Similarity of Ribosomal Proteins Studied by Two-Dimensional Coelectrophoresis for Identification of Gram-Positive Bacteria

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A two-dimensional electrophoresis method was devised for comparing ribosomal proteins from different bacteria. First-dimension separation of the proteins was carried out by nonequilibrium pH gradient gel electrophoresis in glass capillary tubes. Next, the first-dimension gels were developed in a slab gel by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Within about 3 h (total running time), 40 to 48 distinct and compact ribosomal protein spots were detected in each of the 34 strains belonging to eight genera of gram-positive bacteria which we studied. Similarity coefficients (S_{AB} values) among the proteins of *Corynebacterium* and *Arthrobacter* strains were calculated by determining the numbers of coincident spots obtained by coelectrophoresis. The S_{AB} values between strains having DNA relatedness values of more than 61% (strains of the same species) were greater than 0.85, whereas the S_{AB} values between strains of different species were less than 0.45. Ribosomal protein analysis by our improved method is a potential tool for species identification of bacteria.

Ribosomes are made up of three separate RNA molecules and more than 50 species of ribosomal proteins. The structural regions essential for function have been conserved during evolution. Therefore, ribosomal constituents have attracted considerable attention in phylogenetic investigations of organisms.

Ribosomal proteins have been analyzed by different methods, including one- and two-dimensional electrophoresis, immunochemical cross-reactions, and amino acid sequencing. Two-dimensional gel electrophoresis has greatly facilitated the resolution of ribosomal proteins and has made it possible to compare the proteins of different organisms. For separation on gels, various systems have been devised for the first and second dimensions. This method has been applied to the taxonomy of species of the family *Enterobacteriaceae* (4) and of the genus *Bacillus* (3) and has been extended to other groups of organisms. Several species of the family *Enterobacteriaceae* and of the genus *Bacillus* were shown to be very similar in their ribosomal protein patterns within the family and within the genus. On the other hand, some *Streptomyces* species were shown to exhibit striking variability in their ribosomal protein patterns (12). Also, certain ribosomal proteins (the AT-L30 proteins) were found by Ochi (13) and Ochi et al. (14) to exhibit electrophoretic mobilities that were specific for each genus of actinomycetes.

Whether analysis of ribosomal protein patterns is useful for species identification of eubacteria is still uncertain, because the currently available information is limited to ribosomal proteins of a few bacterial species and also is not based on exact comparisons of individual protein spots. In addition, to obtain similarity coefficients for the proteins from many strains, it would be desirable to develop the previously described methods, especially with respect to spot size resolved, sensitivity, and running time.

In this report, we describe an improved two-dimensional coelectrophoretic analysis method for obtaining the similarity coefficients of total ribosomal proteins. We also discuss

the relationship between ribosomal protein similarity and DNA-DNA complementarity.

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1.

Preparation of ribosomes. *Lactobacillus delbrueckii* was grown in a lactobacillus culture medium (Nissui) at 37°C, and *Escherichia coli* was grown in a brain heart infusion medium (Difco) at 37°C. The other strains used in this study were grown in nutrient broth medium (Kyokuto), which contained 0.5% yeast extract, at 28°C. After cultivation for 4 to 6 h (mid-exponential phase), cells from each 500-ml culture were collected by centrifugation, washed in TSM buffer (10 mM Tris-HCl, 3 mM succinic acid, 10 mM magnesium chloride; pH 8.0), and resedimented. Then they were resuspended in TSM buffer containing 10 µg of DNase per ml. The cells were disrupted in a Braun model MSK cell homogenizer with glass beads (diameter, 0.1 to 0.11 mm) for 3 min. The cell debris was removed by centrifugation at 25,000 × g for 30 min. The ribosomes in the supernatant were purified by using the method of Kurland (9), and the purified ribosomes were used as the 70S ribosome preparation for subsequent experiments.

Preparation of total ribosomal proteins. Ribosomal proteins were prepared as described below by the method of Hardy et al. (6). The ribosomal preparation described above was dissolved in TSM buffer, and then 2 volumes of glacial acetic acid and 0.1 volume of 1 M magnesium chloride (based on the original volume) were added simultaneously. The extraction mixture was stirred vigorously for 45 min in an ice bath. The precipitate, which contained rRNA, was removed by centrifugation at 12,000 × g for 10 min. For further extraction of the ribosomal proteins, the precipitated RNA was dissolved in 3 ml of 67% (vol/vol) acetic acid containing 33 mM magnesium chloride, and the preparation was stirred for 20 min in an ice bath. After centrifugation at 12,000 × g for 10 min, the supernatant was collected, combined, and dialyzed overnight against 2% (vol/vol) glacial acetic acid. The dialysate was frozen, lyophilized, and stored at -80°C.

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TABLE 1. Strains used in this study

Species	Strain ^a
<i>Arthrobacter atrocyaneus</i>	KY 3209 ^T (= IFO 12956 ^T = ATCC 13752 ^T)
<i>Arthrobacter citreus</i>	KY 3155 ^T (= ATCC 11625 ^T)
<i>Arthrobacter citreus</i>	KY 3170 (= ATCC 15170)
<i>Arthrobacter citreus</i>	KY 3171 (= ATCC 17775)
<i>Arthrobacter globiformis</i>	KY 3156 ^T (= ATCC 8010 ^T)
<i>Arthrobacter nicotianae</i>	KY 3213 ^T (= IFO 14234 ^T = ATCC 15236 ^T)
<i>Arthrobacter oxydans</i>	KY 3169 ^T (= ATCC 14358 ^T)
<i>Arthrobacter polychromogenes</i>	KY 3214 ^T (= JCM 2523 ^T = ATCC 15216 ^T)
<i>Arthrobacter protophormiae</i>	KY 3475 ^T (= CCEB 282 ^T = ATCC 19271 ^T)
<i>Arthrobacter sulfureus</i>	KY 3218 ^T (= IAM 1488 ^T = ATCC 19098 ^T)
<i>Arthrobacter ureafaciens</i>	KY 3152 ^T (= IAM 1658 ^T = ATCC 7562 ^T)
<i>Arthrobacter ureafaciens</i>	KY 6722
<i>Arthrobacter ureafaciens</i>	KY 6725
<i>Bacillus subtilis</i>	KY 7522 ^T (= IAM 12118 ^T = ATCC 6051 ^T)
" <i>Brevibacterium divaricatum</i> "	KY 3461 (= NRRL B-2311 = ATCC 14020)
" <i>Brevibacterium lactofermentum</i> "	KY 10123 (= ATCC 13869)
<i>Brevibacterium linens</i>	KY 3431 ^T (= IAM 12437 ^T = ATCC 9172 ^T)
" <i>Brevibacterium roseum</i> "	KY 10124 (= ATCC 13825)
<i>Corynebacterium ammoniagenes</i>	KY 3453 ^T (= ATCC 6871 ^T)
<i>Corynebacterium flavesces</i>	KY 3821 ^T (= IAM 1642 ^T = ATCC 10340 ^T)
<i>Corynebacterium glutamicum</i>	KY 9002 ^T (= ATCC 13032 ^T)
<i>Corynebacterium glutamicum</i>	KY 10535
<i>Corynebacterium glutamicum</i>	KY 10865
<i>Corynebacterium glutamicum</i>	KY 10231
<i>Corynebacterium glutamicum</i>	KY 10694
" <i>Corynebacterium lilium</i> "	KY 3509 (= NRRL B-2243 = ATCC 15990)
<i>Corynebacterium pseudodiphtheriticum</i>	KY 3540 (= CCM 1930)
<i>Corynebacterium renale</i>	KY 3028 (= JCM 1322 = ATCC 10848)
<i>Corynebacterium vitrum</i>	KY 3005 ^T (= NCIB9291 ^T = ATCC 10234 ^T)
<i>Corynebacterium xerosis</i>	KY 3029 ^T (= JCM 1971 ^T = ATCC 373 ^T)
<i>Escherichia coli</i>	KY 3590 (= ATCC 11303)
<i>Lactobacillus delbrueckii</i>	KY 3682 ^T (= IFO 3202 ^T = ATCC 9649 ^T)
<i>Micrococcus luteus</i>	KY 3758 ^T (= ATCC 4698 ^T)
<i>Rhodococcus erythropolis</i>	KY 3885 ^T (= ATCC 4277 ^T)
<i>Staphylococcus aureus</i>	KY 4279 (= ATCC 6538P)

^a Abbreviations: KY, Tokyo Research Laboratories, Kyowa Hakko Co., Ltd., Machida, Japan; ATCC, American Type Culture Collection, Rockville, Md.; CCEB, Culture Collection of Entomogenous Bacteria, Institute of Entomology, Prague, Czechoslovakia; CCM, Czechoslovak Collection of Microorganisms, J. E. Purkyně University, Brno, Czechoslovakia; IAM, Institute of Applied Microbiology, University of Tokyo, Tokyo, Japan; IFO, Institute for Fermentation, Osaka, Japan; JCM, Japan Collection of Microorganisms, Wako, Japan; NCIB, National Collection of Industrial Bacteria, NCIMB Ltd., Aberdeen, Scotland; NRRL, Agricultural Research Service Culture Collection, Northern Regional Research Center, Peoria, Ill.

Electrophoresis in the first dimension. The nonequilibrium pH gradient gel electrophoretic method described by O'Farrell et al. (15) was modified as described below. The separation gel mixture contained (per 4 ml) 1.96 g of urea, 20 µl of Nonidet P-40, 100 µl of ampholine (pH 3.0 to 10.0), 0.16 g of acrylamide, 4 mg of *N,N'*-methylene-bisacrylamide, 9 µl of *N,N,N',N'*-tetramethylethylenediamine, and 20 µl of 10% (wt/vol) ammonium persulfate. For first-dimension electrophoresis, three glass capillary tubes (length, 42 mm; inner diameter, 1.3 mm) were used. The prepared gels could be kept refrigerated for several months with no adverse effects in tightly sealed tubes by wrapping them with a polyethylene sheet. Ribosomal protein samples were dissolved at concentrations of 100 to 200 µg/10 µl in sample buffer containing (per 100 ml) 36 g of urea, 1 ml of Nonidet P-40, and 5 ml of 2-mercaptoethanol. Usually, 2 to 4 µl of a sample was applied. The upper and lower electrode solutions were 0.01 M H₃PO₄ and 0.04 M NaOH, respectively. Electrophoresis was carried out at 0.1 to 0.15 mA per gel at room temperature for 15 to 20 min with a model 3500 computerized programmable power supply (TEFCO).

Electrophoresis in the second dimension. After the first run, the gels were removed from the glass capillary tubes by careful extrusion with injection and were dialyzed immedi-

ately for 5 min against 1 ml of dialyzing buffer containing (per 100 ml) 25 mM Tris-HCl, 2 g of sodium dodecyl sulfate, 5 ml of 2-mercaptoethanol, and 10 ml of glycerol. Three dialyzed gels (length, 42 mm) were applied side by side to a second-dimension slab gel which was 138 mm wide, 125 mm long, and 1 mm thick. Second-dimension electrophoresis was performed with a 14% sodium dodecyl sulfate-polyacrylamide gel at 30 mA per gel until the tracking dye reached the bottom (about 130 min). The gels were stained with 0.5% Coomassie brilliant blue R-250 in 45% methanol-10% acetic acid-water for 2 h at room temperature and destained with 40% methanol-10% acetic acid-water for several hours.

RESULTS

Resolution of two-dimensional gel electrophoresis. The electrode conditions of the nonequilibrium pH gradient gel electrophoresis used in the first dimension with glass capillary tubes were examined with respect to the current and running time. When first-dimension electrophoresis was performed at 0.1 to 0.15 mA per gel for 17 to 22 min, the total ribosomal proteins from *E. coli* KY 3590 (= ATCC 11303) were separated into 46 compact spots on the two-dimensional electrophoresis gel, although several proteins mi-

FIG. 1. Two-dimensional electrophoresis of total ribosomal proteins from *E. coli*. The first dimension was run at 0.15 mA per gel for 17 min. The second-dimension electrophoretic conditions are described in Materials and Methods. The ribosomal proteins migrated from the left side to the right (cathode) side. O, origin in the first dimension.

grated to the same positions (Fig. 1). With a running time of less than 10 min and a current of 0.1 mA per gel, the proteins failed to migrate, whereas with electrophoresis for more than 40 min at a current of 0.1 mA per gel or for more than 20 min at a current of 0.4 mA per gel many proteins migrated so far to the right side that the number of spots detected decreased markedly (data not shown).

Reproducibility of two-dimensional gel electrophoresis. Reproducible separation of the ribosomal proteins from *Corynebacterium glutamicum* KY 9002^T (= ATCC 13032^T) was confirmed under several conditions. First-dimension electrophoresis was performed with a current of 0.1 mA per gel for 22 min. The total ribosomal proteins were separated into 43 compact, clear spots and 6 small, pale spots (Fig. 2A). When several first-dimension gels were prepared at the same time, the protein patterns were not affected, even if some gels were stored at 5°C for several months. Furthermore, the level of reproducibility was very high when second-dimension electrophoresis was performed after the first-run gels were stored at -20°C for 1 week (Fig. 2B). However, the migration patterns of a few basic ribosomal proteins, which had molecular weights less than about 11,000 and pI values greater than 12, were not constant under all conditions.

Application to analysis of ribosomal proteins from gram-positive bacteria. Our method was applied to an analysis of the ribosomal proteins from species belonging to eight genera of gram-positive bacteria. Figure 3 shows the patterns obtained for *Arthrobacter globiformis* KY 3156^T (= ATCC

FIG. 2. Reproducibility of separation of ribosomal proteins. Ribosomal proteins from *C. glutamicum* KY 9002^T were used. The first-dimension gels were prepared at the same time. (A) First-dimension electrophoresis was performed at 0.1 mA per gel for 22 min. (B) Gel electrophoresed at a different time under the same conditions as those used for the gel in panel A, after the first-dimension electrophoretic gel was stored at -20°C for 1 week.

8010^T), *Bacillus subtilis* KY 7522^T (= IAM 12118^T), *Brevibacterium linens* KY 3431^T (= IAM 12437^T), *C. glutamicum* KY 9002^T (= ATCC 13032^T), *L. delbrueckii* KY 3682^T (= IFO 3202^T), *Micrococcus luteus* KY 3758^T (= ATCC 4698^T), *Rhodococcus erythropolis* KY 3885^T (= ATCC 4277^T), and *Staphylococcus aureus* KY 4279 (= ATCC 6538P). Distinct spots, ranging in number from 43 to 46, were detected. About six to nine proteins migrated into the regions corresponding to molecular weights of 25,000 to 33,000, while most of the rest of the proteins appeared in the regions corresponding to molecular weights of less than 18,000.

Similarity coefficients of ribosomal proteins. Two individual ribosomal protein preparation electrophoretograms and a coelectrophoretogram of a mixture of two ribosomal protein preparations run on the same slab gel were compared. For example, in the comparison of *C. glutamicum* KY 9002^T and "*Brevibacterium lactofermentum*" KY 10123 (= ATCC 13869), 44 of 46 proteins from each strain migrated to the same positions, whereas 2 proteins from *C. glutamicum* KY 9002^T and 3 proteins from "*Brevibacterium lactofermentum*" KY 10123 migrated to different positions (Fig. 4B, arrows). As shown in Fig. 4E, more than 70 proteins were detected by coelectrophoresis of ribosomal proteins from *A. globiformis* KY 3156^T and *E. coli* KY 3590. About 30 proteins from *A. globiformis* KY 3156^T did not share spots with the proteins from *E. coli* KY 3590. Thus, similarity coefficients for the ribosomal proteins from two strains were obtained easily by our method. Similarity (S_{AB}) values were determined as follows: 2(number of coincident spots)/total number of spots for both proteins. The S_{AB} value for the ribosomal proteins of *C. glutamicum* KY 9002^T and "*Brevibacterium lactofermentum*" KY 10123 was 0.96, and the

FIG. 3. Two-dimensional electrophoresis of total ribosomal proteins from gram-positive bacteria. (A) *A. globiformis* KY 3156^T. (B) *Bacillus subtilis* KY 7522^T. (C) *Brevibacterium linens* KY 3431^T. (D) *C. glutamicum* KY 9002^T. (E) *L. delbrueckii* KY 3682^T. (F) *M. luteus* KY 3758^T. (G) *R. erythropolis* KY 3885^T. (H) *S. aureus* KY 4279.

S_{AB} value for the ribosomal proteins of *E. coli* KY 3590 and *A. globiformis* KY 3156^T was 0.26.

Influence of mutations on conservation of ribosomal proteins. The ribosomal proteins of amino acid-producing mutants, which were auxotrophic and/or antimetabolite-resistant strains of *C. glutamicum* and *Arthrobacter ureafaciens*, were compared with the ribosomal proteins of the respective wild-type strains. For example, as shown in Fig. 5, most of the spots for the ribosomal proteins from strain KY 10231, which was a lysine-producing mutant of *C. glutamicum* KY 9002^T, migrated to the same positions as the spots for strain

KY 9002^T proteins when coelectrophoresis was performed. As shown in Table 2, the S_{AB} values of six mutants ranged from 0.95 to 0.99. Thus, alterations in ribosomal proteins by sequential mutations were not obvious on two-dimensional electrophoresis gels.

Correlation between ribosomal protein similarity and DNA-DNA relatedness. Whether ribosomal protein similarity was useful for identification of bacterial species was investigated. It has been proposed that the phylogenetic definition of a species should be as follows: a species comprises strains that exhibit approximately 70% or greater DNA-DNA related-

FIG. 4. Comparison of ribosomal protein spots by two-dimensional coelectrophoresis. (A) *C. glutamicum* KY 9002^T. (B) Coelectrophoresis of *C. glutamicum* KY 9002^T and "*Brevibacterium lactofermentum*" KY 10123. (C) "*Brevibacterium lactofermentum*" KY 10123. (D) *E. coli* KY 3590. (E) Coelectrophoresis of *E. coli* KY 3590 and *A. globiformis* KY 3156^T. (F) *A. globiformis* KY 3156^T.

FIG. 5. Two-dimensional coelectrophoresis of mutant KY 10231 and wild-type strain KY 9002^T of *C. glutamicum*. The arrows indicate the spot which was observed in mutant KY 10231. (A) Strain KY 9002^T. (B) Coelectrophoresis. (C) Strain KY 10231.

ness. Therefore, the S_{AB} values were compared with the levels DNA relatedness. Ten species of the genus *Corynebacterium* and nine species of the genus *Arthrobacter* were selected because DNA relatedness data were available (8, 16). The S_{AB} values of these organisms are shown in Tables 3 and 4. "*Brevibacterium divaricatum*" KY 3461 (= NRRL

TABLE 2. Ribosomal protein similarities of mutants of *C. glutamicum* KY 9002^T and *A. ureafaciens* KY 3152^T

Strain	Phenotype ^a	Product ^a	Ribosomal proteins	
			Total no.	<i>S</i> _{AB} ^b
<i>C. glutamicum</i>				
KY 9002 ^T			46	1.00
KY 10535	Phe Tyr 5MT ^r TrpHx ^r	Trp	46	0.99
KY 10865	Phe Tyr 5MT ^r TrpHx ^r 6FT 4MT ^r PFP ^r TyrHx ^r PheHx ^r Rif ^r AS ^r DR ^r DON ^r	Trp	46	0.96
KY 10694	PFP ^r MFP ^r	Phe	46	0.95
KY 10231	Hom Leu PC ^r AEC ^r	Lys	46	0.96
<i>A. ureafaciens</i>				
KY 3152 ^T			46	1.00
KY 6722	Lys	LL-DAP	47	0.99
KY 6725	Met	Ala	48	0.98

^a Abbreviations: Phe, phenylalanine; Tyr, tyrosine; Hom, homoserine; Leu, leucine; Lys, lysine; Met, methionine; Ala, alanine; LL-DAP, LL-diaminopimelic acid; 5MT^r, resistant to 5-methyltryptophan; TrpHx^r, resistant to tryptophan hydroxamate; 6FT^r, resistant to 6-fluorotryptophan; 4MT^r, resistant to 4-methyltryptophan; PFP^r, resistant to *para*-fluorophenylalanine; TyrHx^r, resistant to tyrosine hydroxamate; PheHx^r, resistant to phenylalanine hydroxamate; Rif^r, resistant to rifampin; AS^r, resistant to azaserine; DR^r, resistant to 2-deoxyribose; DON^r, resistant to 6-diazo-5-oxo-L-norleucine; MFP^r, resistant to *meta*-fluorophenylalanine; PC^r, resistant to penicillin; AEC^r, resistant to aminoethylcysteine.

^b S_{AB} was calculated as described in the text.

B-2311), "*Brevibacterium lactofermentum*" KY 10123 (= ATCC 13869), "*Brevibacterium roseum*" KY 10124 (= ATCC 13825), and "*Corynebacterium lilium*" KY 3509 (= NRRL B-2243), which exhibited DNA relatedness values of more than 70% to *C. glutamicum* KY 9002^T, were found to have very high S_{AB} values when they were compared with *C. glutamicum* (range, 0.96 to 0.98). Five other species of the genus *Corynebacterium* gave low S_{AB} values (range, 0.15 to 0.27) and exhibited DNA relatedness values of less than 24% in comparisons with *C. glutamicum*. *Arthrobacter citreus* KY 3170 (= ATCC 15170), which exhibited 73% DNA relatedness to *Arthrobacter sulfureus* KY 3218^T (= IAM 1488^T), gave an S_{AB} value of 0.89 in a comparison with strain KY 3218^T. *A. citreus* KY 3171 (= ATCC 17775), which exhibited 72% DNA relatedness to *Arthrobacter protophormiae* KY 3475^T (= CCEB 282^T), gave an S_{AB} value of 0.92 in a comparison with strain KY 3475^T. The results with *Arthrobacter polychromogenes* KY 3214^T (= JCM 2523^T) and *A. globiformis* KY 3156^T are worth noting. These organisms are type strains, but they gave relatively high S_{AB} values (Table 4) in comparisons with *Arthrobacter oxydans* KY 3169^T (= ATCC 14358^T). The DNA relatedness value for *A. polychromogenes* KY 3214^T and *A. oxydans* KY 3169^T was 61%, and the DNA relatedness value for *A. globiformis* KY 3156^T and *A. oxydans* KY 3169^T was 43%. The S_{AB} values for other combinations, whose DNA relatedness values were less than 31%, ranged from 0.35 to 0.48. Consequently, as shown in Fig. 6, the results of ribosomal protein similarity and DNA relatedness experiments were found to be highly correlated.

DISCUSSION

The two-dimensional polyacrylamide gel electrophoresis method developed by Kaltschmidt and Wittmann (7) has been successfully applied to analysis of bacterial ribosomal

TABLE 3. Correlation of DNA-DNA relatedness and ribosomal protein similarity for species of the genus *Corynebacterium*

Strain	Comparison with reference strain KY 9002 ^T			
	% of DNA relatedness		Ribosomal proteins	
	Method of Suzuki et al. ^a	Method of Komatsu and Kaneko ^b	Total no.	S _{AB} value
<i>Corynebacterium glutamicum</i> KY 9002 ^T	100	100	46	1.00
" <i>Corynebacterium lilium</i> " KY 3509	99		46	0.96
" <i>Brevibacterium lactofermentum</i> " KY 10123		102.1	46	0.96
" <i>Brevibacterium divaricatum</i> " KY 3461		93.4	46	0.98
" <i>Brevibacterium roseum</i> " KY 10124		73.5	46	0.96
<i>Corynebacterium ammoniagenes</i> KY 3453 ^T	24		46	0.20
<i>Corynebacterium renale</i> KY 3028 ^T	22		40	0.21
<i>Corynebacterium flavesces</i> KY 3821 ^T	20		43	0.27
<i>Corynebacterium vitrum</i> KY 3005 ^T	20		44	0.15
<i>Corynebacterium xerosis</i> KY 3029 ^T	15		43	0.22

^a See reference 17.^b See reference 8.

proteins, especially *E. coli* ribosomal proteins. One of the methods used for more quantitative comparisons, which combines the first-dimension separation system of Mets and Bogorad (11) with the second-dimension system of Kaltschmidt and Wittmann (7), was described by Böck in 1985 (1). Böck's system includes (i) a visual comparison of protein spots, (ii) measurements of the R_f value of each protein, (iii) monitoring of gels which are run in the same chamber assembly as that devised by Kaltschmidt and Wittmann on a light box, and (iv) electrophoresis of protein mixtures. These techniques are too complicated to allow a comparison of the similarity of spots without a special apparatus for the second dimension. Moreover, the detection of different spots by superimposing two gel patterns is influenced by changes in gel expansion and contraction. For these reasons, it was thought that routine use of the method for taxon identification would require further improvements. Therefore, we examined different previously described procedures and devised an improved system by combining nonequilibrium pH gradient gel electrophoresis in the first dimension in glass capillary tubes with sodium dodecyl sulfate-polyacrylamide gel electrophoresis in the second dimension, using a slab gel onto which the contents of three glass capillary tubes could be applied. Our method has the following advantages over previous procedures: (i) the first-

dimension run requires only about 17 min, compared with about 2 h for the procedure of Dipak et al. (2) or 7 h for the procedure of Böck (1), and the second-dimension run requires only about 130 min; and (ii) the individual ribosomal proteins from two organisms can be compared on one slab gel. Thus, an S_{AB} value for two organisms can be obtained by coelectrophoresis. Although our method did not completely separate all of the ribosomal proteins from each other, more than 40 proteins were detected as single distinct spots, which corresponded closely with the values obtained by the methods of Kaltschmidt and Wittmann (7) and other workers.

Our improved method was used to analyze a number of gram-positive bacteria (Table 1), and our results indicated that the ribosomal protein patterns were specific for each taxon. Organisms have been classified into taxonomic groups (taxa) on the basis of similarities or genetic relationships; these two approaches are phenetic classification (aiming at similarity) and phylogenetic classification (aiming at genuine relatedness). Recently, the Ad Hoc Committee on Reconciliation of Approaches to Bacterial Systematics reported that phylogeny based on the complete DNA sequence should determine taxonomy (18). This committee also proposed a recommended phylogenetic definition for a species, which is the basis of the taxonomic hierarchy, which gener-

TABLE 4. Correlation of DNA-DNA relatedness and ribosomal protein similarity among species of the genus *Arthrobacter*

Strain	Total no. of ribosomal proteins	Strain KY 3281 ^T		Strain KY 3475 ^T		Strain KY 3169 ^T		Strain KY 3155 ^T	
		% of DNA relatedness ^a	Ribosomal protein S _{AB} value	% of DNA relatedness ^a	Ribosomal protein S _{AB} value	% of DNA relatedness ^a	Ribosomal protein S _{AB} value	% of DNA relatedness ^a	Ribosomal protein S _{AB} value
<i>Arthrobacter sulfureus</i> KY 3218 ^T	46	100	1.00					12	0.42
<i>Arthrobacter citreus</i> KY 3170	44	73	0.89					10	0.42
<i>Arthrobacter protophormiae</i> KY 3475 ^T	44			100	1.00			16	0.48
<i>Arthrobacter citreus</i> KY 3171	42			72	0.93			16	0.48
<i>Arthrobacter oxydans</i> KY 3169 ^T	43					100	1.00	20	0.43
<i>Arthrobacter polychromogenes</i> KY 3214 ^T	42					61	0.89	23	0.43
<i>Arthrobacter citreus</i> KY 3155 ^T	46					17	0.43	100	1.00
<i>Arthrobacter ureafaciens</i> KY 3152 ^T	46					31	0.45	16	0.39
<i>Arthrobacter globiformis</i> KY 3156 ^T	46					43	0.72	23	0.48
<i>Arthrobacter atrocyaneus</i> KY 3209 ^T	42					16	0.35	16	0.45
<i>Arthrobacter nicotianae</i> KY 3213 ^T	41					15	0.41		

^a See reference 16.

FIG. 6. Relationship between ribosomal protein similarity and DNA-DNA relatedness. Symbols: ▲, *Arthrobacter* strains; ○, *Corynebacterium* strains.

ally includes strains that exhibit approximately 70% or greater DNA-DNA relatedness and differences between the melting temperatures of homologous hybrids and the melting temperatures of heterologous hybrids of 5°C or less. In our study, ribosomal protein similarity and DNA-DNA relatedness were compared. Strains belonging to the genera *Corynebacterium* and *Arthrobacter* were found to have high correlations between the two indices. It is quite obvious that ribosomal proteins have been conserved within species during evolution. In contrast to DNA-DNA hybridization, two-dimensional analysis of ribosomal proteins can reveal differences among strains of the same species. Our improved method is a potential tool for strain identification.

Recently, "*Brevibacterium divaricatum*," "*Brevibacterium lactofermentum*," "*Brevibacterium roseum*," and "*C. lilium*" were proposed as synonyms of *C. glutamicum* on the basis of the results of a restriction fragment length polymorphism analysis of ribosomal DNA, as well as DNA-DNA hybridization, chemotaxonomic, and biochemical studies (10). These organisms were found to have S_{AB} values in comparisons with ribosomal proteins of *C. glutamicum* (range, 0.96 to 0.98). Hence, our results support the proposal by Lieble et al. (10). It has been suggested that *A. polychromogenes* KY 3214^T is a subspecies of *A. oxydans* on the basis of the high level of DNA-DNA relatedness exhibited by these organisms (16). In our study, the S_{AB} value for the *A. polychromogenes* KY 3214^T and *A. oxydans* ribosomal proteins was 0.89; this value indicated that these organisms were closely related taxonomically. *A. globiformis* KY 3156^T is known to exhibit a DNA relatedness value of 43% in comparisons with *A. oxydans* KY 3169^T (16). The S_{AB} value of the ribosomal proteins of these strains was 0.72, which was significantly higher than the S_{AB} value for any other different *Arthrobacter* species (range, 0.35 to 0.48). As a

result, the taxonomic relationship between *A. globiformis* and *A. oxydans* based on ribosomal protein comparisons has not been resolved.

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