ribosomes from several isolates of four different species were compared.

E. coli. Six *E. coli* isolates, each representing a different somatic antigen group, were chosen so that comparisons could be made with a known taxonomic scheme. Antigen for injection was obtained from isolate 01a; intact ribosomes were from 01a, 04, and 06; and crude ribosomes were from 01b, 02, and 02b. The results showed that a precipitin line identical to that of the homologous system occurred only with 01b ribosomes. No R-1 reaction occurred with ribosomes from any of the other strains (Fig. 6A). These results indicate that the R-1 determinant is subspecies specific and appears to parallel differences in somatic antigens.

S. typhimurium. Ribosomes from two *S. typhimurium* isolates and one *Salmonella cholerae-suis* isolate (Table 1) were tested against a single antiserum to *S. typhimurium* ribosomes. An identical line of precipitin occurred with both *S. typhimurium* strains, whereas no reaction occurred with ribosomes from *S. cholerae-suis* (Fig. 6B).

P. carotovora. Antigens from isolates 34 and 150 were used for injection, and four additional isolates (Table 1) were used for antigens. The results showed that only in the homologous system did an R-1 reaction occur (Table 4 and Fig. 6C). These results suggest that *P. carotovora*, like *E. coli*, has a highly evolved R-1 determinant or that the strains, even though pectolytic, may not belong to *P. carotovora*.

E. rubrifaciens. Two isolates (533 and 540) were used to prepare antisera. When intact ribosomes from cultures 533, 540, and 602-C were tested against either antiserum, identical lines of precipitin occurred. To test this apparent homogeneity among strains, crude

TABLE 3. Reactions of the R-2 determinant as detected by Ouchterlony immunodiffusion^a

Ribosomes from	Ribosomal antisera						
	<i>Esche- richia coli</i>	<i>Salmo- nella typhi- murium</i>	<i>Klebsiella pneumoniae</i>	<i>Pectobacterium carotovora</i>		<i>Erwinia rubri- faciens</i>	<i>Xantho- monas campestris</i>
	01a	13311	20	34	150	533	BBs
<i>Escherichia coli</i> 01a	+	+	+	+	+	+	—
<i>E. coli</i> 01b	+	—	+	+	+	+	—
<i>E. coli</i> 02a	+	—	+	—	—	+	—
<i>E. coli</i> 02b	+	—	+	—	—	+	—
<i>E. coli</i> 04	+	—	+	+	+	—	—
<i>E. coli</i> 06	+	—	+	+	—	—	—
<i>Salmonella typhi- murium</i> 13311	+	+	+	+	+	+	—
<i>S. typhi- murium</i> 4066	+	+	—	—	+	+	—
<i>S. cholerae-suis</i>	+	+	+	—	+	+	—
<i>Klebsiella pneumoniae</i> 15	+	—	+	+	+	—	—
<i>K. pneumoniae</i> 20	+	+	—	—	—	+	—
<i>K. pneumoniae</i> 30	+	—	+	+	—	—	—
<i>Pectobacterium carotovora</i> 34	+	—	+	+	—	—	—
<i>P. carotovora</i> 14	+	—	+	+	+	+	—
<i>P. carotovora</i> 105	+	+	+	+	—	—	—
<i>P. carotovora</i> 150	+	+	+	+	+	+	—
<i>P. carotovora</i> 495	+	—	—	—	+	+	—
<i>P. carotovora</i> A-8	+	+	—	+	—	—	—
<i>Erwinia rubrifaciens</i> 533	+	+	+	—	+	+	—
<i>E. rubrifaciens</i> 540	+	+	+	—	+	+	—
<i>E. rubrifaciens</i> 602-C	+	+	+	+	—	+	—
<i>E. rubrifaciens</i> 605-C	+	+	+	—	+	+	—
<i>E. rubrifaciens</i> 613	+	—	—	—	—	+	—
<i>E. rubrifaciens</i> 668	+	+	+	—	+	+	—
<i>Xanthomonas campestris</i> BBs	—	—	—	—	—	—	+
<i>X. campestris</i> B-24	—	—	—	—	—	—	+
<i>Pseudomonas syringae</i> B-3	—	—	—	—	—	—	—
<i>Staphylococcus epidermidis</i> 14990	—	—	—	—	—	—	—

^a +, Reaction of identity or partial identity; —, no reaction.

extracts from three additional isolates (Table 1) were obtained. These isolates also gave identical lines of precipitin when tested against anti-serum to 540 (Fig. 6D) or 533 ribosomes. These results are in contrast to those obtained with *E. coli* and *P. carotovora* and may indicate a highly conserved ribosomal genome in *E. rubrifaciens*.

DISCUSSION

The data presented above show that the analysis of 50S ribosomal subunit proteins by disc gel electrophoresis and 70S ribosomes by Ouchterlony double-diffusion have certain values and limitations as taxonomic tools. Whereas the gel electrophoresis data varied somewhat

and were difficult to interpret, the immunological data were consistent and required little explanation. That immunodiffusion was found to be more sensitive than gel electrophoresis was not entirely unexpected. It is known, for example, that immunodiffusion can detect a 4.0% divergence in an amino acid sequence (30).

Disc gel electrophoresis. The electrophoresis

data failed to differentiate clearly the closely related *E. coli*, *S. typhimurium*, *K. pneumoniae*, and *Enterobacter agglomerans*. Whether this failure was due to inadequate resolution of certain proteins or a true similarity in the size and charge of 50S ribosomal proteins of different species is not known. The data did, however, suggest that enterobacteria could be differentiated according to animal or plant

TABLE 4. Reactions of the R-1 determinant as detected by Ouchterlony immunodiffusion^a

Ribosomes from	Ribosomal antisera							
	<i>Esche- richia coli</i>	<i>Salmo- nella typhi- murium</i>	<i>Klebsiella pneumoniae</i>	<i>Pectobacterium carotovora</i>		<i>Erwinia rubrifaciens</i>		<i>Xantho- monas campestris</i>
	01a	13311	20	34	150	533	540	BBs
<i>Escherichia coli</i> 01a	++	—	—	—	—	—	—	—
<i>E. coli</i> 01b	++	—	—	—	—	—	—	—
<i>E. coli</i> 02a	—	—	—	—	—	—	—	—
<i>E. coli</i> 02b	—	—	—	—	—	—	—	—
<i>E. coli</i> 04	—	—	—	—	—	—	—	—
<i>E. coli</i> 06	—	—	—	—	—	—	—	—
<i>Salmonella typhi- murium</i> 13311	—	++	—	—	—	—	—	—
<i>S. typhi- murium</i> 4066	—	++	—	—	—	—	—	—
<i>S. cholerae-suis</i>	—	—	—	—	—	—	—	—
<i>Klebsiella pneumoniae</i> 15	—	—	—	—	—	—	—	—
<i>K. pneumoniae</i> 20	—	—	—	—	—	—	—	—
<i>K. pneumoniae</i> 30	—	—	—	—	—	—	—	—
<i>Pectobacterium carotovora</i> 34	—	—	—	++	—	—	—	—
<i>P. carotovora</i> 14	—	—	—	—	—	—	—	—
<i>P. carotovora</i> 105	—	—	—	—	—	—	—	—
<i>P. carotovora</i> 150	—	—	—	—	++	—	—	—
<i>P. carotovora</i> 495	—	—	—	—	—	—	—	—
<i>P. carotovora</i> A-8	—	—	—	—	—	—	—	—
<i>Erwinia rubrifaciens</i> 533	—	—	—	—	—	++	++	—
<i>E. rubrifaciens</i> 540	—	—	—	—	—	++	++	—
<i>E. rubrifaciens</i> 602-C	—	—	—	—	—	++	++	—
<i>E. rubrifaciens</i> 605-C	—	—	—	—	—	++	++	—
<i>E. rubrifaciens</i> 613	—	—	—	—	—	++	++	—
<i>E. rubrifaciens</i> 668	—	—	—	—	—	++	++	—
<i>Xanthomonas cam- pestris</i> BBs	—	—	—	—	—	—	—	++
<i>X. campestris</i> B-24	—	—	—	—	—	—	—	++
<i>Pseudomonas syringae</i> B-3	—	—	—	—	—	—	—	—
<i>Staphylococcus epidermidis</i> 14990	—	—	—	—	—	—	—	—

^a ++, Complete identity; —, no reaction; partial identity reaction (+) not observed.

FIG. 6. Immunodiffusion reactions of ribosomes from several isolates of the same species. Center well contained antiserum to ribosomes from: (A) *Escherichia coli* 01a, (B) *Salmonella typhimurium* 13311, (C) *Pectobacterium carotovora* 150, and (D) *Erwinia rubrifaciens* 540. Outer wells of A contained ribosomes from (1) *E. coli* 01a, (2) 01b, (3) 06, (4) 02b, (5) 02a, and (6) 04. Outer wells of B contained ribosomes from *S. typhimurium*: (1) 13311, (2) 13311, (3) 4066, (5) 4066, and (6) 13311. Well 4 of B contained ribosomes from *Salmonella cholerae-suis*. Outer wells of C contained ribosomes from *P. carotovora*: (1) 150, (2) 14, (3) 34, (4) 105, (5) 495, and (6) A-8. Outer wells of D contained ribosomes from *E. rubrifaciens*: (1) 540, (2) 533, (3) 602-C, (4) 605-C, (5) 613, and (6) 668.

origin. Both plant pathogenic organisms studied had two additional faster-migrating proteins. These differences were sufficiently large and reproducible to warrant further investigations to determine whether these particular proteins might serve to differentiate plant and animal enterobacteria. There were minor differences in profiles of different strains, but these differences were not reproducible and therefore were not considered to be taxonomically valuable.

Even though profiles of several strains of a different family were distinguishable from enterobacteria profiles, all had similar over-all profiles. These results conflict with earlier results which showed that ribosomal proteins of *erwiniae* distributed into definitive over-all patterns which did not exist in strains of a different family (19).

Several researchers (19, 26, 27) have suggested that acrylamide gel electrophoresis of ribosomal proteins might be a useful taxonomic

tool. The results here suggest that disc gel electrophoresis data of 50S ribosomal proteins alone are not adequate taxonomic criteria. On the contrary, the data suggest that ribosomal proteins of similar size and charge may exist among unrelated organisms. Even if small differences in protein size and charge could be measured statistically, the taxonomic significance of such small differences would be doubtful. More research is needed to identify each protein band. For example, the use of specific 50S antiribosomal protein(s) in combination with gel profiles might allow more accurate gel comparisons.

Immunology. Ribosomes of most of the strains tested had two immunogenic determinants: one specific at the subspecies level, designated R-1, and one partially specific at the family level, designated R-2. Whereas the R-2 determinant was of questionable taxonomic value because of inconsistencies and variability, the R-1 determinant was extremely reliable and was either present or absent. The failure of ribosomes of one strain to react with antiserum to ribosomes of a strain from a different species demonstrates the unique specificity of ribosomal topography.

Research into the specificity of ribosomal topography is recent, and the results sometimes conflict. Ribosomes were first thought to be immunogenically species specific (14, 31); however, subsequent investigations have shown otherwise. For example, antisera to *E. coli* ribosomal proteins cross-reacted with ribosomal proteins of *Neurospora crassa* (1, 32). Also, antisera to ribosomes of *E. coli*, their subunits, core particles, or split protein reacted as strongly with *Proteus*, *Salmonella*, or *Serratia* ribosomes as in the homologous system and to a lesser degree even with ribosomes from *Azotobacter*, *Lactobacillus*, or *Sarcina* strains (49). With the exception of a positive reaction between mouse and chicken ribosomes, Noll and Bielka (24) found that intact ribosomes from different animal tissues reacted only with homologous antisera. The discovery by Noll and Bielka that ribosome structure was more specific than extracted proteins may explain some of the discrepancies in the literature. I have found that intact ribosomes are not specific at the species level as suggested by Noll and Bielka but instead are specific at the subspecies level. My results agree favorably with molecular genetic studies that used several of the same isolates. For example, using deoxyribonucleic acid (DNA) relatedness, Brenner et al. (3) found a 17% divergence in the DNAs of *E. coli* 01a and 02a. Since I found that *E. coli*

01a and 02a ribosomes did not react specifically, one could conclude that ribosome immunology is capable of detecting a DNA divergence of less than 17%. The lack of immunological relatedness between the six *P. carotovora* isolates agrees with the confused taxonomy of this species (16) and with the finding that *P. carotovora* has a guanine plus cytosine (G:C) base ratio which ranges from 51.0 to 54.0 (38). However, another interpretation could be that all the isolates studied were not *P. carotovora*. Finally, the immunological identity of the six *E. rubrifaciens* strains agrees with the biochemical homogeneity of *E. rubrifaciens* (Ewing, personal communication) and also with other genetic data. It has been reported that isolates 533, 540, and 602-C have a G:C base ratio with a range of less than 0.6% (38).

That *E. rubrifaciens* might have a highly conserved genome is also supported by ecological data. *E. rubrifaciens* was discovered in 1962 (48) and has not been reported outside California. Also, each isolate used in this study was obtained from a different geographic location over a period of 8 years (Table 1). In addition, the only known host of *E. rubrifaciens*, Persian walnut, cultivar Hartley, was originally propagated in 1940 and not extensively planted until the 1950's. The apparent limitation within California is supported by an extensive survey conducted in 1970 by C. I. Kado and myself, in cooperation with the Oregon State University Agricultural Extension Service, which included almost all mature Hartley walnut orchards known to exist outside California. Furthermore, the pathogen is limited to the nonfunctional phloem tissue of mature trees; it will not cause a disease in 3-year-old trees nor in primary tissue of mature trees (33, 34). Finally, *E. rubrifaciens* is not capable of surviving as a saprophyte (34).

It is suggested that ribosomal topography, as revealed by immunodiffusion, is an excellent indicator of the evolutionary state of an organism. It is suggested further that because of its apparently conserved genome, *E. rubrifaciens* is an ideal organism for studying the influence environment has on genetic variability of an organism. Finally, ribosomal topography appears to be a more accurate indicator of intraspecific phylogenetic relatedness than do individual ribosomal components, such as subunit proteins.

Besides being a valuable taxonomic tool, ribosomal immunology should also help in elucidating the ribosome's topography. Little is known about the structural relationships be-

tween ribosomal RNA (rRNA) and protein. Proposals of topographical models of the ribosome suggest a helical rRNA packed with globular molecules of protein (10) or each subunit having an equally exposed protein and RNA moiety (8). The most recent investigations on ribosomal topography show, however, that proteins are more exposed on the 30S subunit (5), whereas the opposite is true for the 50S subunit (6). These contradictory results are in line with the extreme immunogenic heterogeneity found in this study between ribosomes of strains of the same species.

Ribosome immunology may have yet another important application, that is, as an identification tool for diagnostic laboratories. For example, an antiserum for a specific pathogen or a pool of several antisera could be used to obtain a quick identification of an isolate, thereby expediting proper treatment.

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