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Methods of Multilocus Enzyme Electrophoresis for Bacterial Population Genetics and Systematics

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INTRODUCTION

Many methods, including serotyping, monoclonal antibody typing, biotyping, bacteriophage typing, fimbriation typing, resistotyping, cell electrophoresis, whole protein extract electrophoresis, outer membrane protein electrophoresis, and various types of carbohydrate, lipid, or other chemical profiling or fingerprinting, have been used to type or characterize strains in bacterial systematics and epidemiology. However, because these methods detect phenotypic variation that is difficult, if not impossible (given the present state of knowledge of the genetic bases of these traits), to relate to allelic variation at specific gene loci, they have not provided the information on frequencies of alleles and multilocus genotypes that is required for analysis of the genetic structure of populations.

Hybridization (reassociation) of the total DNA of the bacterial cell, which yields estimates of total nucleotide sequence divergence (7, 64, 77), has been widely used to define species limits and relationships (see studies cited in reference 32), but for the following reasons this technique has made little contribution to the study of genetic variation within species. (i) Because of the relatively large experimental error associated with DNA hybridization, the technique lacks the precision required for the analysis of genetic relationships of closely related strains. Results obtained in different laboratories or even in replicate experiments in the same laboratory may be very discordant (see examples in reference 67). (ii) Reciprocal experiments often yield nonisomorphic values. (iii) Variation among strains in type and amount of extrachromosomal DNA is potentially a source of error in estimating genealogical (phylogenetic) relationships (9). (iv) In practical application, DNA hybridization experiments have provided information on the degree of similarity of a set of strains to one or a few reference strains rather than a complete matrix of coefficients of genetic relatedness between all pairs of strains (but see reference 75).

Multilocus enzyme electrophoresis, which has long been a standard method in eucaryotic population genetics (3, 34, 46, 49, 60, 69) and systematics (58), has recently been used in large-scale studies to estimate the genetic diversity and structure in natural populations of a variety of species of bacteria (Table 1). This research has established basic population genetic frameworks for the analysis of variation in serotypes and other phenotypic characters and has provided extensive data for systematics and useful marker systems for epidemiology.

In response to requests from several of our colleagues in other microbiological laboratories, we have prepared the present compendium of methods of enzyme extraction, gel electrophoresis, and specific enzyme staining used in our laboratory to study genetic variation in *Escherichia coli* and other bacteria (Table 1). The procedures described can be applied, with only minor modifications, to any species of bacterium.

BACKGROUND ON ENZYME ELECTROPHORESIS

Description and rationale of the method

Standard laboratory methods for studying polymorphic variation in enzymes by gel electrophoresis are described in detail by Manwell and Baker (37), Smith (74), and Harris and Hopkinson (24). Most of the methods used for studying bacteria in our laboratory were modified from those earlier described for studying mammals (68).

Isolates are characterized by the relative electrophoretic mobilities of a large number of water-soluble cellular enzymes (Fig. 1). Because the net electrostatic charge and, hence, the rate of migration of a protein during electrophoresis are determined by its amino acid sequence, mobility variants (electromorphs or allozymes) of an enzyme can be directly equated with alleles at the corresponding structural gene locus. (In the interest of clarity, we avoid the use of the generic term isoenzyme [or isozyme], which applies to all multiple molecular forms of a given type or species of enzyme, including those encoded by separate loci, variants [allozymes] of the same enzyme, and secondary enzymes, such as those produced by posttranslational modification of protein structure [24].) Recent studies of several proteins of known sequence indicate that gel electrophoresis can detect a large proportion (80 to 90%) of amino acid substitutions (38, 59, 71). However, because some substitutions do not affect electrophoretic mobility, electromorphs may be sequentially heterogeneous (4, 17, 40), and at the level of the nucleotide sequence of the gene itself, there is even greater heterogeneity, owing primarily to silent substitutions (31). Although electromorph profiles over loci can be equated with multilocus genotypes and electromorph frequencies can be equated with allele frequencies, it is with the understanding that the alleles recognized may actually be groups of isoalleles.

Posttranslational modification is a potential source of error in the application of the multilocus enzyme technique to population genetics and systematics (16, 28). Natural populations of *E. coli* have been shown to be weakly polymorphic (3 variant strains in a sample of 104 from diverse animal and human sources) for activity of the *iap* gene that effects an electrophoretically detectable posttranslational modification of alkaline phosphatase (19), but there is no evidence that

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TABLE 1. Bacterial species to which the technique of multilocus enzyme electrophoresis has been applied

Species	References
Escherichia coli (including *Shigella* spp.)[a]	1, 12–15, 41, 42, 53–56, 65, 66
Salmonella newport	J. M. Musser (unpublished data)
Bordetella spp.	45a
Haemophilus influenzae	44, 45
Neisseria meningitidis	11a, 57; B. A. Crowe et al., Semin. Infect. Dis. (in press).
Neisseria gonorrhoeae	J. M. Musser (unpublished data)
Legionella spp.	67; T. S. Whittam et al., Mol. Biol. Evol. (in press), P. H. Edelstein (unpublished data)
Pseudomonas aeruginosa	33
Streptococcus spp.	M. N. Gilmour (unpublished data)
Yersinia ruckeri	63
Klebsiella oxytoca	27
Rhizobium spp.	85

[a] The methods used for *E. coli* are also suitable for *Enterobacter* and *Klebsiella* spp. (R. K. Selander and D. A. Caugant, unpublished data).

posttranslational modification of enzymes occurs frequently enough in any organism to seriously bias estimates of genetic variation derived from the electrophoresis of proteins (10, 18, 73).

For pairs of isolates of *E. coli* (including *Shigella* spp.) and *Legionella* spp., we have demonstrated that estimates of genetic distance based on multilocus enzyme electrophoresis are strongly correlated with estimates of divergence in nucleotide sequence obtained from hybridization experiments (55, 67; T. S. Whittam, D. J. Brenner, and R. K. Selander, Mol. Biol. Evol., in press). Concordant results from multiple enzyme electrophoresis and DNA hybridization experiments on the relationships of species have also been reported for several other types of bacteria, including *Gluconobacter* spp. (84). On the basis of this evidence that the 15 to 25 enzymes routinely assayed in our laboratory are a representative sample of the structural genes of the bacterial genome, we have used the data on multilocus genotypes of isolates and allele frequencies in samples to estimate the levels of genetic variation within populations and the degrees of overall genetic relatedness among isolates, populations, and species.

When many polymorphic enzymes, each represented by several or many alleles, are examined, the number of possible genotypic combinations is enormous (66). However, in practice, no more than a few hundred genotypes have been encountered in any species, owing partly to the limited number of isolates examined and partly to the circumstance that bacterial species are, in general, clonal, with only a fraction of all possible genotypes being commonly represented in populations (1, 45, 53, 67).

Analysis of the genetic structure of natural populations of bacteria requires an efficient method of determining genotypes at large numbers of chromosomal loci (1, 53). Although it may soon be possible to obtain DNA sequence data, either directly (42) or by restriction fragment analysis (25), in quantities sufficient for extensive use in bacterial population genetics, at present the only feasible way to determine multilocus genotypes in the large samples of isolates required for the analysis of the genetic structure of natural populations is by the electrophoresis of enzymes. The special advantage of enzyme electrophoresis is that variation in mobility can be directly related to allelic variation at specific genes encoding specific proteins. Another attractive feature is the likelihood that much of the electrophoretically demonstrable polymorphic variation in enzymes is selectively neutral or nearly so (26) and, therefore, minimally subject to evolutionary convergence (29).

History of application

Following its introduction to population genetics, with applications to *Drosophila* spp. (35) and humans (23), multilocus enzyme electrophoresis quickly became a standard technique in eucaryotic evolutionary biology. Gel electrophoresis was used as early as 1963 to distinguish strains of bacteria (51), but as a population genetic and systematic technique it failed to have as significant an impact on the study of procaryotes as it did on the study of eucaryotes. Apart from a brief early account by Norris (50) and a review by Williams and Shah (81), there has been little or no treatment of the technique in texts and reviews of molecular and other methods in bacterial genetics and taxonomy (see, e.g., references 21, 32, 43, and 62).

Early studies of enzyme polymorphism in bacteria were not particularly enlightening; only one or a few enzymes were assayed in small numbers of strains, and inferior laboratory techniques sometimes resulted in limited resolving power (5). Moreover, early microbiological applications were primarily concerned with the prosaic problem of iden-

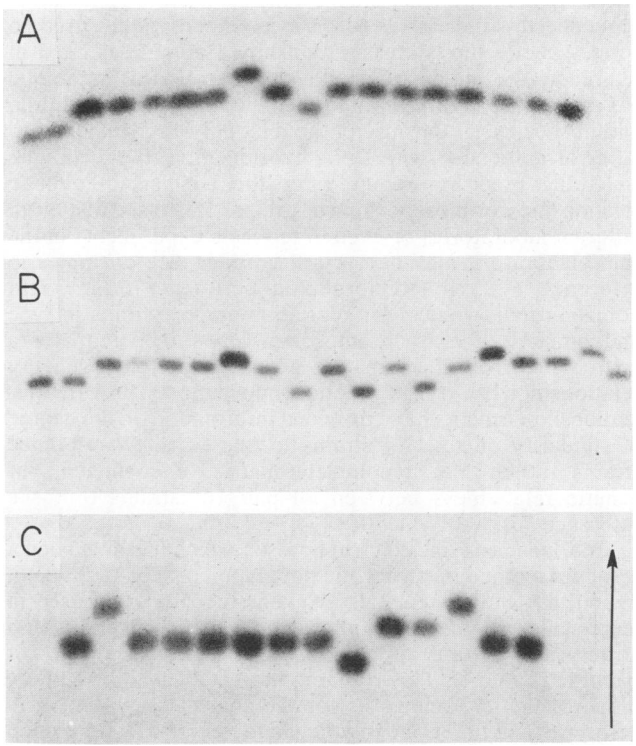

FIG. 1. Gels illustrating electrophoretic variation in three enzymes. (A) Mannitol 1-phosphate dehydrogenase in *E. coli*; 18 isolates. (B) Glucose 6-phosphate dehydrogenase in *N. meningitidis*; 19 isolates. (C) Malate dehydrogenase in *E. coli*; 14 isolates. Anodal direction of migration from the origin is indicated by the arrow.

TABLE 2. Buffer systems for electrophoresis of bacterial enzymes

System	Electrode buffer	Gel buffer	Voltage (V)
A	Tris-citrate (pH 8.0) (83.20 g of Tris [T 1378[a]], 33.09 g of citric acid monohydrate, 1.00 liter of water)	Tris-citrate (pH 8.0) (electrode buffer diluted 1:29)	130
B	Tris-citrate (pH 6.3) (27.00 g of Tris, 18.07 g of citric acid monohydrate, 1.00 liter of water; pH adjusted with NaOH)	Tris-citrate (pH 6.7) (0.97 g of Tris, 0.63 g of citric acid monohydrate, 1.00 liter of water; pH adjusted with NaOH)	150
C	Borate (pH 8.2) (18.50 g of boric acid, 2.40 g of NaOH, 1.00 liter of water)	Tris-citrate (pH 8.7) (9.21 g of Tris, 1.05 g of citric acid monohydrate, 1.00 liter of water)	250
D	Lithium hydroxide (pH 8.1) (1.20 g of $LiOH \cdot H_2O$, 11.89 g of boric acid, 1.00 liter of water)	Lithium hydroxide (pH 8.3) (electrode buffer mixed 1:9 with solution of 6.20 g of Tris, 1.60 g of citric acid monohydrate, and 1.00 liter of water)	325
E	Tris-maleate (pH 7.4) (12.10 g of Tris, 11.60 g of maleic acid, 3.72 g of disodium ETDA, 2.03 g of $MgCl_2 \cdot 6H_2O$, 1.00 liter of water; pH adjusted with 5 g of NaOH)	Tris-maleate (pH 7.4) (electrode buffer diluted 1:9)	100
F	Tris-maleate (pH 8.2) (same as system E, but with pH adjusted with 5.15 g of NaOH)	Tris-maleate (pH 8.2) (electrode buffer diluted 1:9	100
G	Potassium phosphate (pH 6.7) (18.14 g of KH_2PO_4, 2.39 g of NaOH, 1.00 liter of water)	Potassium phosphate (pH 7.0) (1.06 g of KH_2PO_4, 0.25 g of citric acid monohydrate, 1.00 liter of water)	100
H	Borate (pH 8.2) (same as system C)	Tris hydrochloride (pH 8.5) (1.2l g of Tris, 1.00 liter of water; pH adjusted with 6 or 7 drops of HCl)	250
I	Tris-borate (pH 8.0) (60.60 g of Tris, 40.00 g of boric acid, 6.00 g of disodium EDTA, 1.00 liter of water)	Tris-borate (pH 8.0) (electrode buffer diluted 1:9)	200

[a] Sigma Chemical Co. product number.

tifying strains to the species level (see, e.g., references 11, 36, 61, and 80), and not, as was the case for much of the work on eucaryotes, with assessing the levels of genetic variation in populations and genetic relatedness among individuals to test important aspects of evolutionary theory (34). A few of the early studies on bacteria, notably the study of Bowman et al. (6) on two enzymes in 38 isolates of 29 species of the family *Enterobacteriaceae*, clearly demonstrated the power of the technique to reveal genetic variation within and among species. However, in the absence of large-scale applications to significant problems in the population genetics and evolution of bacteria, enzyme electrophoresis understandably was perceived as merely another typing method. Application of the technique of enzyme electrophoresis may have been further discouraged by an over-cautious and rather negative review by Williams and Shah (81), who were largely concerned with its value in demonstrating species-diagnostic traits. Hence, the occurrence of extensive electrophoretic variation in an enzyme among isolates of a species was regarded by these and some other workers as a mark against the technique as a tool in taxonomy rather than as a rich source of information for estimating genetic relationships.

Several misconceptions are expressed in the review by Williams and Shah (81). It is, for example, simply not true, as they claim (p. 30), that "electrophoretic patterns of enzymes have not been significant in classification" of animals (see references 2, 20, 58, and 83). Also, the suggestion that the electrophoresis of enzymes and other proteins somehow fails as a systematic method because chimpanzees and humans are indistinguishable on the basis of electrophoretic mobility of hemoglobins and certain enzymes misses the whole point of recent discoveries in evolutionary genetics that demonstrate a very close genetic and phylogenetic relationship between these primates (72, 82).

Although enzyme electrophoresis continues to be used to a limited extent in the systematics of bacteria (see, e.g., references 8, 22, 30, 52, and 70), the first serious study of the genetics of natural populations of a bacterium was made in 1973 by Milkman (40, 41), who measured allelic variation at five enzyme loci in more than 800 isolates of *E. coli* to test the neutral theory of molecular polymorphism and evolution (29). This pioneering work in evolutionary genetics was extended by Selander and Levin (66) as well as in a series of studies of *E. coli* and *Shigella* spp. that ultimately involved several thousand isolates (see references in Table 1). More recently, multilocus enzyme electrophoresis has been applied in large-scale studies of the genetic structure of populations of *Legionella pneumophila*, *Haemophilus influenzae*, and other species listed in Table 1. The primary objective of most of these investigations was to estimate the overall genomic relationships among strains within species. For this purpose, large numbers of enzyme loci were assayed to minimize the error in estimates of relatedness resulting from

TABLE 3. Enzymes assayed and recommended buffer systems for various species

Enzyme			Buffer system (species)[a]
EC no.	Name	Symbol	
Oxidoreductases			
1.1.1.1	Alcohol dehydrogenase	ADH	C (*E. coli*, *N. meningitidis*, *Pseudomonas aeruginosa*)
1.1.1.17	Mannitol 1-phosphate dehydrogenase	M1P	B (*E. coli*, *Streptococcus* spp.)
1.1.1.30	3-Hydroxybutyrate dehydrogenase	HBD	C (*Bordetella* spp., *L. pneumophila*)
1.1.1.37	Malate dehydrogenase	MDH	A (*E. coli*, *P. aeruginosa*, *Bordetella* spp., *H. influenzae*, *L. pneumophila*) G (*N. gonorrhoeae*)
1.1.1.40	Malic enzyme	ME	A (*N. meningitidis*, *H. influenzae*, *Bordetella* spp.) G (*N. gonorrhoeae*)
1.1.1.42	Isocitrate dehydrogenase	IDH	A (*N. meningitidis*, *N. gonorrhoeae*, *Bordetella* spp., *L. pneumophila*) B (*E. coli*)
1.1.1.44	6-Phosphogluconate dehydrogenase	6PG	A (*E. coli*, *N. meningitidis*, *Streptococcus* spp.) E (*N. gonorrhoeae*, *H. influenzae*)
1.1.1.49	Glucose 6-phosphate dehydrogenase	G6P	A (*Bordetella* spp., *N. meningitidis*, *L. pneumophila*, *Streptococcus* spp.) C (*E. coli*) E (*N. gonorrhoeae*, *H. influenzae*)
1.2.1.12	Glyceraldehyde-phosphate dehydrogenase (NAD)	GP1	A (*Streptococcus* spp.) B (*E. coli*, *N. meningitidis*) G (*H. influenzae*)
1.2.1.13	Glyceraldehyde-phosphate dehydrogenase (NADP)	GP2	B (*Streptococcus* spp.)
1.1.1.27	L-Lactate dehydrogenase	LDH	B (*E. coli*, *Streptococcus* spp.)
1.1.1.x[b]	Threonine dehydrogenase	THD	C (*L. pneumophila*)
1.2.3.2	Xanthine dehydrogenase	XDH	B (*P. aeruginosa*)
1.4.1.1	Alanine dehydrogenase	ALD	A (*L. pneumophila*)
1.4.1.2	Glutamate dehydrogenase (NAD)	GD1	C (*N. meningitidis*)
1.4.1.4	Glutamate dehydrogenase (NADP)	GD2	A (*L. pneumophila*) B (*P. aeruginosa*) C (*N. meningitidis*, *H. influenzae*) D (*N. gonorrhoeae*, *Streptococcus* spp.) G (*Bordetella* spp.)
1.4.3.2	Leucine dehydrogenase	LED	C (*L. pneumophila*)
1.4.3.x	Aspartate dehydrogenase	ASD	C (*L. pneumophila*)
1.4.3.x	Lysine dehydrogenase	LYD	C (*L. pneumophila*)
1.x.x.x	Unidentified dehydrogenase	UDH	A (*L. pneumophila*)
1.11.1.6	Catalase	CAT	B (*P. aeruginosa*) G (*Bordetella* spp., *H. influenzae*)
1.15.1.1	Indophenol oxidase (superoxide dismutase)	IPO	B (*E. coli*, *N. meningitidis*) C (*Bordetella* spp., *L. pneumophila*, *H. influenzae*) D (*Streptococcus* spp.) G (*N. gonorrhoeae*)

Continued on following page

TABLE 3—*Continued*

Enzyme			Buffer system (species)[a]
EC no.	Name	Symbol	
Transferases			
2.4.1.11	Glucosyltransferase	GTF	D (*Streptococcus* spp.)
2.4.2.1	Nucleoside phosphorylase	NSP	A (*E. coli*, *Streptococcus* spp.)
2.6.1.1	Glutamic-oxalacetic transaminase	GOT	A (*Bordetella* spp., *H. influenzae*) B (*P. aeruginosa*) C (*L. pneumophila*) D (*E. coli*) *E* (*N. meningitidis*)
2.6.1.2	Glutamic-pyruvic transaminase	GPT	B (*Streptococcus* spp.)
2.7.1.1	Hexokinase	HEX	D (*Streptococcus* spp.)
2.7.4.3	Adenylate kinase	ADK	A (*E. coli*, *L. pneumophila*) B (*Streptococcus* spp.) G (*Bordetella* spp., *H. influenzae*) H (*N. meningitidis*, *Streptococcus* spp.)
2.7.5.1	Phosphoglucomutase	PGM	A (*H. influenzae*, *Streptococcus* spp.) D (*N. gonorrhoeae*, *Streptococcus* spp.) E (*Bordetella* spp., *N. meningitidis*) F (*E. coli*)
Hydrolases			
3.1.1.1	Esterases	EST	A (*Bordetella* spp., *Streptococcus* spp.) C (*P. aeruginosa*) D (*E. coli*, *L. pneumophila*) G (*L. pneumophila*) I (*L. pneumophila*)
3.1.3.1	Alkaline phosphatase	ALP	A (*N. gonorrhoeae*) D (*N. meningitidis*) E (*Bordetella* spp.) F (*E. coli*)
3.1.3.2	Acid phosphatase	ACP	F (*E. coli*)
3.2.1.23	β-Galactosidase	BGA	A (*E. coli*)
3.4.1.1	Leucine aminopeptidase	LAP	A (*E. coli*, *N. meningitidis*, *L. pneumophila*, *Streptococcus* spp.) B (*Streptococcus* spp.) E (*H. influenzae*, *Bordetella* spp.) H (*N. gonorrhoeae*)
3.4.x.x	Peptidases	PEP	A (*E. coli*, *N. meningitidis*, *L. pneumophila*, *Streptococcus*, spp.) E (*Bordetella* spp., *H. influenzae*) H (*N. gonorrhoeae*)
Lyases			
4.1.2.13	Aldolase	ALD	A (*N. gonorrhoeae*, *Streptococcus* spp.)
4.2.1.2	Fumarase	FUM	C (*Bordetella* spp., *H. influenzae*) D (*N. meningitidis*)
4.2.1.3	Aconitase	ACO	A (*L. pneumophila*, *N. meningitidis*) B (*E. coli*, *Streptococcus* spp.)
Isomerases			
5.3.1.8	Mannose phosphate isomerase	MPI	C (*E. coli*)
5.3.1.9	Phosphoglucose isomerase	PGI	A (*H. influenzae*) B (*E. coli*) D (*P. aeruginosa*, *Streptococcus* spp.) G (*Bordetella* spp., *N. gonorrhoeae*)

[a] See explanation of buffer systems in Table 2.
[b] x, Undefined in *Enzyme Nomenclature*.

TABLE 4. Staining solutions for oxidoreductases[a]

Enzyme	Substrate and coupling enzyme	Amt of buffer (ml)		Salt	Coenzyme[d]
	Compound or solution (amt)	Tris hydrochloride (pH 8.0)[b]	Sodium phosphate (pH 7.0)[c]	Type (amt)	
Dehydrogenases[d]					
ADH	Ethanol (3 ml)	50			NAD
	Isopropanol (2 ml)				
M1P	Mannitol 1-phosphate (M4250; 5 mg)	50			NAD
HBD	DL-β-Hydroxybutyric acid (sodium salt) (H 6501) (100 mg)	50		0.1 M $MgCl_2$[e] (2 ml) NaCl (200 mg)	NAD
MDH	2.0 M malic acid[f] (6 ml)	40			NAD
ME	2.0 M malic acid[f] (6 ml)	40		0.1 M $MgCl_2$ (2 ml)	NADP
IDH	1.0 M isocitric acid[g] (2 ml)	50		0.1 M $MgCl_2$ (2 ml)	NADP
6PG	6-Phosphogluconic acid (barium salt) (P 7627) (10 mg)	20		0.1 M $MgCl_2$ (10 ml)	NADP
G6P	Glucose 6-phosphate (disodium salt hydrate) (G 7250) (100 mg)	50		0.1 M $MgCl_2$ (1 ml)	NADP
GP1	Fructose 1,6-diphosphate (F 0752) (100 mg)	40		$Na_2HAsO_4 \cdot 7H_2O$ (50 mg)	NAD or NADP
GP2	Aldolase (A 6253) (10 U)				
LDH	DL-Lithium lactate (330 mg)	0.1 M Glycyl-glycine (pH 7.5[h]), 50			NAD
	Fructose 1,6-diphosphate (10 mg)				
THD	L-Threonine (50 mg)		50		NAD
XDH	Hypoxanthine (100 mg)	50			NAD
ALD	DL-Alanine (50 mg)		50		NAD
GD1	L-Glutamic acid (200 mg)	50			NAD
GD2	L-Glutamic acid (200 mg)	50			NADP
LED	L-Leucine (50 mg)		50		NAD
ASP	L-Aspartic acid (50 mg)		50		NAD
LYS	L-Lysine (50 mg)		50		NAD
Other					
IPO	None[i]	40		0.1 M $MgCl_2$ (2 ml)	NAD
CAT[j]					

[a] Sigma Chemical Co. product numbers in parentheses, except as noted.
[b] 0.2 M Tris hydrochloride (pH 8.0) buffer: 24.2 g Tris in 1 liter of water; adjust pH with HCl.
[c] Sodium phosphate (pH 7.0) buffer: mix equal parts of 27.6 g of $NaH_2PO_4 \cdot H_2O$ (monobasic) in 1 liter of water and 53.6 g of $Na_2HPO_4 \cdot 7H_2O$ in 1 liter of water, then dilute the mixture 1:25 with water.
[d] To stain dehydrogenases, dissolve substrate in buffer, then add 1.0 ml of dimethylthiazol tetrazolium (MTT; M 2128) solution (1.25 g in 100 ml of water) and 0.5 ml of phenazine methosulfate (PMS; P 9625) solution (1 g in 100 ml of water) and either 2.0 ml of NAD (Boehringer 127-981)* solution (1 g of NAD-free acid in 100 ml of water) or 1.0 ml of NADP (Boehringer 128-058)* solution (1 g of disodium NADP in 100 ml water), as indicated. Keep solutions refrigerated, and keep MTT and PMS solutions in the dark. Asterisks refer to Boehringer-Mannheim Biochemicals product numbers.
[e] Magnesium chloride solution: 2.03 g of $MgCl_2 \cdot 6H_2O$ in 100 ml of water.
[f] Malic acid solution: 268 g of DL-malic acid and 160 g of NaOH in 1 liter of water. Caution: potentially explosive reaction.
[g] Isocitric acid solution: 2.94 g of DL-isocitric acid $\cdot H_2O$ (trisodium salt) in 100 ml of water.
[h] Glycyl-glycine (pH 7.5) buffer: 11.3 g of glycine (G 7126) in 1 liter of water. Adjust pH with 1 M KOH.
[i] Stain in the light at room temperature.
[j] Catalase: incubate gel slice for 15 min in 50 ml of a solution containing 1.5 ml of a 50% solution of hydrogen peroxide and 750 mg of sodium sulfite. Pour off solution, rinse gel slice with water, and immerse it in a freshly made 1.5% solution of potassium iodide (750 mg in 50 ml of water). Mix gently and remove stain solution when white zones appear on dark-blue background.

interlocus variance in allelic diversity and evolutionary rate of change (46, 48). In certain studies (see, e.g., reference 33), the objective has been merely to distinguish strains for epidemiological tracing. For this purpose, the analysis of only a few polymorphic loci may be sufficient, depending on the number and relationships of the strains involved in particular epidemiological situations.

LABORATORY PROCEDURES

Preparation of enzyme extracts

To obtain sufficient concentrations of enzymes in lysate preparations, 10^{11} cells of each isolate are grown. For example, *E. coli* is grown overnight at 37°C in 100 ml of nutrient broth, and *Neisseria gonorrhoeae* is grown overnight on 10 GC agar base (Difco Laboratories) with 1% IsoVitalex (BBL Microbiology Systems) plates.

Whether cells are grown in broth or on agar plates is unimportant, since growth conditions do not affect the electrophoretic mobilities of enzymes. However, some enzymes may vary in activity (but not in mobility) on gels, depending on the type of medium in which the cells were grown. The activity level of certain enzymes may also vary with the composition of the culture medium, but, again, electrophoretic mobilities are not affected.

Cells harvested by centrifugation of broth cultures (15,000 × *g* for 10 min) or by being scraped from plates are suspended in 2 ml of buffer solution. For most bacteria the buffer is 10 mM Tris–1 mM EDTA–0.5 mM NADP (pH 6.8); for *Streptococcus* spp. it is 40 mM potassium phosphate (pH

TABLE 5. Staining solutions for transferases

Enzyme	Substrate, coupling enzyme, and coenzyme[a]	Buffer and salt	Dye and catalyst
	Compound (amt)	Type (amt)	Type (amt)
GTF[b]	Sucrose (5 g)	0.1 M KH_2PO_4 (pH 6.8) (100 ml) Sodium azide (12 mg)	
NSP[b]	Inosine (I 4125) (20 mg) Xanthine oxidase (2 U)	Sodium phosphate (pH 7.0)[c] (25 ml)	MTT[d] (1.0 ml) PMS[d] (0.5 ml)
GOT	L-Aspartic acid (50 mg) Pyridoxal 5'-phosphate (P 9255) (1 mg) α-Ketoglutaric acid (100 mg)	0.2 M Tris hydrochloride (pH 8.0) (50 ml)	Fast Blue BB salt (100 mg)
GPT[b]	L-Alanine (50 mg) Glutamate dehydrogenase (G 2501) (40 mg) α-Ketoglutaric acid (100 mg) NAD (2 mg) NADP (1 mg)	0.2 M Tris hydrochloride (pH 8.0) (50 ml)	MTT (1.0 ml) PMS (0.5 ml)
ADK[b]	Glucose (100 mg) ADP (Boehringer 127-507) (25 mg) Hexokinase (H 5125) (1 mg) Glucose 6-phosphate dehydrogenase (G 8878) (15 U) NADP (1 mg)	0.2 M Tris hydrochloride (pH 8.0) (25 ml) 0.1 $MgCl_2$ (1 ml)	MTT (0.6 ml) PMS (0.6 ml)
PGM	Glucose 1-phosphate (G 1259) and Glucose 1,6-diphosphate[e] (5 mg) Glucose 6-phosphate dehydrogenase (G 8878) (50 U) NADP (0.5 mg)	0.2 M Tris hydrochloride (pH 8.0) (5 ml) 0.1 M $MgCl_2$ (5 ml) Water (25 ml)	MTT (1.0 ml) PMS (0.5 ml)
HEX	D-Glucose (200 mg) ATP (50 mg) NADP (10 mg) Glucose 6-phosphate dehydrogenase (10 U)	0.1 M Glycyl-glycine (pH 7.5)[f] 0.1 M $MgCl_2$ (2 ml)	MTT (1.0 ml) PMS (0.5 ml)

[a] Sigma Chemical Co. product numbers in parentheses, except as noted.
[b] Agar overlay: 500 mg of agar in 25 ml 0.2 M Tris hydrochloride buffer (pH 8.0). Boil, cool to 60°C, add to stain solution, mix, and pour evenly over gel slice.
[c] See footnote *c* to Table 4.
[d] See footnote *d* to Table 4.
[e] The glucose 1-phosphate preparation (G 7250) contains sufficient glucose 1,6-diphosphate for the staining reaction.
[f] See footnote *h* to Table 4.

7.5) with 3 mM dithiothreitol, 10 mM L-cysteine hydrochloride, and 0.06 mM $MnSO_4$; and for *H. influenzae* it is a phosphate-buffered saline solution. Cells are normally lysed by sonication with a model 200 Branson Cell Disrupter with microtip for 30 to 60 s, with ice-bath cooling. However, any method of lysis that does not denature proteins may be used, e.g., freezing at −25°C for 48 to 72 h (*Neisseria meningitidis*) or sonication with glass beads (diameter, 5 μm) for 3 min, with dry ice-methanol cooling (*Streptococcus* spp.).

After lysis and centrifugation at 30,000 × *g* for 20 min, aliquots of the several milliliters of lysate (supernatant) are transferred to three or four culture tubes and stored at −70°C until used for electrophoresis. At that temperature, lysates of *E. coli* and some other species can be stored for several years without significant loss of activity of most enzymes. However, the stability of the enzymes varies markedly among species of bacteria: for *L. pneumophila*, there may be conspicuous loss of activity within a few weeks, and in lysates of all species of bacteria, enzyme activity gradually diminishes with repeated thawing and freezing.

Electrophoresis

Apparatus for horizontal starch-gel electrophoresis is used. Starch gels are preferred over polyacrylamide gels because of the ease with which horizontal slices can be cut for independent assays of several different enzymes (see reference 39 for an alternative polyacrylamide gel method).

To prepare a gel, a suspension of 48 g of starch (no. 2901-027; Connaught Laboratories) in 420 ml of gel buffer (Table 1) in a 1-liter Erlenmeyer flask is heated over a Bunsen burner to just beyond the boiling point, with constant vigorous swirling. The suspension is then aspirated for 1 min (or until very large bubbles appear) and immediately poured into a 9 by 190 by 210 mm lucite gel mold. After the gel has cooled at room temperature for 2 h, it is wrapped in plastic film to prevent desiccation. Gels are used within 24 h of preparation.

In loading a gel, pieces of Whatman no. 3 filter paper (9 by 6 mm) are individually dipped into samples of lysate, blotted on filter paper to remove excess liquid, and then inserted at 3-mm intervals in a continuous slit cut in the gel. Up to 20 lysates can be electrophoresed on a single gel. Pieces of filter paper dipped in amaranth dye are inserted at one or both ends of the slit to mark the migration front of the buffer line.

During electrophoresis, a constant voltage is maintained (Table 2) and the gel is cooled by a pan of ice supported above the gel mold on a thin plate of glass. (Gels may be run in cold rooms or refrigerators at 4°C with or without ice-pan

TABLE 6. Staining solutions for hydrolases

Enzyme	Substrate and enzyme[a]	Buffer and salt	Dye
	Compound (amt)	Type (amt)	Type (amt)
EST	α-/β-Naphthyl acetate or α-/β-Naphthyl propionate (1% solution in acetone) (1.5 ml)	Sodium phosphate (pH 7.0)[b] (40 ml)	Fast Blue RR salt (25 mg)
ALP	β-Naphthyl acid phosphate (50 mg) Polyvinylpyrrolidone (100 mg)	0.05 M Tris hydrochloride (pH 8.5) (50 ml) NaCl (1 g) 0.1 M $MgCl_2$ (2 ml) 0.25 M $MnCl_2$[c] (2 ml)	Fast Blue BB salt (50 mg)
ACP	α-Naphthyl acid phosphate (50 mg) β-Naphthyl acid phosphate (50 mg)	0.05 M sodium acetate (pH 5.0)[d] (50 ml)	Black K salt (20 mg)
BGA	6-Bromo-2-naphthyl-β-D-galactopyraniside (B 7627) (dissolved in 5 ml of methanol) (10 mg)	Phosphate-citrate[e] (pH 5.0) (8.5 ml) Water (30 ml)	Tetrazotized *o*-dianisidine (D 3502) (30 mg)
LAP	L-Leucine-β-naphthylamide hydrochloride (L 0376) (30 mg)	0.1 M KH_2PO_4 (pH 5.5)[f] (50 ml) 0.1 M $MgCl_2$ (1 ml)	Black K salt (30 mg)
PEP[g]	Peptide[h] (20 mg) Peroxidase (P 8125) (10 mg) Snake venom (V 7000) (10 mg)	0.2 M Tris hydrochloride (pH 8.0) (50 ml) 0.25 M $MnCl_2$ (0.5 ml)	*o*-Dianisidine dihydrochloride (10 mg)

[a] Sigma Chemical Co. product numbers in parentheses.
[b] See footnote *c* to Table 4.
[c] Manganese chloride solution: 4.90 g $MnCl_2 \cdot 4H_2O$ in 100 ml of water.
[d] Sodium acetate buffer: 6.8 g of sodium acetate · $3H_2O$ in 1 liter of water; adjust pH to 5.0 with about 2 ml of HCl.
[e] Phosphate-citrate buffer: mix 10.2 ml of 1.0 M phosphoric acid, 10.2 ml of 2.0 M NaOH, and 1.03 g of citric acid monohydrate in 76.9 ml of water. To stain for BGA, incubate gel slices in the substrate solution at 37°C for 15 min, then stain for 5 min if BGA was induced (or for 1 h if it was not induced) with a solution of 30 mg of dye in 30 ml of water, with pH adjusted to 7.8 with $NaHCO_3$.
[f] Potassium phosphate buffer: 13.6 g KH_2PO_4 in 1 liter of water; adjust pH to 5.5 with NaOH.
[h] Peptides: L-leucyl-L-alanine (Leu-Ala), L-phenylalanyl-L-leucine (Phe-Leu), etc.
[g] Agar overlay: 500 mg of agar in 25 ml of 0.2 M Tris hydrochloride buffer (pH 8.0).

cooling, but under these conditions there is some loss of resolution.) Following electrophoresis, three or four horizontal slices (1 to 2 mm thick) are cut from the gel with a thin wire and incubated individually at 37°C in various enzyme-staining solutions (see Tables 4 through 8).

The optimal electrophoretic conditions for each enzyme for a given bacterial species are determined by testing various buffer systems and other variables (59). The activity level and degree of separation of electromorphs of an enzyme may depend on the type and pH of the buffer system used and, to some extent, on the concentration of starch in the gel. Thus, an enzyme may appear to be monomorphic or polymorphic for only two or three electromorphs in one buffer system but exhibit 5 to 10 electromorphs in another buffer system. Occasionally, the relative mobilities of certain electromorphs are reversed in different buffer systems.

Staining for specific enzymes

The enzymes routinely stained in our laboratory are listed in Table 3, with the appropriate buffer system in which each enzyme is electrophoresed for various bacterial species.

Stain recipes are given in Tables 4 through 8. Each recipe is for a volume of solution sufficient to stain a single gel slice. For some enzymes, as noted, the stain solution is applied to the gel in an agar overlay. Gels are incubated at 37°C in the dark until bands appear; this, for different enzymes and species of bacteria, may be from 10 min to several hours. The stain solution is then poured off, and the gel slice is rinsed with water (except when the stain has been applied in an agar overlay) and fixed in a 1:5:5 mixture of acetic acid, methanol, and water.

Properly electrophoresed and stained enzymes appear on gels as narrow, sharply defined bands (Fig. 1); for some particularly active enzymes it may be necessary to dilute aliquots of lysate to obtain satisfactory resolution of electromorphs. For many enzymes in any given bacterial species, activity can be demonstrated on gels, but poor resolution precludes the accurate scoring of polymorphic variation.

Reading the gels

Comparisons of the mobilities of enzymes from different isolates are made visually against one another on the same gel slice. It is not sufficient to compare relative mobilities of an enzyme in different strains by measuring distances of migration from the origin, even when the enzymes have been electrophoresed on the same gel. For weakly polymorphic populations or species consisting of small numbers of clones, only 40 to 50 gels may be required to score 20 different enzymes in 100 isolates. However, a similar analysis of a highly polymorphic species, such as *E. coli* or *L. pneumo-*

TABLE 7. Staining solutions for lyases

Enzyme	Substrate and enzyme[a]	Buffer and salt	Dye and catalyst
	Compound (amt)	Type (amt)	Type (amt)
ALD	Fructose 1,6-diphosphate (F 0752) (100 mg) Glyceraldehyde 3-phosphate dehydrogenase (G 5126) (50 U) Triosphosphate isomerase (T 7877) (100 U) NAD (Boehringer 127-981) (20 mg)	0.1 M Tris acetate (pH 7.5)[b] (50 ml) $Na_2HAsO_4 \cdot 7H_2O$ (100 mg)	PMS (0.5 ml) MTT (1.0 ml)
FUM	Fumaric acid (potassium salt) (F 4633) (50 mg) Malate dehydrogenase (M 7508) (50 U)[c] NAD (20 mg)	0.2 M Tris hydrochloride (pH 8.0) (50 ml)	PMS (0.5 ml) MTT (1.0 ml)
ACO[d]	*cis*-Aconitic acid (A 7251) (20 mg) Isocitrate dehydrogenase (I 2002) (5 U) NADP (10 mg)	0.2 M Tris hydrochloride (pH 8.0) (15 ml) 0.1 M $MgCl_2$ (10 ml)	PMS (0.5 ml) MTT (1.0 ml)

[a] Sigma Chemical Co. product numbers in parentheses, except as noted.
[b] Tris acetate buffer: dissolve 12.11 g of Tris in 1 liter of water, adjust pH to 7.5 with glacial acetic acid, and add 19.63 g of potassium acetate, 333 mg of cobalt chloride, and 35.2 mg of L-cysteine hydrochloride.
[c] For *H. influenzae*, use 32 U.
[d] Agar overlay: 500 mg of agar in 25 ml of 0.2 M Tris hydrochloride buffer (pH 8.0).

TABLE 8. Staining solutions for isomerases

Enzyme[a]	Substrate and enzyme[b]	Buffer and salt	Dye and catalyst
	Compound (amt)	Type (amt)	Type (amt)
MPI	Mannose 6-phosphate (M 8754) (10 mg) Glucose 6-phosphate dehydrogenase (G 8878) (10 U) Phosphoglucose isomerase (P 9010) (50 U) NAD (20 mg) NADP (10 mg)	0.2 M Tris hydrochloride (pH 8.0) (25 ml) 0.1 M $MgCl_2$ (1.0 ml)	PMS (0.5 ml) MTT (1.0 ml)
PGI	Fructose 6-phosphate (F 3627) (10 mg) Glucose 6-phosphate dehydrogenase (G 8878) (3 U) NADP (6 mg)	0.2 M Tris hydrochloride (pH 8.0) (25 ml) 0.1 M $MgCl_2$ (0.3 ml)	PMS (0.5 ml) MTT (1.0 ml)

[a] Agar overlay: 500 mg of agar in 25 ml of 0.2 M Tris hydrochloride buffer (pH 8.0).
[b] Sigma Chemical Co. product numbers in parentheses.

phila, in which some enzymes are represented by a dozen or more electromorphs, may require several hundred gels.

For each enzyme, distinctive electromorphs are numbered in order of decreasing anodal migration. The absence of enzyme activity is scored as a null character state and allele, but in all such cases it should be determined that a given "null" does not merely reflect inadequate enzyme concentration in the lysate or denaturation caused by cell lysis or storage.

Care must also be taken to avoid scoring conformational bands of certain enzymes and misidentifying enzymes. For example, in *N. meningitidis*, an unidentified "nothing" dehydrogenase appears on gels stained for a variety of dehydrogenases and even on gels stained with only a solution of PMS and MTT (dye and intermediary catalyst) (B. A. Crowe, T. Olyhoek, and M. Achtman, Semin. Infect. Dis., in press).

Each isolate is characterized by its combination of electromorphs over the number of enzymes assayed, and distinctive profiles of electromorphs, corresponding to unique multilocus genotypes, are designated electrophoretic types (ETs), which are equivalent to allele profiles.

Analyzing data

A hypothetical example of data analysis is given in Tables 9 through 11. For a sample of isolates (Table 9), genetic diversity may be expressed separately for each enzyme locus and as the mean allelic diversity over loci, calculated from allele frequencies at individual loci among either iso-

TABLE 9. Hypothetical example showing electrophoretic type for five isolates

Isolate no.	Electromorph (allele) at enzyme locus:			
	A	B	C	D
1	1	3	4	2
2	1	3	3	2
3	1	3	5	1
4	1	2	1	3
5	1	1	2	3

TABLE 10. Allele frequencies in sample of five isolates[a]

Enzyme locus	Frequency of allele:				
	1	2	3	4	5
A	1.00				
B	0.20	0.20	0.60		
C	0.20	0.20	0.20	0.20	0.20
D	0.20	0.40	0.40		

[a] See Table 9.

TABLE 11. Genetic distance (*D*) between pairs of isolates[a]

Strain	1	2	3	4	5
1	0.00				
2	0.25	0.00			
3	0.50	0.50	0.00		
4	0.75	0.75	0.75	0.00	
5	0.75	0.75	0.75	0.50	0.00

[a] Genetic distances are based on the alleles at the four enzyme loci (see Table 9).

lates or ETs (Table 10). Genetic diversity for a locus is calculated as $h = 1 - \Sigma x_i^2[n/(n-1)]$, where x_i is the frequency of the ith allele at the locus, n is the number of isolates or ETs in the sample, and $n/(n-1)$ is a correction for bias in small samples (48). (Genotypic diversity may be calculated by the same formula, in which x_i is the frequency of the ith ET and n is the number of ETs.) Mean diversity per locus ($\bar{h}$) is the arithmetic average of h over all loci assayed, including monomorphic ones. For the hypothetical example, genetic diversities for the four loci are 0.00, 0.45, 0.75, and 0.55 for A through D, respectively; the mean diversity for the loci is 0.437.

Genetic distance (*D*) between pairs of isolates or ETs may be expressed by any of several types of coefficients (46, 76). We normally calculate genetic distance between pairs of isolates as the proportion of loci at which dissimilar alleles occur, i.e., the proportion of mismatches (Table 11). In some cases, we have used a weighted coefficient, with the contribution of each locus to *D* being weighted by the reciprocal of the mean genetic diversity at the locus in the total sample being analyzed (67). In this manner, greater weight is given to differences at less variable loci than to those at highly polymorphic loci.

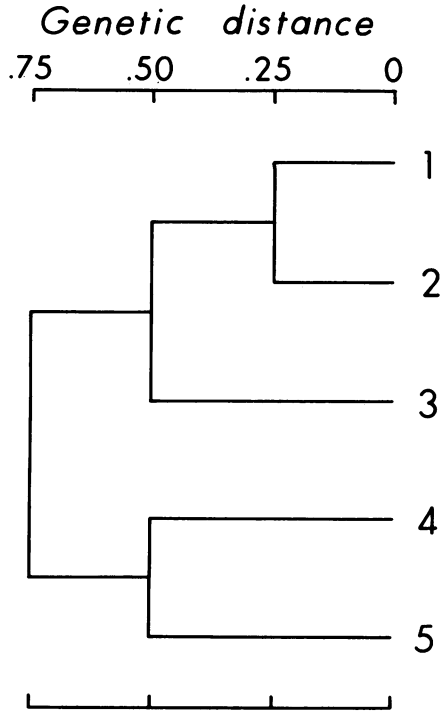

FIG. 2. Dendrogram generated from the matrix of genetic distance (*D*) in Table 11.

From a matrix of coefficients of genetic distance, various multivariate statistical methods may be used to represent overall genetic relatedness among isolates or ETs (76), including principal components, principal coordinates, and clustering (Fig. 2).

G-statistics (47) may be used to apportion genetic diversity within and between groups of ETs or isolates.

Statistical methods of analyzing linkage disequilibrium (nonrandom association of alleles) are illustrated by Whittam et al. (78, 79).

A computer program of statistics for population genetics, written especially for use with bacterial data, is available, upon request, from T.S.W.

Various applications of genetic data obtained by multilocus enzyme electrophoresis to bacterial population genetics, systematics, and epidemiology are illustrated in the papers cited in Table 1.

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