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Phenotypical Divergences between Populations of Soil Bacteria Isolated on Different Media

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Abstract. Bacterial strains were randomly isolated from soil using three different media with glucose (TG), Tryptone Soya Broth (TTS), and succinate (TS) as carbon sources. Plate counts obtained were 12.0×10^7, 4.5×10^7, and 1.5×10^7 g^{-1} soil dry weight, respectively. The strains were characterized phenotypically by the API 20B test system. A cluster analysis of all isolates revealed 40 biotypes at 80% similarity, 23 in TG, 29 in TTS, and 27 in TS. Each of the 10 most common biotypes contained 10 to 2.5% of the isolates, and 17 biotypes contained one or two isolates. The common biotypes were unevenly distributed among the isolates from the different media. About 20% of the isolates from TG and TTS were unique for the particular medium, whereas among the isolates from TS, about 60% were unique. Thirty percent of the isolates belonged to biotypes that were common to all three populations. All media gave approximately the same high diversity measured as Shannon index and Equitability, indicating no direct correlation between plate count and diversity.

Introduction

It is well established that plate counts of bacteria in soil give values that are only a fraction of those obtained by microscopic counts. The plate count values depend on the type of medium, even when purportedly nonselective media are used [6]. In soil microbiology, references to a large number of reports on comparative plate counts on different media can be found [8, 9, 12, 17, 22]. Several authors have studied the microbial diversity in soil. Among the various measures of diversity that have been employed are listing of identified genera [3, 23], diversity indices [3, 11, 21], and multivariate statistical methods [13, 20]. We have found no studies on the effects of culture media on such data. It is conceivable that two media giving roughly the same plate count select for different bacterial types, and thus give different diversity indices.

Using various diversity measures based on phenotypic characters, we have explored the divergence between plate count populations isolated on media giving high and intermediate counts. The intention was to clarify whether there is a relationship between plate count values and microbial diversity, and between medium composition and dominating biotypes.

Materials and Methods

Soil Type

The soil was from the natural beech forest at Seim, about 20 km north of Bergen, Norway. Samples from the humic layer (0–10 cm) were sieved (mesh size 4 mm), mixed, and stored at 4°C in polythene bags for 1 day prior to analysis. Soil organic matter was 31% of dry weight, water content was 161% of dry weight, and pH in distilled water was 4.8. The direct bacterial count using acridine orange was 1.7×10^{10} g^{-1} soil dry weight, and the total hyphal length [4], 1600 m g^{-1} soil dry weight.

Sampling and Media

Ten grams of soil (wet weight) was homogenized (3 × 1 min) with 90 ml sterile-filtered, cold Winogradsky's salt solution diluted 1:20 [15] in a sterile Waring blender at low speed. After settling for 5 min, tenfold dilutions from 10^2 to 10^5 were made. Within 2 hours, 0.1 ml of each dilution was spread on plates (×5) containing different media. The plates were wrapped in plastic bags and incubated in the dark at 22°C. Colonies were enumerated every second day until maximal plate counts were obtained.

For preliminary testing, and with the aim of selecting media giving different plate counts, the media formulations given in Table 1 were used. The media were based on either the mineral salts of Thornton's medium (media 1–6) or Winogradsky's salt solution (media 7–11). Different C and N sources were used in both series, but the concentrations in 7–11 were only 1–3% of those in the 1–6 series. The use of activated charcoal (Merck art. 2186) in medium 2 is based on the suggestion [16] that toxic components may be released in media during autoclaving. The activated charcoal is supposed to adsorb these components [5]. Effect of catalase (Sigma C10) on the plate count was tested on three media (1, 7, and 8). It has been reported that addition of catalase to the surface of medium plates permits increased enumeration of injured bacteria by preventing accumulation of hydrogen peroxide [10]. In this study, injury of cells may be caused by the homogenization and dilution of the soil samples.

Selection of Isolates

About 100 colonies from one plate or a particular area of one plate were streaked out on new plates of the same medium. After incubation for 14 days at 22°C, colonies from the isolates were streaked on new plates. After three such transfers, the isolates were microscopically examined for purity. A few cultures still seemed impure and these were discarded. The isolates were stored at 5°C and subcultured every third month.

Cultivation for Phenotypic Testing

Liquid cultures (40 ml in 100 ml Erlenmeyer flasks) were inoculated from new plates and incubated in a shaker at 22°C. Satisfactory growth in liquid media of some strains was only obtained when the original media were fortified with 0.5% yeast extract, and the glassware carefully washed (boiling sodium carbonate followed by hot HCl and distilled water). The cultures were harvested when the turbidity indicated that they were at the end of the exponential growth phase.

Test System

The main test system was the API 20B system (API #2040, API System S.A., France), which includes the following tests: proteolysis of gelatin (GEL); action of nitrate reductase (NIT); action

Table 1. Composition of the different media

Medium	Symbol	Composition
1		Thornton's medium with 10% soil extract
2		As medium 1, with activated charcoal
3		Thornton's salt solution with 10% soil extract and 0.25 g liter^{-1} NB
4	TTS	As medium 3, but with 0.3 g liter^{-1} TSB instead of NB
5	TG	As medium 3, but with 1 g liter^{-1} glucose instead of NB
6	TS	As medium 3, but with 1 g liter^{-1} Na-succinate instead of NB
7		Winogradsky's salt solution with 0.01 g liter^{-1} NH_4NO_3 and 0.01 g liter^{-1} NB
8		As medium 7, but with 0.01 g liter^{-1} mannitol and 0.01 g liter^{-1} asparagine instead of NB
9		As medium 7, but with 0.01 g liter^{-1} Na-succinate instead of NB
10		As medium 7, but with 0.01 g liter^{-1} peptone and 0.01 g liter^{-1} yeast extract instead of NB
11		Winogradsky's salt solution with 0.01 g liter^{-1} NH_4NO_3
12		40% soil extract

The following media formulations were used: soil extract: 1 kg garden soil mixed with 1.5 liter distilled water, autoclaved 40–50 min at 121°C, filtered, the volume adjusted to 1 liter with distilled water and reautoclaved. Amended salt solution from Thornton's medium: K_2HPO_4, 1 g; NH_4NO_3, 5 g; $MgSO_4 \cdot 7H_2O$, 2 g; $CaCl_2 \cdot 2H_2O$, 1 g; NaCl, 1 g; $Fe(Cl)_3 \cdot 6H_2O$, 0.1 g in 1,000 ml distilled water; pH adjusted to 6.7 before autoclaving. Diluted 1:10 in the final medium. Winogradsky's salt solution: K_2HPO_4, 5.0 g; $MgSO_4 \cdot 7H_2O$, 2.5 g; NaCl, 2.5 g; $Fe(Cl)_3 \cdot 6H_2O$, 0.04 g; $MnSO_4 \cdot 7H_2O$, 0.05 g in 1,000 ml distilled water; pH is adjusted to 6.7. Diluted 1:20 with distilled water and filter-sterilized before use. The media were made up in either of the two salt solutions and with different C (and N) sources. All solid media had 1.5% agar added. TSB = tryptone soya broth (Oxoid code CM129, NB = nutrient broth (Oxoid code CM1)

of β-galactosidase (ONPG); production of acid metabolites from carbohydrates, i.e., 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) and tryptophanase (IND); H_2S formation (H_2S); acetoin production (VP); utilization of citrate as sole carbon source (CIT); and presence of cytochrome oxidase (OX) and catalase (CAT).

The tests were executed according to the manufacturer, but with the following modifications: Instead of using bacterial colonies suspended in the API medium (some colonies were very difficult to disperse) as inoculum, liquid cultures were aseptically centrifuged at 6,000 *g* for 15 min in a Sorvall SS-3 centrifuge, using a SS-34 rotor. A sufficient amount of the pellet was transferred aseptically to an ampule of API medium to give the recommended turbidity. The test plates were incubated at 22°C for 3 days before reading. CAT and OX tests were carried out directly on colonies from Petri dishes 2–3 days after the appearance of visible colonies. Oxidative or fermentative metabolism (OFF) was tested according to the description of API OF (API #5011).

Gram reactions (GRAM) were recorded on 2- to 3-day-old colonies, using the KOH method [2]. Morphology (COCC), motility (MOT), and presence of endospores (SPOR) were recorded using a phase contrast microscope; COCC and MOT on liquid cultures; endospore formation on colonies at least 2 weeks old. Ammonification (AMM) was tested in the following medium: 0.1 g $MgSO_4$, 0.2 g K_2HPO_4, 0.1 g NaCl, and 2.0 g casamino acids were dissolved in 1,000 ml of distilled water; pH was adjusted to 6.2. A few grains of phenol red were added until a clear yellow color appeared. Portions of 5 ml were distributed into tubes that were capped and autoclaved. The tubes were

Table 2. Plate counts of microorganisms from soil on the different media

Medium	Symbol	Plate count × 10^{-7} g^{-1} dry soil ± standard deviation
1		2.6 ± 0.1
1a		3.1 ± 0.4
2		2.2 ± 0.3
3		4.0 ± 0.1
4	TTS	4.5 ± 0.8
5	TG	12.0 ± 1.0
6	TS	1.5 ± 0.3
7		3.6 ± 0.4
7a		4.0 ± 0.3
8		2.2 ± 0.2
8a		2.2 ± 0.5
9		5.0 ± 0.9
10		3.9 ± 0.3
11		10.0 ± 1.0
12		1.7 ± 0.1

Dilutions were made in Winogradsky's salt solution. In the experiments with catalase (1a, 7a, and 8a), 0.1 ml of a sterile filtered solution of 0.15 g catalase in 5 ml Winogradsky's salt solution was added to the dilution before plating. Colonies on three plates were counted and standard deviation calculated

inoculated with one drop from a liquid culture which was at the end of exponential growth phase. The result was recorded after a three-day incubation period at 22°C.

Cluster Analysis

All tests were scored as presence/absence and the results stored in the computer as 1 or 0. All isolates scored negative on IND and H_2S, and these tests were omitted from further analyses.

Test results from all the isolated bacteria were submitted to cluster analysis. This was performed on an Olivetti M24 personal computer with software developed by S. Norland of this department. Proximity was measured using Simple Matching Coefficient (SMC) [19]. SMC takes into account all possible states of the characters (i.e., +/+, +/−, and −/−). The clusters were agglomerated by means of the Complete Link (furthest neighbor) method [7]. The combination of SMC and complete link introduces the possibility of relating the distances between clusters to the number of characters separating them.

Clusters showing similarity above 80% were defined as belonging to the same biotype. This corresponds to five characters separating the biotypes.

Calculation of Diversity Indexes

The Shannon index [18], $H' = -\Sigma(p_i \cdot \log_2 p_i)$ was calculated with biotype replacing the species concept. Thus, p_i is the number of isolates in a biotype divided by the total number of isolates in the sample being analyzed. As previously pointed out [11], the Shannon index is an unsuitable measure of diversity when comparing populations of unequal size. We therefore computed the

Equitability (J) [14] for the three populations and the combination of them. It is defined as $J = H'/H'_{max}$ where H'_{max} is the value of H' when all isolates are evenly distributed among the biotypes.

Results

The amended Thornton's salt solution with soil extract supplemented with glucose (medium TG) and Winogradsky's salt solution agar (medium 11) gave significantly higher counts than the other media (Table 2). Supplementing with succinate (TS), and soil extract alone (medium 12) gave values in the low range. For comparison of population divergences, the three media with Thornton's salt solution and soil extract (media 1-3) and those with glucose (TG), Tryptone Soya Broth (TTS), or succinate (TS) as carbon sources were chosen. Among the media tested, they gave high, intermediate, and low plate counts.

After isolation and purification, 114 strains were obtained from TG, 97 from TTS, and 95 from TS. Fifteen of the strains from TG had colonial and morphological appearances indicating that they belonged to the streptomycete group. These are jointly considered as a "biotype" and are referred to in the Discussion, but inclusion of the streptomycetes in the numerical analysis would require additional tests that would then have to be carried out on all isolates. This was not considered feasible. After transfer to liquid medium, some strains were difficult to grow, and the final number of strains entering the phenotypic test program were 70 from TG, 79 from TTS, and 79 from TS. The majority of the colonies on TS were larger than colonies on the two other media. Many of the colonies on TG were hard and/or small. In general, isolates from TG and TTS grew more slowly than those isolated from TS.

Figure 1 illustrates the frequencies of positive scores in the three populations. The tests are ordered by decreasing frequencies of positive scores in the population from TS. In all populations, Gram-negative, aerobic, or facultatively anaerobic rods dominated. This, together with the other test results, indicate that the majority of the colony-forming bacteria in the soil were of the *Pseudomonas/Alcaligenes* type [1]. The mol% G+C of most isolates were in the range of 58–63% (R. Sørheim, unpublished). The frequency of endospore-forming bacteria was very low. In general, the isolates from TG had less ability to score positive than those from TTS, which gave fewer positive scores than the TS isolates.

With biotype separation at 80% similarity, cluster analysis of the isolates from all three populations in combination revealed 40 biotypes (Fig. 2). The number of different biotypes in the populations TG, TTS, and TS were 23, 29, and 27, respectively. No biotype in population TG contained more than 21% of the isolates, whereas in the populations TTS and TS the corresponding percentages were 13 and 20 (Table 3). Ten biotypes contained eight or more isolates, and 17 consisted of only one or two isolates. Thirteen biotypes were present in all populations. Three biotypes were found only in TG, six only in TTS, and five only in TS. None of these was among the dominant biotypes; only one contained three isolates, the rest one or two. An impression of the divergence between the three populations can be obtained by the distribution of the 10 most common biotypes. Table 4 shows how the isolates belonging

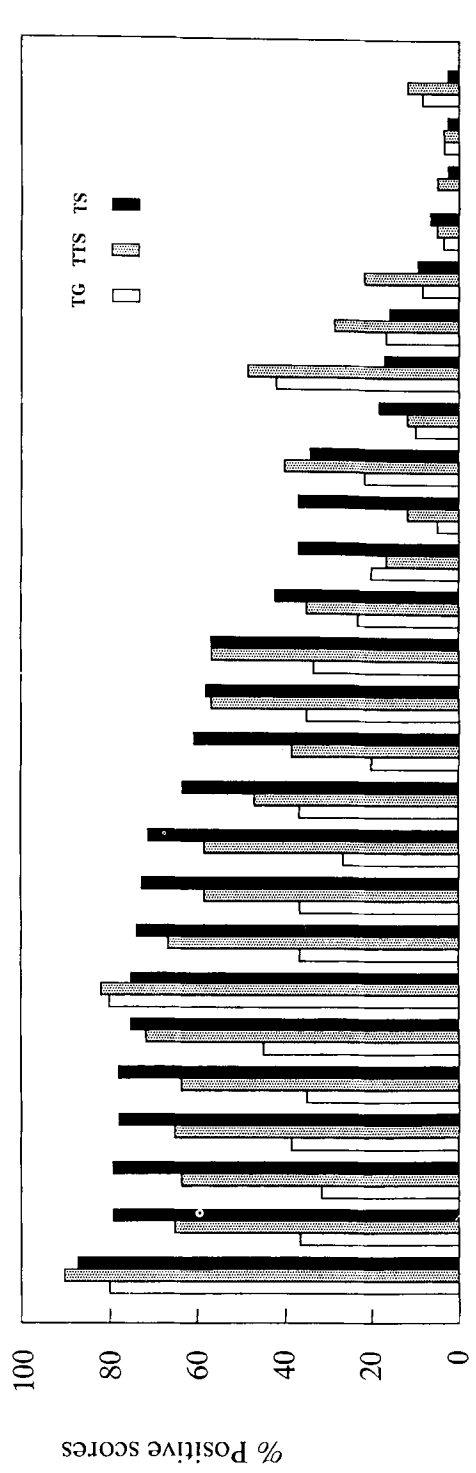

Fig. 1. Percent positive test scores of the isolates in the three populations. The tests are ordered in decreasing percentages for the isolates from TS. H_2S and IND are not included as all isolates were determined negative for these tests. TG: isolates from a medium with glucose; TTS: isolates from a medium with tryptone soya broth; TS: isolates from a medium with succinate.

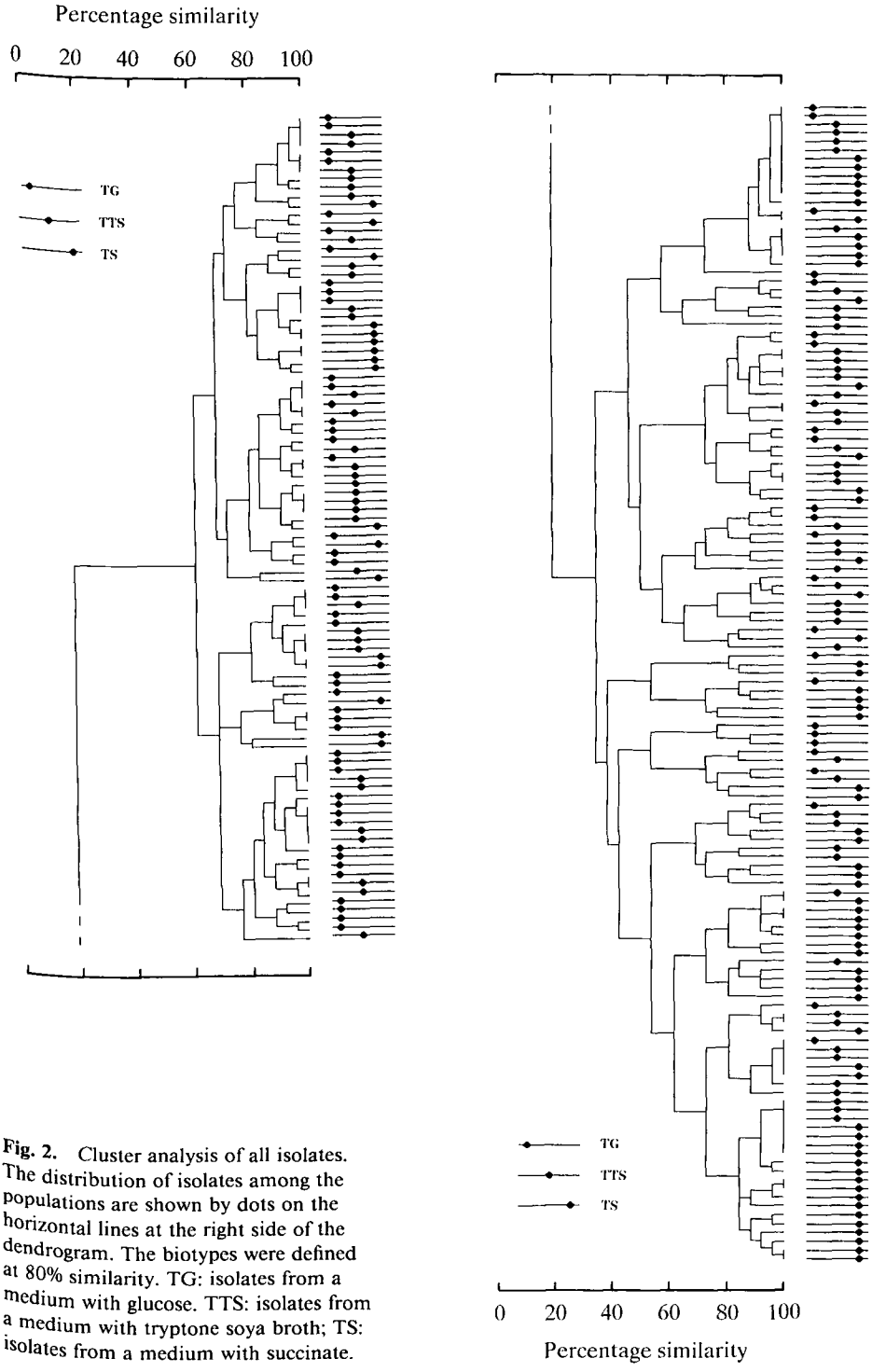

Fig. 2. Cluster analysis of all isolates. The distribution of isolates among the populations are shown by dots on the horizontal lines at the right side of the dendrogram. The biotypes were defined at 80% similarity. TG: isolates from a medium with glucose. TTS: isolates from a medium with tryptone soya broth; TS: isolates from a medium with succinate.

Table 3. Number of isolates belonging to the different biotypes (80% similarity)

Biotype number	Medium (total number of strains)			
	TG (70)	TTS (79)	TS (79)	Sum (228)
4	10	10	2	22
9	15	6	0	21
11	3	5	11	19
40	0	3	16	19
3	4	4	7	15
6	6	4	2	12
1	4	6	1	11
16	3	7	1	11
39	2	6	3	11
37	0	1	7	8
7	4	0	1	5
18	0	3	2	5
19	3	2	0	5
34	1	2	2	5
38	0	1	4	5
2	2	1	1	4
17	2	1	1	4
22	1	2	1	4
13	1	1	1	3
24	1	1	1	3
25	1	0	2	3
26	1	0	2	3
36	0	0	3	3
5	0	1	1	2
8	0	0	2	2
14	0	2	0	2
20	0	1	1	2
23	0	2	0	2
30	2	0	0	2
31	1	1	0	2
32	1	1	0	2
33	0	0	2	2
35	0	2	0	2
10	0	1	0	1
12	1	0	0	1
15	0	1	0	1
21	0	1	0	1
27	0	0	1	1
28	0	0	1	1
29	1	0	0	1

to these biotypes scored on the test system. The populations from TG and TSS were fairly similar, with about 23% of the isolates unique for TG and 17% unique for TTS. TS was more divergent, with 56% of the isolates unique for that population (Table 5).

The Equitability of all populations and their combination was about 90%

Table 4. The number of positive test scores for the 10 most dominant biotypes

Test	Biotype									
	1	3	4	6	9	11	16	37	39	40
GEL	0	0	0	1	0	0	0	5	0	19
NIT	0	4	1	0	1	0	0	2	0	0
ONPG	6	0	2	12	3	0	6	2	1	0
SAC	1	0	0	0	0	18	11	8	0	0
ARA	1	0	0	3	0	18	11	8	11	19
MAN	1	0	0	0	0	18	10	8	11	19
FRU	1	0	0	0	0	18	11	8	11	19
GLU	1	0	0	0	0	18	11	8	11	19
MAL	1	0	0	0	0	18	11	8	11	19
AMD	0	0	0	0	0	0	5	0	0	0
RHA	1	0	0	0	2	18	11	6	11	19
GAL	1	0	0	0	0	18	11	8	11	19
MNE	1	0	0	0	0	18	11	7	7	11
SOR	0	0	0	0	0	0	11	1	0	0
GLY	1	0	0	0	0	18	11	0	2	0
URE	3	2	13	0	1	16	1	0	0	7
VP	0	1	4	11	0	0	11	8	11	19
CIT	11	6	1	0	4	18	0	8	11	16
OX	8	5	16	12	17	18	3	6	11	15
CAT	11	0	20	10	15	17	8	8	11	19
OFF	0	0	0	1	0	0	0	0	0	0
MOT	11	12	22	6	1	5	11	8	10	19
GRAM	0	2	8	0	1	0	9	0	1	0
AMM	1	0	0	1	2	18	0	8	10	16
SPOR	0	0	0	0	0	0	0	0	0	0
COCC	0	0	0	0	1	1	1	0	0	0
Number of isolates	11	15	22	12	21	19	11	8	11	19

Abbreviations under Test System in text

(Table 6). This shows that the isolates were evenly distributed among the biotypes.

Discussion

The distribution of isolates among the biotypes indicates that by increasing the number of isolates, the probability that a new isolate belongs to a new biotype is fairly high with all media. The presence of rare biotypes accordingly does not give information about the divergence of the three populations.

On the other hand, an impression of the divergence can be obtained by the distribution of the 10 most common biotypes (having eight or more isolates in total). The populations from TG and TTS were fairly similar, with an overlap of about 80%, and about 20% unique isolates in each population (Table 5). However, it should be noted that the TG population in addition contained 15 streptomycete isolates. There was also a greater loss of isolates during the

Table 5. Divergence among the populations, based on the isolates in the 10 most common biotypes

Population (medium)	TG	TTS	TS
Number of isolates			
In each population	47	52	50
Similar in all populations	15	15	15
Similar in TG and TTS	21	21	
Similar in TTS and TS		7	7
Similar in TG and TS	0		0
Unique in each population	11	9	28

Table 6. Shannon index (H') and Equitability (J), based on 80% similarity

Population	H'	J
TG	3.94	0.87
TTS	4.42	0.91
TS	4.09	0.86
TG + TTS + TS	4.69	0.88

purification of strains from TG. Thus, the difference between TG and TTS is greater than indicated by the numerical biotype distribution. TS was more divergent than the other populations, with 56% unique isolates.

All isolates can be grouped into two main clusters (similarity about 20%). This grouping corresponded to the break in the most dominating biotypes between numbers 9 and 11 (Table 4). The biotypes to the right of this break have the ability to produce acid from many carbohydrates, whereas those to the left lack this ability. Most of the isolates from TS belong to the former group, and somewhat surprisingly, the majority of isolates from TG belong to the latter group. The most common biotype was 4 with 22 isolates. It was common in TG and TTS, but rare in TS. The isolates belonging to this biotype gave mainly negative tests for acid production from carbohydrates and most of the other tests. A striking feature of this biotype was, however, that more than half of the isolates were urease positive. Biotype 9 (21 isolates) was present only in TG and TTS, with most of the isolates belonging to TG. The only tests for which the members of this biotype scored positive were CAT and OX. Biotype 16 (11 isolates) with most of its representatives isolated on TTS can be characterized as motile, Gram-positive, nonsporulating bacteria able to produce acid from many carbohydrates. Biotype 40 was also unevenly distributed between the populations, with no representative in TG, and 16 of the 19 isolates in TS. This biotype was characterized as Gram-negative, motile rods with an oxidative carbohydrate metabolism, capable of producing acid from many carbohydrates. All isolates in this biotype were able to liquify gelatin. In fact, half of the gelatinase-positive isolates were classified in this biotype. The other half were spread through nine other biotypes.

The diversity, measured as Shannon index and Equitability, was high for all

populations. There seems to be no correlation between plate count and diversity, as should be expected if lower plate count in a medium is a result of selection pressure.

This study confirms that the bacterial populations obtained through plating are dependent on the isolation media used. With three nonselective media, only 30% of the biotypes were found on all media at 80% similarity. This can be understood if the soil contains a large number of strains but that each medium selects for a small proportion of those strains.

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