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Comparison of Phenotypic Diversity and DNA Heterogeneity in a Population of Soil Bacteria

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The phenotypic diversity of about 200 bacterial strains isolated from soil was compared with the genotypic diversity of the same population. The strains were phenotypically characterized by the API 20B test system. The results of these tests were subjected to cluster analysis, which revealed 41 biotypes at 80% similarity. The five dominating biotypes contained 43% of the strains. The phenotypic diversity as determined by the Shannon index, equitability, rarefaction, and cumulative differences was high, but indicated some dominant biotypes. The genetic diversity was measured by reassociation of mixtures of denatured DNA isolated from the bacterial strains (C_0t plots). The observed genetic diversity was high. Reassociation of DNA from all bacterial strains together revealed that the population contained heterologous DNA equivalent to 20 totally different bacterial genomes (i.e., genomes that have no homology). This study showed that reassociation of DNA isolated from a collection of bacteria gave a good estimate of the diversity of the collection and that there was good agreement with different phenotypic diversity measures. The Shannon index in particular has features in common with the genetic diversity measure presented here.

Microbial diversity has been analyzed mainly by methods revealing phenotypic features (1). The phenotypic approach has several limitations. One is that bacterial strains have to be isolated from the biotype and cultivated in the laboratory before phenotypic testing can be performed. Another is that only a restricted part of the genetic information is revealed through phenotypic testing. To reveal information about the entire genetic material, it is necessary to investigate the genome in a more direct manner. A diversity measure based on the genetic composition of the organisms will also make it possible to measure the diversity of the nonculturable part of various bacterial communities.

Britten and Kohne (3) have demonstrated how reassociation of denatured (single-stranded) DNA can be used to determine the genome sizes of different organisms, i.e., the amount of distinct DNA that is present in an organism. Variants of the same principle have also been suggested for defining genetic (phylogenetic) relationships between organisms (19).

In a taxon-oriented study of diurnal fluctuations in river bacteria, Holder-Franklin et al. (7) compared numerical taxonomy and DNA homology by hybridization methods, but to our knowledge this is the only study in which DNA comparisons have been used in population analysis.

Reassociation studies of genomes have so far been performed only with mixtures of DNA from a maximum of two organisms present. In this work we have explored whether reassociation of denatured DNA from a mixture of organisms can be used to obtain a measure of genotypic diversity in a bacterial plate count population. For comparison, the population was analyzed phenotypically, and a variety of diversity parameters have been calculated.

MATERIALS AND METHODS

Soil type. The soil in a natural beech forest at Seim, about 20 km north of Bergen, Norway, was sampled. Samples from the humic layer (0 to 20 cm) were mixed and air dried at 22°C for 30 h. The soil was then sieved (mesh size, 4 mm) prior to analysis. The following results were obtained after air drying; soil organic matter, 49% of the dry weight; water content, 213% of the dry weight; pH in distilled water, 4.1. These values are all quite typical for soils in western Norway. The direct bacterial count obtained by using acridine orange was 1.5×10^{10} g (dry weight) of soil^{-1}, and the plate count was 4.3×10^7 g (dry weight) of soil^{-1} on Thornton medium (18) with 10% soil extract.

Isolation of bacterial strains. A 10-g (wet weight) soil sample was homogenized at low speed (three times for 1 min each) with 90 ml of sterile-filtered, cold Winogradsky salt solution diluted 1:20 (12) in a sterile Waring blender. Samples (0.1 ml) of 10-fold dilutions were spread on plates containing Thornton medium with 10% soil extract. The plates were wrapped in plastic bags (to retain moisture) and incubated at 22°C for 8 days. Then 250 isolates from the 10^{-4} dilution were picked randomly (all colonies from one and one-half plates) and subcultured on the same medium three times. The isolates were then assumed to be pure. Some strains failed to grow after a few transfers to new plates, and some were microscopically determined to be yeasts. Altogether 187 strains were phenotypically characterized, and 206 strains were included in the genetic analysis.

Test system. The isolates were examined by the API 20B test system (API 2040; API System S.A., Montalieu-Vercieu, France). The following tests were used: proteolysis of gelatin (GEL); action of nitrate reductase (NIT); action of β-galactosidase (ONPG); production of acid metabolites from the carbohydrates saccharose (SAC), L-(+)-arabinose (ARA), mannitol (MAN), fructose (FRU), glucose (GLU), maltose (MAL), starch (AMD), rhamnose (RHA), galactose (GAL), mannose (MNE), sorbitol (SOR), and glycerol (GLY); action of urease (URE); action of tryptophanase (IND); H_2S formation (H_2S); utilization of citrate as the sole carbon source (CIT); presence of cytochrome oxidase (OX); and presence of catalase (CAT). API 20B also includes the Voges-Proskauer test. We chose to exclude this test, as we had problems in scoring it for many isolates. The tests were

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performed as specified by the manufacturer, but with the following modifications. The tests were incubated at 22°C for 3 days before reading. CAT and OX were carried out directly on 4-day-old colonies. Oxidative or fermentative glucose metabolism (OFF) was tested as recommended in the description of API OF (API 5011). Gram staining (GRAM) was performed on 4-day-old colonies. Morphology (COCC), motility (MOT), and presence of endospores (SPOR) were examined by using suspensions of 4-day-old colonies and a phase-contrast microscope. Ammonification (AMM) was tested by using a medium consisting of 0.1 g of $MgSO_4$, 0.2 g of K_2HPO_4, 0.1 g of NaCl, and 2.0 g of Casamino Acids (Difco Laboratories, Detroit, Mich.) dissolved in 1,000 of ml distilled water, with the pH adjusted to 6.2. A few grains of phenol red were added until a clear yellow color appeared. Portions (5 ml) were distributed into tubes that were then capped and autoclaved. The tubes were inoculated with 1 drop from a liquid culture at the end of the exponential growth phase. The results were recorded after a 3-day incubation period at 22°C.

All tests were recorded as 1 or 0 (i.e., presence/positive or absence/negative, respectively). Tests on which all isolates scored 0 were omitted from subsequent data analyses.

Phenotypic diversity measures. Cumulative differences (d) between pairs of isolates were calculated by the following formula:

$$d = \sum_{i=1}^{n} | X_{ij} - X_{ik} |$$

where n is the number of tests and X_{ij} and X_{ik} are the score (1 or 0) of isolates j and k on the ith test. The isolates were randomly sequenced and added one by one. The smallest difference between a newly added isolate and the preceding isolates represents the new phenotypic information in the strain, and was added cumulatively. To account for erroneous scoring of tests, a margin of error can be included. We have used margins of 0 and 2. The same sequence of isolates was used as in the DNA renaturation experiments.

Cluster analysis of all isolates was performed as described by Sørheim et al. (17). Proximity was measured by using the simple matching coefficient (16), and clusters were grouped by the complete link (farthest-neighbor) method (8). Clusters showing >80% similarity were defined as a biotype. This corresponds to four characters separating the biotypes.

Rarefaction was performed by the method of Simberloff (14). The method is a theoretical rarefying of a population on the basis of number of biotypes and the number of isolates belonging to each biotype. The program allows an estimation of the expected number of biotypes in a sample of a given size.

The Shannon index, H' (13), was calculated ($\log_2$) on the basis of biotypes defined in the cluster analysis. The equitability, J (11), was also calculated. In addition, H' was calculated for subsamples of the 187 isolates. This was to compare the phenotypic diversity with the genotypic diversity revealed by reassociation of DNA (see below). First, a cluster analysis was performed for five isolates. After addition of the next five isolates, a new cluster analysis was performed. Addition of new isolates continued until cluster analyses had been performed on all isolates. The shannon index was calculated from the biotypes identified among the different subsamples.

Determination of genotypic diversity. Under appropriate conditions (e.g., cation concentration, temperature, length of fragments, and concentration), the reassociation rate of DNA depends on the concentration of homologous DNA (10). The reassociation of single-stranded DNA follows a second-order reaction, in which the rate, k, is proportional to the square of the concentration of homologous DNA. For a constant total DNA concentration, the reassociation rate will thus be an inverse function of DNA diversity. Britten and Kohne (3) introduced the term $C_0t_{1/2}$ as an expression of genomic size; C_0 denotes the molecular concentration of base pairs in single-stranded DNA at the start of the experiment, and $t_{1/2}$ denotes the time in seconds when half of the DNA has reassociated ($C_0t_{1/2} = k^{-1}$). They could demonstrate that the $C_0t_{1/2}$ value of DNA isolated from one species is proportional to the genome size of this species if there are no repeating DNA sequences.

The fraction of DNA which remains single stranded at any time after initiation of reassociation is given by Britten et al. (2) as

$$C/C_0 = 1/(1 + kC_0t) \quad (1)$$

where C is the concentration of single-stranded DNA at any time and t is the reaction time in seconds.

When the reaction is half completed (i.e., $C/C_0 = 1/2$), C_0t is termed $C_0t_{1/2}$ and equation 1 can be written as

$$k = 1/(C_0t_{1/2}) \quad (2)$$

When equal amounts of DNA from different bacteria (without homology) are mixed, it may be expected that the reaction constant, k_n, of the DNA mixture is equal to the mean of the reaction constants, k_i, of the individual DNAs in the mixture divided by the number of strains:

$$k_n = \frac{1}{n^2} \sum_{i=1}^{n} k_i \quad (3)$$

where n is the number of strains. Inserting equation 3 into equation 2 gives

$$\frac{1}{C_0t_{1/2(n)}} = \frac{1}{n^2} \sum_{i=1}^{n} \frac{1}{C_0t_{1/2(i)}} \quad (4)$$

Assuming that $C_0t_{1/2}$ values for DNA from different bacteria are approximately equal, equation 4 may be simplified as follows:

$$C_0t_{1/2(n)} = n \times C_0t_{1/2(i)}$$

The observed $C_0t_{1/2}$ of reassociation of DNA from a mixture of bacteria thus represents the amount of different DNA in the mixture.

Isolation of DNA. The 206 isolates were batch cultured to approximately the same biomass and assembled in groups containing between 5 and 25 isolates. The isolates were placed in the same sequence as for calculation of the cumulative difference. DNA was isolated from these groups by Marmur's method (9), except that the lysis conditions were those of Chassy and Giuffrida (4). The DNA was deproteinized until A_{260}/A_{230} and A_{260}/A_{280} were between 1.8 and 2.3 and between 1.5 and 2.0, respectively.

Reassociation experiments. Before reassociation, the DNA isolates were diluted in 0.1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) (3) to a concentration of approximately 100 $\mu g\ ml^{-1}$ and sheared in a French pressure cell at 20,000 lb in^{-2}. The mean molecular mass of the DNA fragments after shearing was 420,000 daltons (18a).

The equipment used and the procedure for the reassocia-

TABLE 1. Percent positive test scores of the 187 isolates[a]

Test[b]	% Positive scores
GEL	5.9
NIT	31.6
ONPG	55.6
SAC	8.0
ARA	18.7
MAN	24.1
FRU	23.0
GLU	30.5
MAL	9.6
AMD	3.7
RHA	14.4
GAL	20.9
MNE	18.7
SOR	11.8
GLY	8.0
URE	28.3
CIT	89.3
OX	54.6
CAT	94.1
OFF	13.4
MOT	50.8
GRAM	10.7
COCC	5.3
AMM	78.1

[a] All isolates scored negative in the H_2S, IND, and SPOR tests.
[b] For an explanation of abbreviations, see the text.

tion experiments are described elsewhere (18a). To perform reassociations at low temperature and avoid thermal degradation of DNA, we performed all reassociation experiments in a buffer of 4× SSC–30% dimethyl sulfoxide (Merck catalog no. 2950) (5) at 53°C. This temperature was determined after melting of sheared DNA from six randomly selected isolates in 4× SSC–30% dimethyl sulfoxide. The mean melting point (T_m) of these DNAs was 77.5°C, and the optimal temperature for reassociation (T_r) is 25°C lower.

The concentration of DNA in all reassociation experiments was between 1.42×10^{-4} and 1.97×10^{-4} mol liter^{-1} and the collapse (i.e., a rapid fall in optical density as DNA is cooled from denaturing temperature to T_r [2]) was between 19.2 and 23.6% of melting hyperchromicity.

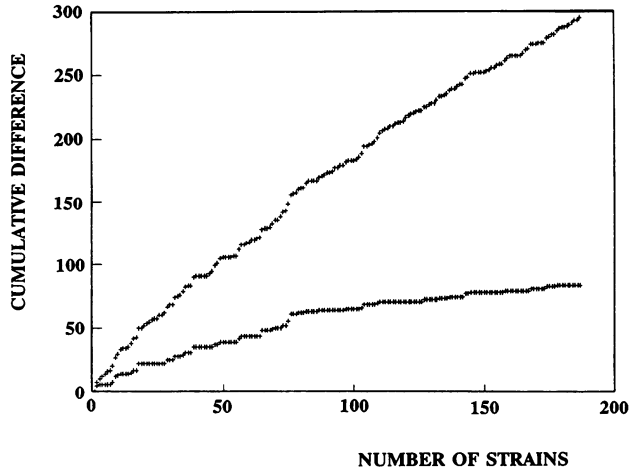

FIG. 1. Cumulative difference. The abscissa gives the number of strains, randomly ordered. The ordinate gives new phenotypic information added by each new isolate. For the upper curve, the margin of error is 0; for the lower curve, the margin of error is 2.

FIG. 2. Cluster analysis of all 187 strains. Biotypes are defined as having 80% similarity.

RESULTS

Total bacterial counts (epifluorescence) and plate counts on Thornton medium with 10% soil extract were of the same order of magnitude as previously reported for this soil (15, 17). The results of the phenotypic tests are presented in Table 1. The three tests H_2S, IND, and SPOR were negative or absent for all isolates. The other tests were positive or present for between 7 (AMD) and 176 (CAT) isolates. The majority of tests were positive or present for a low proportion (<40%) of the isolates.

The ratio between the cumulative difference (Fig. 1) and the number of strains was nearly constant when no tests were allowed to be equal for two isolates. It reached a maximum of 294 for 187 isolates, which is approximately 1.6 units per isolate. Even with as many as 187 isolates, the

TABLE 2. Distribution of isolates among biotypes

n^a	No. of biotypes with n isolates[b]
27	1
15	1
14	2
11	1
8	2
6	1
5	5
4	3
3	7
2	8
1	10

[a] n is the number of isolates in one biotype.
[b] Biotypes include isolates with ≥80% similarity.

curve was still increasing. When the added difference was reduced by 2 (i.e., allowing two tests to be different), the total cumulative difference of the population was reduced to 84. The cumulative difference indicates that the population is composed of bacteria that differ only slightly from each other.

Cluster analysis of the isolated strains (Fig. 2) revealed 41 biotypes of strains showing more than 80% similarity. All isolates were grouped at 17% similarity. That is equivalent to 20 different characteristics in the isolates that are least similar. The distribution of strains among biotype is shown in Table 2. Five biotypes contained more than 10 isolates each, and 43% of the population was classified into one or another of these biotype. The dominating biotype included 14% of the strains (i.e., 27 strains). Four biotypes included between 11 and 15 strains each, and the rest (106 strains) was spread among the remaining 36 biotypes, each containing 1 to 8 isolates. The isolates that belong to the five dominating biotypes are mainly characterized as unable to produce acid from carbohydrates (Table 3). Only two gram-positive isolates belonged to the dominant biotype. The rest were spread among six of the nondominant biotypes.

Figure 3 shows rarefaction of the population. At a sample size of 40 isolates, the estimated number of biotypes was 21, which is half the maximal number of biotypes in the population. The maximal number was not reached before the sample size was near maximum. This results from the skewing of the population. The diversity of the population was high and gave a Shannon index of 4.73. The equitability was 0.88, indicating the presence of some dominant biotypes.

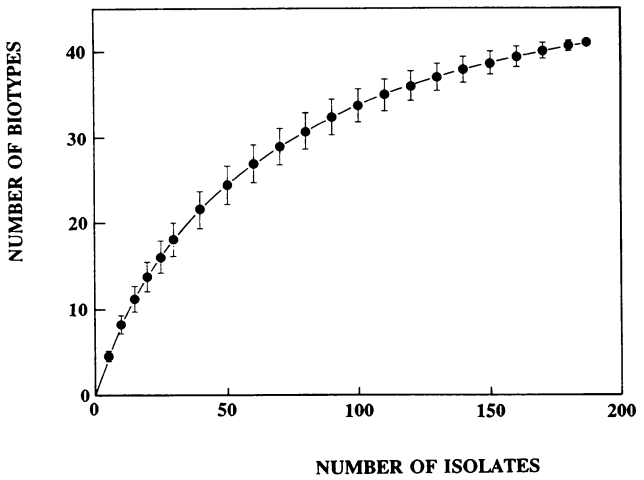

FIG. 3. Rarefaction of all isolates, based on the biotypes obtained from the cluster analysis. The ordinate gives the expected number of biotypes for a given number of isolates.

The Shannon index on subsamples of the original population (Fig. 4) showed a rapid increase with increasing population size, until a near-maximal value was obtained at approximately 90 isolates, whereafter the curve flattened out. This demonstrates that the diversity as measured by the Shannon index would have been equally high with 90 isolates as with 187. The rather high values of the Shannon index, combined with the shape of the curve, also illustrate that the population is composed of many biotypes, some of which are more dominant than others.

Typical reassociation curves are presented in Fig. 5. The experiments were performed until approximately 50% of the DNA had reassociated. Reassociation curves can be plotted as the inverse concentration of nonreassociated DNA against the time (in seconds) of the reassociation (20). An

TABLE 3. Number of isolates with positive test scores in the five dominating biotypes

Test	No. of positive scores in biotypes containing following no. of isolates:				
	27	15	14	14	11
GEL	1	1	0	0	0
NIT	12	15	0	13	3
ONPG	18	15	6	14	4
SAC, ARA, FRU, GLU, MAL, AMD, RHA, MNE, SOR, GLY	0	0	0	0	0
MAN, GAL	0	1	0	0	0
URE	1	0	2	14	11
CIT	27	15	12	14	11
OX	11	10	14	7	1
CAT	27	15	13	11	11
OFF	0	1	3	0	0
MOT	24	0	2	4	1
GRAM	2	0	0	0	0
COCC	0	0	0	0	1
AMM	25	15	0	13	7

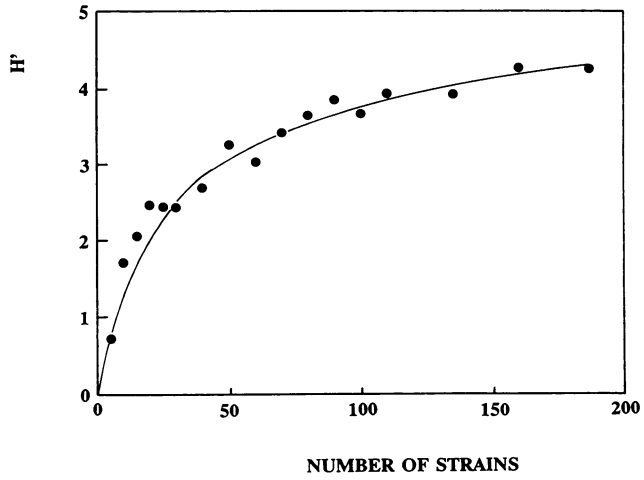

FIG. 4. Shannon index obtained for subpopulations of different sizes.

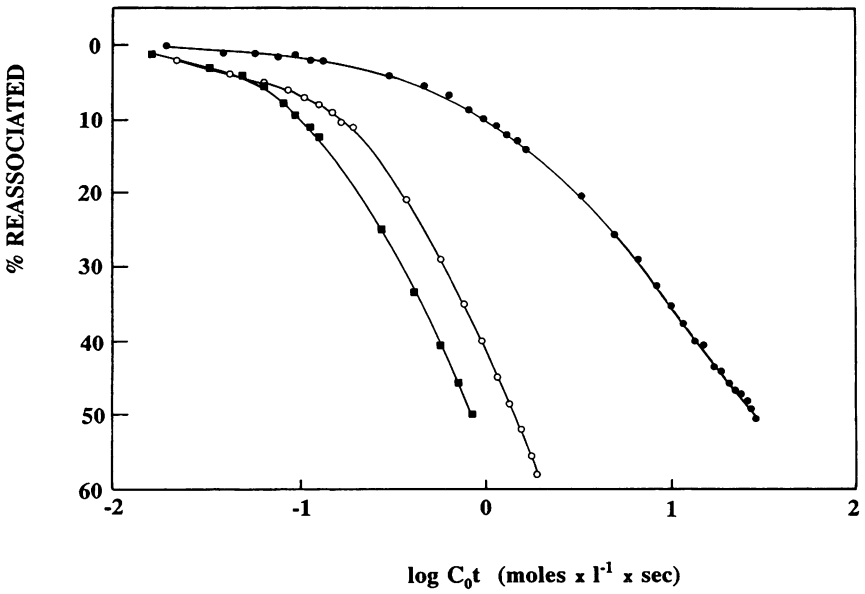

FIG. 5. C_0t plots for *E. coli* B (■), soil isolate 23A (○), and the 206 soil bacterial isolates in the mixture (●).

ideal second-order reaction would then appear as a straight line. The three curves in Fig. 5 have been plotted in this way (not shown), and the correlation coefficient were 0.9997 for the two DNAs isolated from single bacteria (i.e., *E. coli* B and the soil isolate 23A) and 0.9826 for the DNA from all 206 isolates. The reduced correlation of the DNA from the 206 isolates can be explained as the effect of an increased amount of homologous relative to heterologous DNA. This is probably due to the skewed distribution of different bacteria in the population, or it may be that parts of the genomes of different bacteria are homologous. This loss of correlation is, however, probably not sufficient to account for the skew observed in the phenotypical results.

Reassociation of DNA individually from six randomly selected soil bacterial strains in 4× SSC–30% dimethyl sulfoxide (Table 4) shows an average $C_0t_{1/2}$ of (1.40 ± 0.27) mol s liter^{-1}, whereas DNA from *E. coli* B gave 0.85 mol s liter^{-1}. The size of the *E. coli* B genome was determined by Gillis and De Ley (6) to be 2.71 × 10^9 daltons (4.1 × 10^6 base pairs). The size of the genome of an average soil bacterium, denoted standard genome, is calculated to be (4.5 ± 0.9) × 10^9 daltons [(6.8 ± 1.3) × 10^6 base pairs].

$C_0t_{1/2}$ increased with increasing numbers of isolates until it approached a maximum when about 90 isolates were blended (Fig. 6). This implies that the heterogeneity of the DNA in dominant bacterial types in the population is observed in the first 90 isolates. Any DNA variation in bacterial types that occurs less frequently is not recorded before more than 50% of the DNA is reassociated, because of the low concentration of homologous DNA. The values of $C_0t_{1/2}$ show some variation between 90 and 206 isolates. This may be explained as a variation in the amount of homologous DNA in the isolates. When new isolates are added, they may have DNA that is homologous or heterologous to the previous isolates. If the concentration of homologous DNA is increased, $C_0t_{1/2}$ is consequently lowered. It seems unlikely that the variation of $C_0t_{1/2}$ is due to major experimental errors, as duplicate experiments of some DNA mixtures showed only minor variations.

TABLE 4. $C_0t_{1/2}$ for *E. coli* B and six of the soil isolates

Bacterium	10^{-4} Concn (C_0) (mol liter^{-1})	$C_0t_{1/2}$ (mol s liter^{-1})
E. coli B	1.42	0.85
Soil isolates		
2	1.88	1.62
10	1.93	1.32
21	1.90	1.78
23A	1.89	1.41
23B	1.86	1.20
28	1.97	1.05

DISCUSSION

The cumulative-difference approach used in this study clearly visualizes a major problem of microbiology, namely, the lack of stringent borders between species. The gradual

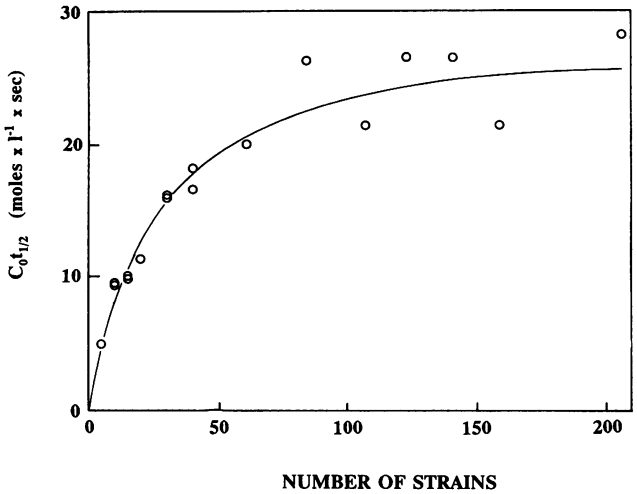

FIG. 6. Relationship between $C_0t_{1/2}$ and the number of isolates from which DNA had been mixed in the reassociation experiment.

shift in characteristics from one strain to another results in increasing numbers of bacterial types even when many strains have already been isolated and characterized. In the treatment of phenotypic results by cluster analysis, this problem is avoided, because the data are simplified in such a way that the continuous variation between different bacteria is obscured.

When the percent reassociated DNA is plotted against the logarithm of t, the curve has a maximal slope at 50% reassociation. The slope is independent of the value of $C_0t_{1/2}$. Britten and Kohne (3) used this to demonstrate the presence of repetitive DNA in eucaryotic cells. Repetitive DNA occurs as multiple short fragments of DNA, thus giving relatively high concentrations of homologous DNA. This appears in the reassociation curves as a separate bump from which a low $C_0t_{1/2}$ value can be deduced. During reassociation of a mixture of DNAs from a bacterial population, similar effects may appear. If the different DNAs are present in the same relative concentrations, the curve will show the features of a second-order reaction, but if some DNAs are present at higher concentrations than the others (some species being dominant), the curve will deviate because it will be a composite of several second-order reactions with different rate constants. This can be seen as a lack of symmetry and a flattening of the slope around $C_0t_{1/2}$. In this case, $C_0t_{1/2}$ has no precise kinetic meaning. It is, however, still a useful parameter for expressing the diversity of DNA in the population, and we use it simply to denote the point at which 50% of the total DNA (C_0) has become reassociated.

Dividing the $C_0t_{1/2}$ of the mixture by the mean $C_0t_{1/2}$ value of genomes of bacteria gives the number of totally different bacteria in the mixture. Reassociation of DNA from 206 bacterial isolates showed a $C_0t_{1/2}$ of 28.2 mol s liter^{-1}. This value corresponds to the $C_0t_{1/2}$ obtained if DNAs from 20 totally different soil bacteria were reassociated. The 206 strains may thus be said to contain heterologous DNA corresponding to 20 totally different bacteria.

However, it must be assumed that in a mixture of randomly sampled environmental bacteria, parts of the genomes of some bacteria are homologous. This will result in a lower $C_0t_{1/2}$ than if the genomes of these bacteria were totally different from each other. $C_0t_{1/2}$ will therefore normally underestimate the number of different bacteria in the mixture. It has been proposed that bacterial species should be defined as having about 70% homologous DNA (19). If the remaining 30% are completely heterologous, the observed $C_0t_{1/2}$ would correspond to 67 different species.

The curves for the Shannon index and $C_0t_{1/2}$ in the population show an increase until about 90 isolates have been included. On the other hand, the cumulative-difference curve shows that new information is added as long as the number of isolates in the population increases. This can be explained by the fact that cumulative difference is a measure of information content, whereas H' and $C_0t_{1/2}$ are also affected by the distribution of the information.

In this study we have shown that reassociation of DNA may be used as a measure of the genetic diversity of bacteria, alternative to the phenotypic approach. C_0t plots for DNA isolated from bacterial populations take into account both the amount of information and its distribution in the population. In this manner C_0t plots resemble some of the classic diversity indices (e.g., the Shannon index) and may be used as an alternative, genetic diversity index.

LITERATURE CITED

1. **Atlas, R. M.** 1984. Diversity of microbial communities. Adv. Microb. Ecol. **7**:1–47.
2. **Britten, R. J., D. E. Graham, and B. R. Neufeld.** 1974. Analysis of repeating DNA sequences by reassociation. Methods Enzymol. **29**:363–418.
3. **Britten, R. J., and D. E. Kohne.** 1968. Repeated sequences in DNA. Science **161**:529–540.
4. **Chassy, B. M., and A. Giuffrida.** 1980. Method for the lysis of gram-positive, asporogenous bacteria with lysozyme. Appl. Environ. Microbiol. **39**:153–158.
5. **Escara, J. F., and J. R. Hutton.** 1980. Thermal stability and renaturation of DNA in dimethyl sulfoxide solutions: acceleration of the renaturation rate. Biopolymers **19**:1315–1327.
6. **Gillis, M., and J. De Ley.** 1975. Determination of the molecular complexity of double-stranded phage DNA from initial renaturation rates. The effect of DNA base composition. J. Mol. Biol. **98**:447–464.
7. **Holder-Franklin, M. A., A. Thorpe, and C. J. Cormier.** 1981. Comparison of numerical taxonomy and DNA-DNA hybridization in diurnal studies of river bacteria. Can. J. Microbiol. **27**:1165–1184.
8. **Lance, G. N., and W. T. Williams.** 1967. A general theory of classificatory sorting strategies. I. Hierarchical systems. Comput. J. **9**:373–380.
9. **Marmur, J.** 1963. A procedure for the isolation of deoxyribonucleic acid from microorganisms. Methods Enzymol. **6**:726–738.
10. **Marmur, J., R. Rownd, and C. L. Schildkraut.** 1963. Denaturation and renaturation of deoxyribonucleic acid. Prog. Nucleic Acid Res. **1**:231–300.
11. **Pielou, E. C.** 1966. The measurement of diversity in different types of biological collections. J. Theor. Biol. **13**:131–144.
12. **Pochon, J.** 1954. Manuel technique d'analyse microbiologique du sol. Masson et Cie., Paris.
13. **Shannon, C. E.** 1948. A mathematical theory of communication. Bell Syst. Technol. **27**:379–423.
14. **Simberloff, D.** 1978. Use of rarefaction and related methods in ecology, p. 150–165. *In* K. L. Dickson, J. Cairns, Jr., and R. J. Livingston (ed.), Biological data in water pollution assessment: quantitative and statistical analyses. ASTM STP 652. American Society for Testing and Materials, Philadelphia.
15. **Skogland, T., S. Lomeland, and J. Goksøyr.** 1988. Respiratory burst after freezing and thawing: experiments with soil bacteria. Soil Biol. Biochem. **20**:851–856.
16. **Sokal, R. R., and C. D. Michener.** 1958. A statistical method for evaluating systematic relationships. Univ. Kansas Sci. Bull. **38**:1409–1438.
17. **Sørheim, R., V. L. Torsvik, and J. Goksøyr.** 1989. Phenotypic divergences between populations of soil bacteria isolated on different media. Microb. Ecol. **17**:181–192.
18. **Thornton, H. G.** 1922. On the development of a standardized agar medium for counting soil bacteria, with especial regard to the repression of spreading colonies. Ann. Appl. Biol. **9**:241–274.

18a. **Torsvik, V., J. Goksøyr, and F. L. Daae.** 1990. High diversity in DNA of soil bacteria. Appl. Environ. Microbiol. **56**:782–787.

19. **Wayne, L. G., D. J. Brenner, R. R. Colwell, P. A. D. Grimont, O. Kandler, M. I. Krichevsky, L. H. Moore, R. G. E. Murray, E. Stackebrandt, M. P. Starr, and H. G. Truper.** 1987. Report of the ad hoc committee on reconciliation of approaches to bacterial systematics. Int. J. Syst. Bacteriol. **37**:463–464.
20. **Wetmur, J. G., and N. Davidson.** 1968. Kinetics of renaturation of DNA. J. Mol. Biol. **31**:349–370.