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Cross-Reactivity of Phenylalanyl-Transfer Ribonucleic Acid Ligases from Different Microorganisms

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The cross-reaction of phenylalanyl-transfer ribonucleic acid (tRNA) ligases from different microorganisms with antibodies raised against the purified enzyme from *Escherichia coli* has been investigated. The results of immunotitration and immunodiffusion experiments and of the sodium dodecyl sulfate-polyacrylamide gel electrophoretic analysis of immunoprecipitates revealed: (i) a high degree of immunochemical identity of this enzyme only within the family *Enterobacteriaceae*; (ii) intermediate-to-weak cross-reaction with the phenylalanyl-tRNA ligases from *Pseudomonadaceae*, *Rhodopseudomonas spheroides*, and *Bacillus stearothermophilus*; (iii) no detectable cross-reaction (with the methods employed) with the enzymes from several gram-positive organisms, *Euglena gracilis*, and several fungi. As revealed by immunochemical analysis, a merodiploid strain of *E. coli* carrying an episome (F148) that covers the *aroD* region of the *E. coli* chromosome possesses at least twice the amount of phenylalanyl-tRNA ligase in comparison with its haploid parent strain. This suggests that the cistrons for both the α and β polypeptides of this enzyme are mapping in this area.

Phenylalanyl-transfer ribonucleic acid (tRNA) ligase (PRS; EC 6.1.1.20) from *Escherichia coli* belongs to that class of aminoacyl-tRNA ligases possessing an apparent $\alpha_2\beta_2$ subunit structure (4, 7). The same type of subunit composition has been demonstrated for this enzyme from *Saccharomyces cerevisiae* (3, 14), rat liver (16), and *Salmonella typhimurium* (P. W. Schiller and A. N. Schechter, Fed. Proc., vol. 34, abstr. 1490). The sizes of the α and β polypeptide chains are very similar in the case of the enzymes from *E. coli* and *S. typhimurium* but differ greatly from those found for the yeast and rat liver enzymes.

The conservation of the $\alpha_2\beta_2$ subunit structure of PRS in these organisms prompted us to study the serological relatedness of this enzyme in different organisms. One of the questions one could ask is whether certain structural features such as the sites for phenylalanine, adenosine 5'-triphosphate, or $tRNA^{Phe}$ binding are maintained in enzymes from different organisms and whether this is revealed by immunological cross-reactivity. Moreover, in case of cross-reaction it should be possible to obtain immunoprecipitates from crude extracts by using antibodies against purified phenylalanyl-tRNA ligase from one organism; an analysis of these immunoprecipitates by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis could then tentatively indicate the type of subunit structure of the cross-reacting enzymes.

Equally, immunochemical analysis of the amount of PRS synthesized in merodiploid strains is supposed to be a valuable tool to detect whether the chromosome segment of *E. coli* coding for the α polypeptide chain (1, 8) also contains the cistron for the β subunit.

MATERIALS AND METHODS

Media and growth conditions. All chemoorganotrophic bacteria and *Euglena gracilis* were grown aerobically at 30 C in a rich medium (TGYE) containing 1% tryptone, 0.5% yeast extract, and 0.2% glucose. *Bacillus stearothermophilus* was cultivated in a medium consisting of 2% tryptone, 1% yeast extract, 0.5% glucose, and 0.5% NaCl at 55 C. For the preparation of crude extracts from yeast, a commercial preparation of bakers' yeast was used. *Rhodopseudomonas spheroides* was grown in medium 27 as recommended by the German Collection of Microorganisms (6). The minimal medium used was salt solution P of Fraenkel and Neidhardt (5) supplemented with 0.2% ammonium sulfate and 0.4% glucose. The cells were harvested in the exponential phase of growth by chilling

and centrifugation. The pellets were washed once in cold PRS buffer containing 20 mM tris(hydroxymethyl)aminomethane-hydrochloride (pH 7.5), 30 mM NH_4Cl, 10 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid, and 6 mM 2-mercaptoethanol. For the isolation of S100 extracts from *E. gracilis*, the PRS buffer was made 40% in glycerol.

Preparation of crude extracts. The cells obtained from 1 liter of growth medium were suspended in 3 ml of PRS buffer containing 5 μg of deoxyribonuclease I (Boehringer Mannheim) per ml. They were broken by passage through a French pressure cell at 18,000 lb/in^2. Cell debris was subsequently removed by centrifugation for 2 h at 45,000 rpm in a Spinco Ti75 rotor at 2 C. The protein contents of these S100 extracts were then determined by the Folin phenol procedure (11), with bovine serum albumin as the standard.

Preparation of antiserum. Rabbits were immunized against PRS from *E. coli* K10 by subcutaneous injection of 300 to 500 μg of purified enzyme or subunit protein (7, 8) that was mixed thoroughly with the complete form of Freund adjuvant in a volume ratio of 1:1. After 5 weeks the same amount of protein was again injected per animal in combination with Freund incomplete adjuvant. One week after this booster injection about 40 ml of blood was collected from the ear vein of each animal. The blood was allowed to clot by standing for 24 h at 4 C. Erythrocytes were then removed by low-speed centrifugation at 4 C (10 min, 3,000 rpm), and the immunoglobulin fraction was obtained by three consecutive ammonium sulfate precipitations, at 33% saturation, performed at room temperature. The sediment of the last precipitation step was taken up in half of the original volume of 1 mM potassium phosphate buffer (pH 8.0) containing 0.9% sodium chloride and was finally dialyzed overnight against the same buffer.

Titration of PRS activity with antibodies. In a typical experiment, 500 μg of crude extract protein from each strain was incubated either with or without antibodies usually for 60 min at 37 C. Total incubation volume was 0.3 ml. The incubation mixture also contained 50 μg of bovine serum albumin. A 1:100 dilution of the immunoglobulin fraction was used for the titration experiments; dilution buffer was 10 mM potassium phosphate (pH 7.5) containing 0.2 mM ethylenediaminetetraacetic acid. The incubation temperature of 37 C caused inactivation of the PRS activity of several strains (e.g., *Streptococcus faecalis* and *B. subtilis*). In this case incubation was done at 0 C. No differences were revealed with the homologous *E. coli* system as the control and an incubation temperature of 37 C.

At the end of this incubation period, suitable samples were taken and assayed for PRS activity in the test system described by Kosakowski and Böck (9). Crude tRNA from *E. coli* was used in the tests for all organisms except yeast; in this case, crude tRNA from brewers' yeast (Boehringer, Mannheim) was employed.

Immunodiffusion. Immunodiffusion was performed in 1% agar (Difco Noble agar) made up in 10 mM sodium borate (pH 7.8) containing 1 mM sodium azide. Plates were allowed to develop for 48 h at 30 C; they were then rinsed with 0.9% sodium chloride at room temperature for 2 days. Staining was performed by the addition of 1% amido black in 7% acetic acid (for 2 h); destaining was with 7% acetic acid.

Formation and SDS-gel electrophoresis of immunoprecipitates. For the isolation of PRS-immunoprecipitates, 8 mg of protein from crude extracts was first reacted for 24 h at 0 C with 0.35 ml of the immunoglobulin fraction of nonimmune gamma globulins to precipitate all unspecifically adsorbing proteins. After clarification of this incubation mixture by centrifugation at 20,000 × *g* for 20 min, 0.20 ml of the globulin fraction of immunoserum was added. The amount of immunoserum that gives quantitative precipitation of the cross-reacting material was determined for each batch of antiserum in preliminary experiments. Addition of an excess of immunoglobulins results in the appearance of unspecifically precipitated protein bands in the SDS-gels. The precipitate was again allowed to form for 24 h at 0 C. It was collected by centrifugation at 20,000 × *g* for 10 min and then washed three times with 5 ml of 10 mM potassium phosphate buffer (pH 7.5). A 0.3-ml volume of SDS-containing cracking buffer (15) was then added to each sediment. The samples were heated in a boiling-water bath for 4 min and cooled to room temperature. Portions (20 μl) were then loaded onto the SDS-gels. A mixture of standard proteins and a sample of purified PRS from *E. coli* were treated in the same way. SDS-slab gel electrophoresis was performed in the apparatus described by Studier (15). Compositions of the stacking and separation gels were as described by Studier (15).

RESULTS

Analysis of PRS cross-reacting material from different strains of E. coli. It has been shown recently that the site on the *E. coli* chromosome (*pheS*) responsible for *p*-fluorophenylalanine-resistant PRS activity (1) codes for the α subunit of PRS (7). To obtain information as to whether the cistron for the β subunit codes in the same area of the chromosome we investigated the specific activity and immunochemical behavior of PRS from *E. coli* KLF48/KL159, which carries an episome (F148) covering part of the *his-aroD* region and thereby possibly also the *pheS* area. Table 1 shows the results of the determination of specific PRS activity of this strain, of its haploid derivative KL159, and of strain K10, which up to now has been used by us for the isolation of PRS. It is clear that the merodiploid strain exhibits about twice the specific PRS activity when grown in a rich medium and more than three times the specific activity of the haploid strain in extracts prepared from minimal medium-grown cells. The difference in specific PRS activity of all

three strains grown in a rich medium or in a minimal medium reflects the metabolic regulation of aminoacyl-tRNA synthetase formation (12).

To determine whether the increase in specific activity in the diploid strain was due to the gene dosage of the α and β cistrons, we titrated crude extracts with antiserum against total PRS and with antisera against purified α and β subunits. The results (Fig. 1) show that the merodiploid strain seems to possess at least twice the amount of cross-reacting material than the haploid strain in all three titration experiments.

Since immunotitration experiments of enzyme activity by PRS-inactivating antibodies are not necessarily a measure for the quantity of cross-reacting material, we determined the amount of immunoprecipitates obtained from equal amounts of crude-extract protein of the three strains by precipitation with three different immunoglobulin concentrations. The precipitates were washed, dissolved in SDS-containing buffer, and analyzed by gel electrophoresis. The α and β polypeptides were clearly separated from the heavy and light chains of the immunoglobulins (Fig. 2). There is one polypeptide band migrating very close to the β chain. The amount of this co-precipitate can be reduced by preadsorption of the extracts with nonimmune gamma globulins and by reduction of the ratio of antiserum to crude-extract protein. Scanning of cylindrical gels run from the same material showed that episome-carrying strain KLF48/KL159 contained exactly twice the amount of α amd β chains than its haploid parent strain KL159. The ratio of the amount of the enzyme from strain K10 to that of KLF48/KL159 was 1:1.4.

TABLE 1. *Specific PRS activity of S100 extracts from E. coli strains K10, KL159, and KLF48/KL159 in glucose minimal medium supplemented with the required metabolites and in a rich medium (TGYE) at 30 C*

Strain	Genotype	PRS activity (nmol/mg per h)	
		Minimal medium	Rich medium
K10	*rel-1 tonA22* T_2^R	100	274
KL159[a]	*thi-1 his-4 aroD5 proA2 recA1 xyl-5*? or *xyl-7*? *nalA12 tsx-1*? or *tsx-29*?λ^- *supE44*?	108	255
KLF48/KL-159	Like KL159 with episome 148	354	570

[a] From the Coli Genetic Stock Center at Yale University. Episome KLF-48 carries a deletion between *his* and *cheA* (10).

Cross-reactivity of phenylalanyl-tRNA ligases from different organisms. The serological relatedness of PRS from a wide range of different microorganisms has been tested by three different techniques. The typical results obtained with these methods are illustrated in Fig. 3 and 4; the results obtained for all the organisms investigated are summarized in Table 2.

The first technique consisted of immunotitration of the PRS activity of these organisms with antibodies induced against *E. coli* PRS. According to their inactivation response, three different groups of microorganisms can be discerned. The first group possesses an enzyme that yields the same type of inactivation curve as that obtained with the PRS from *E. coli*; the enzyme from *Serratia marcescens* is a representative of this group (Fig. 3). Table 2 indicates that the enzymes from all *Enterobacteriaceae* tested react in the same way.

The second group of organisms is characterized by intermediate reactivity. *Pseudomonas aeruginosa* and *B. stearothermophilus* (Fig. 3) belong to this group, as well as *P. testosteroni*, *P. acidivorans*, and *R. spheroides*; the latter

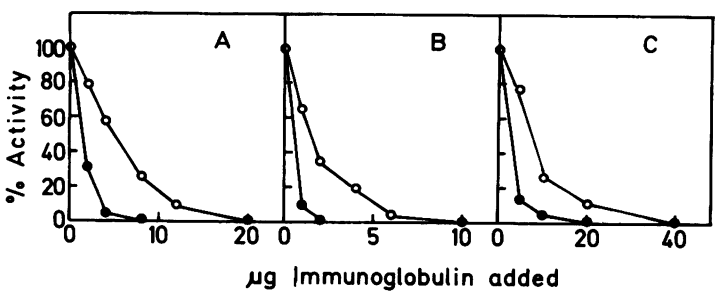

FIG. 1. *Immunotitration of PRS activity from E. coli strains KLF48/KL159 (○) and KL159 (●) with antiserum against wild-type PRS (A), purified α subunits (B), and purified β subunits (C). Cell strains were grown on glucose minimal medium containing the required supplements.*

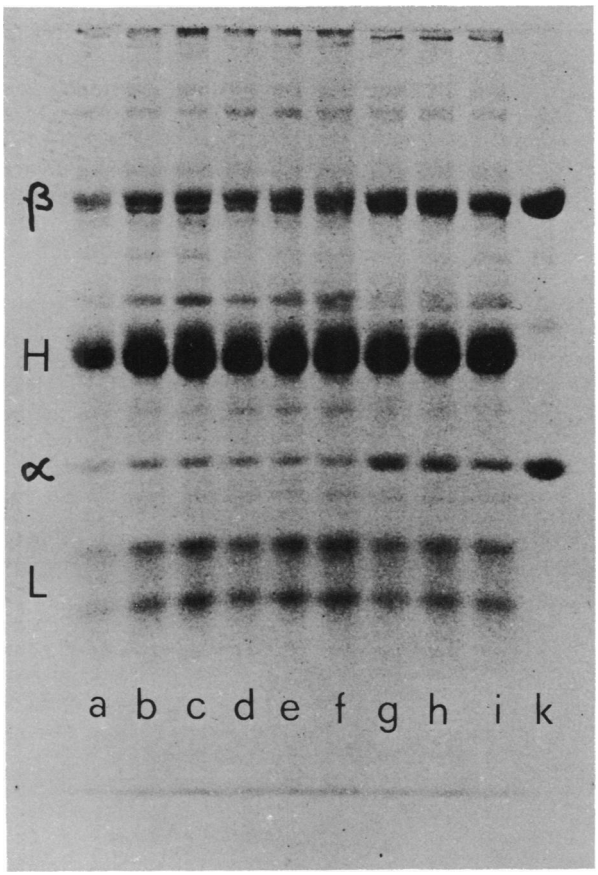

FIG. 2. *SDS-slab gel electrophoresis of immunoprecipitates from E. coli strain K-10, KL159, and KLF48/KL159 and of purified PRS. Crude-extract protein (8 mg) of each strain was incubated with three different concentrations of antiserum, and the washed precipitates were subjected to SDS-gel electrophoresis. (a, b, c) Precipitates from strain K10; (d, e, f) from strain KL159; and (g, h, i) from strain KLF48/KL159 formed with 0.30, 0.40, and 0.50 ml of antiserum, respectively. In this experiment, the crude extracts were not preabsorbed with nonimmune gamma globulins. Gel k contains 10 μg of E. coli PRS. H and L refer to the immunoglobulin heavy and light chains, respectively.*

three strains, however, exhibit a very weak intermediate reactivity.

The third group of organisms possesses an enzyme with no apparent cross-reactivity. *Micrococcus lysodeikticus* is shown as an example (Fig. 3). Members of this group are *B. subtilis*, *S. faecalis*, *Sarcina exigna*, *E. gracilis*, *S. cerevisiae* (bakers' yeast), *Neurospora crassa*, and *Blastocladiella* sp. Since crude *E. coli* tRNA has been used in these experiments for the determination of the residual PRS activity, we must keep in mind that in this heterologous system, in addition to the aminoacylation of *E. coli* $tRNA^{Phe}$, other tRNA species might have been charged. This has already been proven in detail for phenylalanyl-tRNA synthetase from *N. crassa* (13) and from brewers' yeast (2). Therefore, the immunological identities of the different enzymes correctly refer only to the recognition of phenylalanine and adenosine 5'-triphosphate and possibly less to the recognition of $tRNA^{Phe}$.

A typical result of the immunodiffusion experiments is given in Fig. 4, indicating that those enzymes not inactivated by antibodies against *E. coli* PRS also do not give a visible precipitin band in the double-diffusion tests. The intermediate cross-reactivity of the *Pseudomonas* enzyme is also revealed in the immunodiffusion pattern. Among the *Enterobacteriaceae* tested, the enzymes from *Citrobacter freundii* and *Klebsiella pneumoniae* seem to be more related to the *E. coli* enzyme than those of *S. marcescens* or *Proteus mirabilis*.

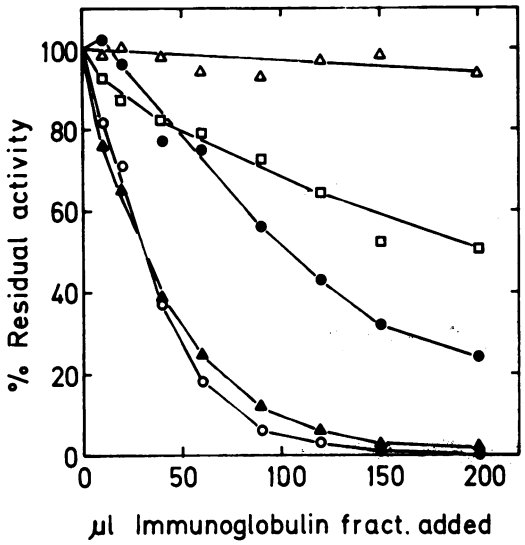

FIG. 3. *Inactivation of the PRS activity from different bacterial organisms by the immunoglobulin fraction of serum raised against E. coli PRS. A 500-µg amount of S100 protein of each strain was preincubated with the antibodies; a portion of this mixture, yielding in each case about 2,500 counts/min per 10 min of incubation at 28 C in the absence of antibodies, was then used for the PRS assays. Symbols: ○, E. coli K10; ●, P. aeruginosa; △, Micrococcus lysodeikticus; ▲, S. marcescens; □, B. stearothermophilus.*

In a further attempt to resolve the immunological resemblance of PRS from different organisms, the immunoprecipitates formed in the reaction of antibodies against *E. coli* PRS with crude extract protein of these organisms were analyzed on SDS-polyacrylamide gels (Fig. 5). The results show that, in addition to polypeptide chains corresponding to the heavy and light chains of immunoglobulins, protein bands are resolved in precipitates from extracts of all *Enterobacteriaceae*, which correspond in position to the α and β bands of *E. coli* PRS. The multiple bands visible in the *Enterobacter cloacae* gels might be the result of unspecific precipitation. No extra bands besides the small amount of heavy and light chains can be seen in the gels from *B. subtilis*, *S. cerevisiae*, or *Euglena*. Immunoprecipitates from *P. aeruginosa* and *B. stearothermophilus*, which show

TABLE 2. *Immunological reaction of phenylalanyl-tRNA ligases from different microorganisms with antibodies against the E coli enzyme*

Organism	Immunotitration[a]	Degree of identity in immunodiffusion	SDS-gel analysis of immunoprecipitates
Escherichia coli B[b]	++	Full	Like purified enzyme
Klebsiella pneumoniae[c]	++	Almost full	Similar to *E. coli*
Citrobacter freundii[c]	++	Almost full	Similar to *E. coli*
Serratia marcescens[c]	++	Partial	Similar to *E. coli*
Proteus mirabilis[b]	++	Partial	Similar to *E. coli*
Erwinia carotovora[b]	++	NT[d]	NT
Enterobacter cloacae[b]	++	NT	Similar to *E. coli* with additional bands
Pseudomonas aeruginosa ATCC 7700[b]	+	Partial, but less than *Serratia* or *Proteus*	Unlike *E. coli*
P. testosteroni[c]	±	NT	NT
P. acidivorans[c]	±	NT	NT
Rhodopseudomonas spheroides[e]	±	NT	NT
Sarcina exigna[b]	−	None	No precipitate
Micrococcus lysodeikticus[b]	−	None	No precipitate
Streptococcus faecalis[c]	−	None	No precipitate
Bacillus subtilis[b]	−	None	No precipitate
B. stearothermophilus[b]	+	Very weak	Unlike *E. coli*
Euglena gracilis[b]	−	None	No precipitate
Neurospora crassa[b]	−	NT	NT
Blastocladiella sp.[b]	−	NT	NT
Saccharomyces cerevisiae	−	None	No precipitate

[a] ++, Inactivation response comparable to *E. coli* PRS; +, intermediate reaction; ±, very weak reaction; −, no inactivation in the range of antibody concentration employed.
[b] Laboratory strains (wild type).
[c] From the German Collection of Microorganisms (6).
[d] Not tested.
[e] From H. Kosakowski.

intermediate reactivity in the immunotitration experiments, do not reveal the polypeptide pattern characteristic for *Enterobacteriaceae*, but do show bands in other positions. It is unclear whether these are constituents of the PRS of these organisms or simply unspecifically precipitated proteins.

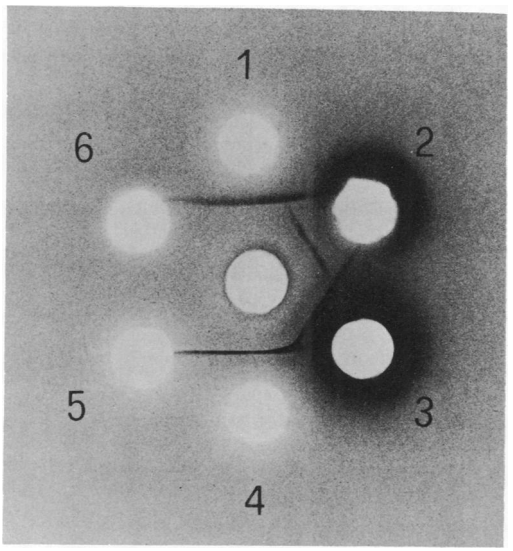

FIG. 4. *Ouchterlony immunodiffusion. Reaction of an antiserum to PRS from E. coli with about 12 μg of purified E. coli PRS (wells 1 and 4) and with about 1.5 mg of S100 extract from P. aeruginosa (well 2), S. marcescens (well 3), S. exigna (well 5), and M. lysodeikticus (well 6).*

DISCUSSION

The comparison of structural features of aminoacyl-tRNA synthetases in different organisms is of considerable interest because of the essential role that these enzymes play in protein synthesis and because of their reaction with a tRNA molecule, which assures the correct translation of the universal genetic code. As a probe for the structural relatedness of one of these enzymes (phenylalanyl-tRNA ligase) in different organisms, the immunological reactivity with immunoglobulins against *E. coli* PRS was used. The results have revealed considerable structural heterogeneity of this enzyme. Only the enzymes of species of the rather precisely defined family *Enterobacteriaceae* seem to react very similarly with inactivating antibodies, and the immunoprecipitates contain the same sizes of polypeptides as revealed by the *E. coli* PRS. On the other hand, double-diffusion experiments show that even within this group the enzymes differ; those of *Klebsi-*

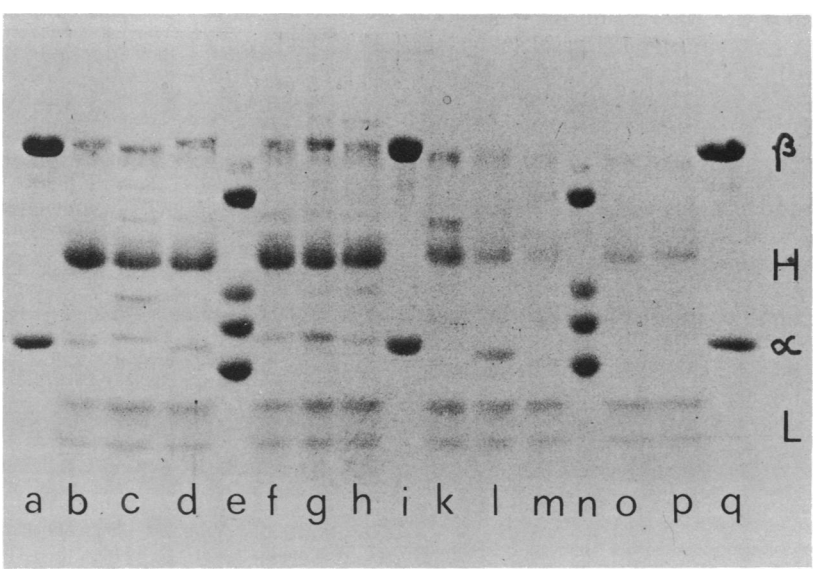

FIG. 5. *SDS-polyacrylamide slab gel electrophoresis of immunoprecipitates from E. coli (b), E. cloacae (c), K. pneumoniae (d), C. freundii (f), P. mirabilis (g), S. marcescens (h), P. aeruginosa (k), B. stearothermophilus (l), B. subtilis (m), S. cerevisiae (o), and E. gracilis (p). Gels (a), (i), and (q) show the electrophoresis pattern of about 6 μg of purified E. coli PRS; gels (e) and (n) show the gel pattern of the following standard proteins (from bottom): lactic dehydrogenase (pig heart), fructose-1,6-diphosphate aldolase (rabbit muscle), ovalbumin, bovine serum albumin, and ovalbumin dimer. α and β indicate the positions of the two constituent PRS polypeptides; H and L give the position of the heavy and light chains of immunoglobulins, respectively.*

ella and *Citrobacter* being more related to the *E. coli* PRS than those of *Serratia* or *Proteus*.

There is a striking similarity between the strength of cross-reactivity of *E. coli* PRS with enzyme from different organisms and that with ribosomes from different organisms as reported by Wittmann and Stöffler (17). No reaction between antiserum against *E. coli* PRS with the enzyme from the gram-positive organisms tested could be detected, and there is only weak or very weak cross-reaction in case of the ribosome (17). In both cases, *B. stearothermophilus*, which shows intermediate reactions, seems to be an exception. Also, there is intermediate cross-reaction between *E. coli* and members of the family *Pseudomonadaceae* and also *R. spheroides*. Furthermore, no cross-reactivity exists between PRS or ribosomes (17) from eukaryotic organisms and from *E. coli*. It must be emphasized in this connection that the lack of cross-reaction refers only to the methods employed in our study; use of more sensitive techniques such as the complement fixation assay could reveal a cross-reaction that might not show up by our methods.

The results obtained upon investigation of the PRS cross-reacting material in different strains of *E. coli* show: (i) that the α and β polypeptides are present in immunoprecipitates of freshly prepared cell extracts and are, therefore, probably not the result of proteolysis during enzyme preparation (SDS-gel analysis of immunoprecipitates, therefore, seems to be a valuable tool for detecting proteolytic modification of an enzyme during preparation); (ii) that episome F148, which covers part of the *E. coli* genome in the *his-aroD* region, possibly carries both structural cistrons for *E. coli* PRS and, thereby, raises the possibility that the α and β cistrons are mapping closely together. Merodiploids carrying F148 should, therefore, be a better strain for the purification of PRS. Recent experiments, indeed, gave a higher yield than had been obtained previously (M. Engelhard, unpublished data).

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