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土壤细菌的培养基、接种量和时间对于可培养效应的影响

Effects of Growth Medium, Inoculum Size, and Incubation Time on Culturability and Isolation of Soil Bacteria

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Soils are inhabited by many bacteria from phylogenetic groups that are poorly studied because representatives are rarely isolated in cultivation studies. Part of the reason for the failure to cultivate these bacteria is the low frequency with which bacterial cells in soil form visible colonies when inoculated onto standard microbiological media, resulting in low viable counts. We investigated the effects of three factors on viable counts, assessed as numbers of CFU on solid media, and on the phylogenetic groups to which the isolated colony-forming bacteria belong. These factors were inoculum size, growth medium, and incubation time. Decreasing the inoculum size resulted in significant increases in the viable count but did not appear to affect colony formation by members of rarely isolated groups. Some media that are traditionally used for soil microbiological studies returned low viable counts and did not result in the isolation of members of rarely isolated groups. Newly developed media, in contrast, resulted in high viable counts and in the isolation of many members of rarely isolated groups, regardless of the inoculum size. Increased incubation times of up to 3 months allowed the development of visible colonies of members of rarely isolated groups in conjunction with the use of appropriate media. Once isolated, pure cultures of members of rarely isolated groups took longer to form visible colonies than did members of commonly isolated groups. Using these new media and extended incubation times, we were able to isolate many members of the phyla *Acidobacteria* (subdivisions 1, 2, 3, and 4), *Gemmatimonadetes*, *Chloroflexi*, and *Planctomycetes* (including representatives of the previously uncultured WPS-1 lineage) as well as members of the subclasses *Rubrobacteridae* and *Acidimicrobidae* of the phylum *Actinobacteria*.

Soils contain phylogenetic groups of bacteria that are globally distributed and abundant in terms of the contributions of individuals of those groups to total soil bacterial communities (3, 10, 23). However, until recently, no representatives of many of these groups were available for detailed study due to their apparent inability to grow in or on laboratory media. Part of the reason for this is that only a few (often only about 1%) of the $>10^9$ bacterial cells in each gram of soil seem able to form colonies on laboratory media (5, 14, 28). This means that many groups of soil bacteria cannot be easily studied due to the inability of microbiologists to grow representatives in the laboratory. Some isolates of these groups have recently been cultured by the use of new culture media and extended incubation periods to increase the numbers of colonies formed and by the selection of isolates from plates receiving only small inocula and yielding only small numbers of colonies (12, 15, 24). These approaches were chosen empirically in previous studies. Among the isolates obtained were many members of the phylum *Acidobacteria* as well as some members of the phyla *Verrucomicrobia* and *Gemmatimonadetes* and of the subclasses *Acidimicrobidae* and *Rubrobacteridae* of the phylum *Actinobacteria* (12, 15, 24). These groups are very poorly studied due to the paucity of cultured representatives from soils.

The aim of the present study was to investigate the effects of growth medium, inoculum size, colony density, and incubation time on the appearance of colonies of members of these poorly studied groups of soil bacteria on plates of solid growth media inoculated with soil.

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MATERIALS AND METHODS

Bacterial strains. *Pseudomonas aeruginosa* 185 (ATCC 10145), *Pseudomonas fluorescens* 192 (ATCC 13525), *Bacillus megaterium* 4R6259 (ATCC 9885), *Bacillus subtilis* (ATCC 11774), *Sphingomonas paucimobilis* CL1/70 (ATCC 29837), *Xanthomonas campestris* GB296, *Chromobacterium violaceum* MK (ATCC 12472), and *Kocuria rhizophila* PCI 1001 (ATCC 9341) were obtained from the collection of the Department of Microbiology and Immunology, University of Melbourne. These cultures were maintained on nutrient agar (see below) and used to test the quality of culture media. Their identities were confirmed by comparisons of parts of their 16S rRNA genes, after amplification and sequencing (see below), to sequences in GenBank (www.ncbi.nlm.nih.gov) by the use of BLAST web-based software (1).

Fifty isolates from our laboratory collection were used in experiments to determine the ability of members of different groups to grow on different culture media. These were isolated from soil from the same sample site during several earlier investigations (12, 15, 24; C. A. Osborne and P. H. Janssen, unpublished data; P. Sangwan and P. H. Janssen, unpublished data) or were isolated during this study. These isolates were affiliated with nine different bacterial phyla and were selected so that, at most, two came from any single family-level group (Table 1).

Soil sampling. Soil cores (100-mm long, 25-mm diameter) were collected by use of a soil corer from control paddock L2 at the Dairy Research Institute, Ellinbank, Victoria, Australia (38°14.55′S, 145°56.11′E). The soil characteristics and management regimen of the paddock have been described elsewhere (25, 27). Intact soil cores were transported to the laboratory in aluminum trays enclosed in polyethylene bags at the ambient temperature and were processed within 3 h of collection. The collection dates were 2 March 2001, 5 April 2001, and 29 May 2003. Unless noted otherwise, experiments were performed with soil from the last sampling time. The upper 30 mm of each core was discarded, and large roots and stones were removed from the remainder, which was then sieved through an autoclave-sterilized brass sieve with a 2-mm aperture size (Endecotts

TABLE 1. Isolates used for experiments to determine the ability of members of different groups to grow on different culture media

Phylum	Class, subclass, or subdivision	Family	Isolates (GenBank accession no.)
Acidobacteria[a]	Subdivision 1	Ellin345/WD217 group	Ellin345 (AF498727)
	Subdivision 1	Ellin323/DA038 group	Ellin7137 (AY673303)
	Subdivision 1	Ellin337/P18 group	Ellin337 (AF498719), Ellin5236 (AY234587)
	Subdivision 1	Ellin351/TM2 group	Ellin408 (AF432237)
	Subdivision 1	UA1/UA2 group	Ellin5413 (AY673140), Ellin7225 (AY673391)
	Subdivision 3	Ellin342/MC9 group	Ellin6076 (AY234728)
Actinobacteria	*Acidimicrobidae*[a]	*"Microthrixaceae"*	Ellin5247 (AY234598), Ellin5273 (AY234624)
	Actinobacteridae	Ellin306/WR160 group	Ellin5004 (AY234421), Ellin5284 (AY234635)
	Actinobacteridae	Ellin5034 group	Ellin5423 (AY673150)
	Actinobacteridae	*Geodermatophilaceae*	Ellin5211 (AY234562)
	Actinobacteridae	*Intrasporangiaceae*	Ellin5432 (AY673159)
	Actinobacteridae	*Microbacteriaceae*	Ellin5407 (AY673134)
	Actinobacteridae	*Micromonosporaceae*	Ellin5430 (AY673157), Ellin6203 (AY673162)
	Actinobacteridae	*Mycobacteriaceae*	Ellin5292 (AY234643), Ellin5409 (AY673136)
	Actinobacteridae	*Nocardiaceae*	Ellin5404 (AY673131)
	Actinobacteridae	*Nocardioidaceae*	Ellin5426 (AY673153), Ellin6206 (AY673165)
	Actinobacteridae	*Nocardiopsaceae*	Ellin5291 (AY234642)
	Actinobacteridae	*Pseudonocardiaceae*	Ellin5419 (AY673146)
	Rubrobacteridae[a]	*"Thermoleophilaceae"*	Ellin7179 (AY673345), Ellin7228 (AY673394)
Bacteroidetes	*Sphingobacteria*	*Flexibacteraceae*	Ellin5402 (AY673129), Ellin5420 (AY673147)
	Sphingobacteria	*Sphingobacteriaceae*	Ellin6201 (AY673160), Ellin6205 (AY673164)
"Deinococcus-Thermus"[a]	*Deinococci*	*Deinococcaceae*	Ellin6033 (AY234685)
Firmicutes	*"Bacilli"*	*Bacillaceae*	Ellin5411 (AY673138)
Gemmatimonadetes[a]	Subdivision 1	*Gemmatimonadaceae*	Ellin5290 (AY234641)
Planctomycetes[a]	*"Isosphaerae"*	*"Isosphaeraceae"*	Ellin6059 (AY234711)
	WPS-1	Ellin7224/WD2101 group	Ellin6207 (AY673166), Ellin7224 (AY673390)
Proteobacteria	*Alphaproteobacteria*	Ellin314/wr0007 group	Ellin5002 (AY234419), Ellin5238 (AY237589)
	Alphaproteobacteria	*Beijerinckiaceae*	Ellin5427 (AY673154)
	Alphaproteobacteria	*Bradyrhizobiaceae*	Ellin5405 (AY673132), Ellin5431 (AY673158)
	Alphaproteobacteria	*Phyllobacteriaceae*	Ellin5412 (AY673139)
	Betaproteobacteria	*Burkholderiaceae*	Ellin5403 (AY673130), Ellin5406 (AY673133)
	Betaproteobacteria	*Comamonadaceae*	Ellin5418 (AY673145), Ellin5428 (AY673155)
	Gammaproteobacteria	*Xanthomonadaceae*	Ellin5401 (AY673128), Ellin5421 (AY673148)
Verrucomicrobia[a]	*"Spartobacteria"*	*"Chthoniobacteraceae"*	Ellin428 (AY388649)

[a] Rarely isolated group.

Ltd., London, United Kingdom), mixed, and used immediately for microscopic investigations, dry weight determinations, or cultivation experiments. The numbers of cells in mixed soil samples were determined by microscopic counts of preparations stained with 4′,6′-diamidino-2-phenylindole plus acridine orange (S. N. Cairnduff and P. H. Janssen, unpublished data). Aliquots of freshly sieved soil were accurately weighed and then dried at 105°C for 3 days. The samples were then reweighed after first being allowed to cool to room temperature in a desiccator. The conversion factor of fresh to dry weight for soil was calculated, and all results are expressed per gram of dry soil.

Media. VL55 medium solidified with gellan and containing different growth substrates was prepared as described by Joseph et al. (15). These growth substrates and their final concentrations in the solidified medium were as follows: 2 mM *N*-acetyl-glucosamine; a mix of D-glucose, D-galactose, D-xylose, and L-arabinose (0.5 mM [each]) (15); a mix of D-galacturonate, D-glucuronate, L-ascorbate, and D-gluconate (0.5 mM [each]) (15); a mix of acetate, benzoate, L-lactate, and methanol (0.5 mM [each]) (15); an amino acid mix (9) with an addition of 0.08 g of L-tryptophan per 100 ml of stock solution, added at 10 ml of stock solution per liter of medium; 0.05% (wt/vol) sodium alginate; 0.05% (wt/vol) xanthan; 0.05% (wt/vol) pectin; 0.05% (wt/vol) xylan (VXylG); and 0.05% (wt/vol) carboxymethylcellulose. VL55 medium with agar as the solidifying agent and with either 0.05% (wt/vol) xylan (medium VXylA) or 10 mM glucose (VGluA) as the growth substrate was prepared as described by Sait et al. (24). Glucose was added from a 1 M stock solution that was sterilized by filtration (0.22-μm-pore-size filter). Dilute nutrient broth, solidified with agar (DNBA) or with gellan (DNBG), was prepared as described by Janssen et al. (12). Cold-extracted soil extract agar (CSEA), Winogradsky's salt-solution agar (WSA), and 10-fold-diluted tryptone soy agar (0.1× TSA) were prepared as described by Joseph et al. (15). Nutrient agar was prepared with 8 g of Difco nutrient broth (BD Diagnostic Systems, Sparks, Md.) and 15 g of bacteriological agar no. 1 (Oxoid) per liter of distilled water and had a final pH of approximately 6.0.

All media were used in 90-mm-diameter polystyrene petri dishes.

Cultivation experiments. An accurately weighed sample of freshly sieved soil (approximately 1 g) was dispersed in 100 ml of sterile distilled water before 1-ml aliquots were treated by sonication as described elsewhere (12). These 10^{-2}-diluted aliquots were serially diluted to 10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, and 10^{-7} (12), and 200 μl each of the last three dilutions was used to inoculate each of 3 or 5 replicate plates at each dilution level to constitute a counting set of 9 or 15 plates. Inocula were spread over the surface of the agar- or gellan-containing medium by the use of sterile glass spreading rods. Three or seven counting sets were prepared on each medium for each soil sample. All 1,170 plates were incubated at 25°C in the dark for 16 weeks in sealed polyethylene bags (40-μm film thickness).

Viable counts are expressed relative to the dry weight of the soil. Expected inoculum sizes (expressed as numbers of cells per plate) were calculated from the microscopically determined total counts of cells in the soil, the dilutions made, and the volumes of diluted inocula spread onto the plates. Culturability was defined as the viable count expressed as a percentage of the microscopically determined total count of cells for the sample used in that particular cultivation experiment.

Colony formation was monitored by examining plates after 3 days, after 7 days, and then at further 7-day intervals by using a magnifying lens with a magnification of ×2. When the rate of visible colony formation was being assessed, the midpoint of the week was deemed to be the time of colony formation (1.5 days if colonies appeared within 3 days). However, almost identical results were obtained if the start or end of these time intervals was used for the calculations.

Student's *t* test (two-tailed), analysis of variance (ANOVA), and the χ^2 test were performed with Excel 2001 software (Microsoft Corp., Redmond, Wash.).

Identification of isolates. Colonies were selected randomly and subcultured on VGluA, 0.1× TSA, WSA, CSEA, or DNBA. Partial 16S rRNA gene sequences (≥400 nucleotides [nt]) were determined as described by Joseph et al. (15).

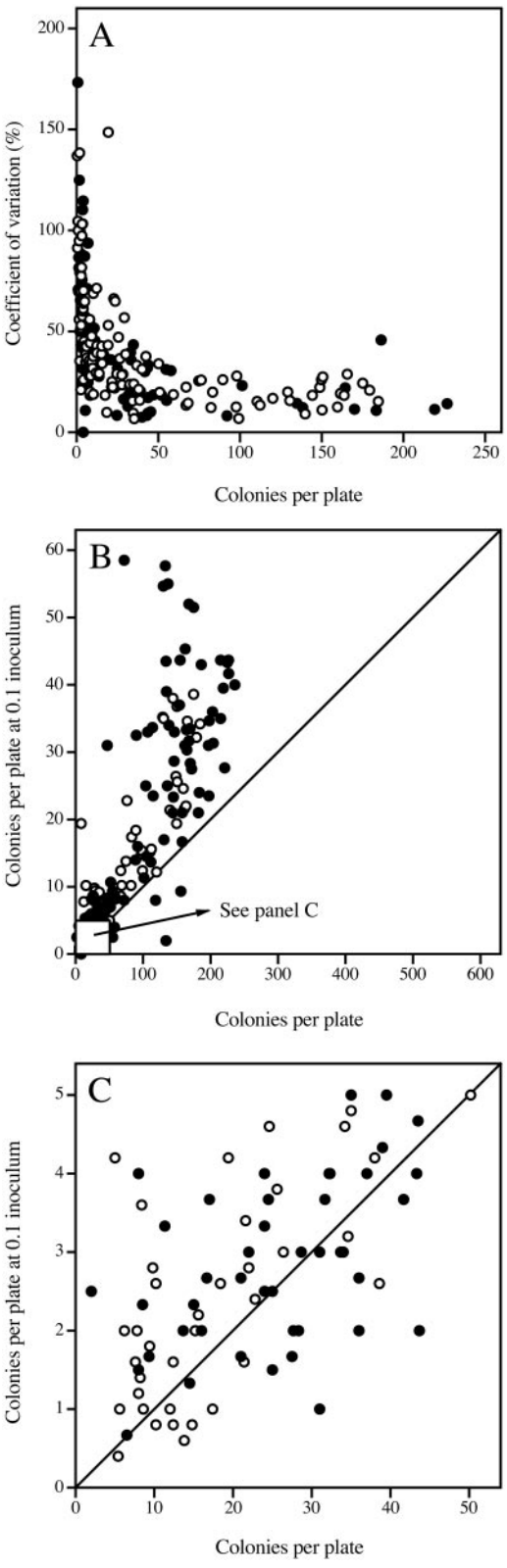

FIG. 1. (A) Variability in colony numbers of replicates within counting sets relative to mean colony number per plate for each counting set. The variability is expressed as the coefficient of variation (SD/mean) for the replicates (three or five) at any one inoculum size within any one counting set. (B) Comparison of colony numbers on plates inoculated with soil suspensions with 10-fold differences in the inoculum size. The numbers of colonies on plates with the larger inoculum are plotted on the *x* axis, and the numbers of colonies on the corresponding plates with a 10-fold smaller inoculum in the same counting set are plotted on the *y* axis. The diagonal line represents the relationship expected between the numbers of colonies forming on the plates, assuming a 10-fold reduction in colony number with a 10-fold smaller inoculum. (C) Enlargement of the data from the blank lower left section of panel B. Symbols for all panels: ●, data from experiments performed in 2001, with each point on all panels representing the result from three replicate plates at each dilution level; ○, data from experiments performed in 2003, with each point representing the result from five replicate plates at each dilution level.

Isolates were identified by obtaining partial 16S rRNA gene sequences (401 to 1,452 nt) and then using BLAST to compare these sequences to those in the GenBank database (24). The identification criteria used were those described by Joseph et al. (15). The nomenclature of phylogenetic and taxonomic groupings generally follows that of Garrity et al. (8), except for the subdivisions of the phyla *Acidobacteria* and *Gemmatimonadetes*, which follow the schemes of Hugenholtz et al. (10) and Zhang et al. (29), respectively, and the class-level groupings (subdivisions) of the phylum *Planctomycetes*, which are based on the scheme of Fuerst et al. (7). The WPS-1 subdivision of the phylum *Planctomycetes* was named by Nogales et al. (20). For simplicity in grouping isolates, we assumed an approximate subphylum rank equivalence of classes and subdivisions and used the subclasses of the phylum *Actinobacteria* as similar subphylum groupings. Family-level groupings follow the scheme used by Joseph et al. (15).

Nucleotide accession numbers. All partial 16S rRNA gene sequences obtained in this study have been deposited in the GenBank database under accession numbers AY673167 to AY673424.

RESULTS

Minimum colony number per plate. We performed counts of CFU of bacteria that were able to form visible colonies within 12 weeks of inoculation. Soil samples were diluted in water, and aliquots from different dilutions were plated onto various media in counting sets with either three (2001 soil samples) or five (2003 soil sample) replicate plates at each inoculum size (dilution level). As expected, the amount of variation in colony numbers between replicate plates in a counting set increased when the mean number of colonies on those three or five plates decreased (Fig. 1A). The coefficient of variation (standard deviation divided by the mean) was relatively constant, with a mean of 19.7% for dilution levels in counting sets in which the mean number of colonies per plate was ≥ 40 ($n = 57$ sets of three or five plates). Dilution levels with mean colony numbers of <40 per plate had larger coefficients of variation (mean = 52%; maximum = 173%; $n = 160$) which were also more variable between different counting sets.

Effect of inoculum size. The expected 10-fold reduction in colony number after 12 weeks of incubation did not occur when 10-fold smaller inocula were used. Instead, there was an approximately 5-fold reduction (5.2-fold for 2001 experiments and 5.7-fold for 2003 experiments) in colony number for each 10-fold reduction in inoculum size. The mean colony number was therefore 2-fold higher than that expected from the numbers of colonies on plates in the same counting set, but with a 10-fold larger inoculum (Fig. 1B). Only at very low colony counts was this effect less apparent (Fig. 1C). As a consequence, viable counts on all media were larger when a more dilute inoculum was used to calculate the viable count, and this result was statistically significant ($P \leq 3 \times 10^{-4}$ for different

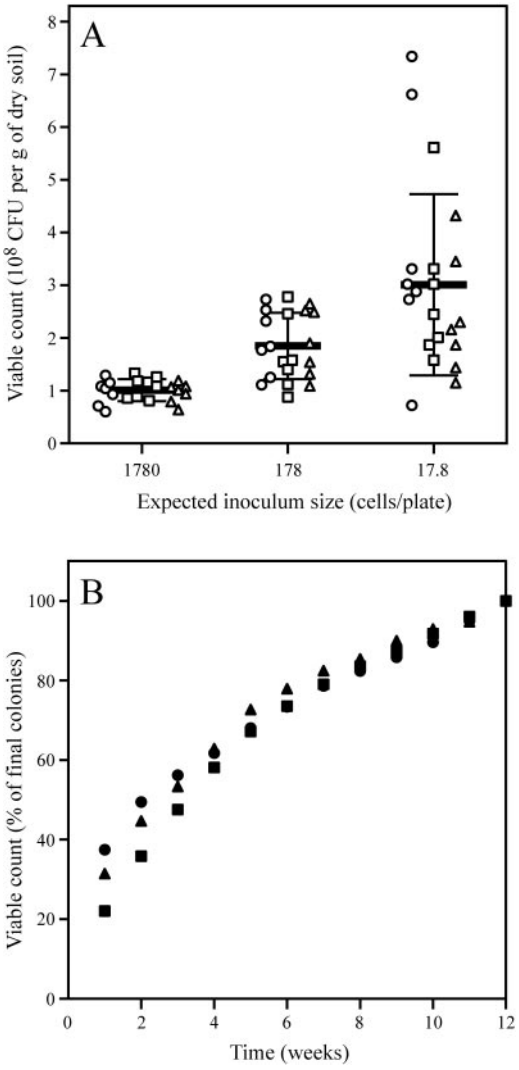

FIG. 2. (A) Viable counts at different inoculum sizes (dilution levels) for three different media after 12 weeks of incubation. Symbols: ○, DNBG; □, VXylA; △, VXylG. Each point represents the mean of five replicate plates. The thick horizontal line indicates the mean, and the vertical lines indicate one standard deviation from the mean. (B) Increase in viable counts with incubation time at three different inoculum sizes. ●, inoculum of 1,780 cells per plate; ■, inoculum of 178 cells per plate; ▲, inoculum of 17.8 cells per plate. Data are pooled results obtained with DNBG, VXylA, and VXylG. The results from each counting set were calculated as a percentage of the 12-week count for that counting set, and each point represents the mean of three media, with each used for seven counting sets, each of which in turn was made of five replicate plates. For clarity, standard errors are not shown; the mean standard error for all points was 12.3% of the values plotted.

inocula in paired t tests and $n = 60$ counting sets of three dilution levels each, with three replicates per dilution level, for data from 2001; $P \leq 4 \times 10^{-4}$ and $n = 42$ counting sets of three dilution levels each, with five replicates per dilution level, for data from 2003).

Figure 2A shows the increased viable counts ($P \leq 5 \times 10^{-6}$ for comparisons of successive dilution levels) with decreasing inoculum sizes for three media that resulted in large counts. Direct epifluorescence microscopic counting of cells (at least 30 fields for each of 15 subsamples of sieved and mixed soil) showed that there were 1.28×10^9 (standard deviation [SD] = 5.03×10^8) cells per g of dry soil in the soil sample used for this experiment. This allowed us to calculate a mean expected inoculum size for each dilution level. The mean culturability, which is the number of CFU expressed as a percentage of the number of cells in the inoculum, increased as the inoculum size decreased (Table 2). The kinetics of colony development, however, were not different for the different inoculum sizes (Fig. 2B).

TABLE 2. Effect of inoculum size on culturability after incubation for 12 weeks and on the number of bacteria affiliated with rarely isolated groups that formed colonies in week 8 or later on plates of DNBG, VXylA, and VxylG

Expected inoculum size (cells/plate)	Mean no. of colonies/plate	Mean culturability (%)	No. of isolates identified	% of isolates affiliated with rarely isolated groups
1,780	140.3	7.9	37	43
178	24.7	13.9	16	25
17.8	4.2	23.6	16	31

Based on these results, we decided to calculate viable counts from plates at the most dilute inoculum that yielded a minimum of 10 colonies per plate, averaged over three or five replicate plates at that dilution level. To overcome the increased variability with these colony numbers, we prepared multiple counting sets for each type of medium.

Selection of growth substrate. The viable counts obtained with VL55 medium, with gellan as the solidifying agent and with each of 10 different additional growth substrates or substrate mixes, ranged from 5.0×10^7 to 6.3×10^8 CFU per g of dry soil in individual counting sets after incubation for 12 weeks at 25°C. The viable counts obtained from two different soil cores (in March and April 2001) were not significantly different ($P = 0.13$ by a paired t test). The choice of growth medium had a detectable effect on the viable counts obtained ($P = 7 \times 10^{-4}$ by single-classification ANOVA). The largest mean count was obtained with xylan as the growth substrate (Fig. 3), and xylan was therefore chosen for further experiments.

Effect of incubation time and medium on viable count. The effects of incubation time and the choice of medium were investigated by determining the numbers of colonies that were visible on plates of six different media at weekly intervals. The viable counts were significantly different between media, even after only 1 week of incubation ($P = 7 \times 10^{-7}$ by single-classification ANOVA), and this continued for the entire 12 weeks (at 12 weeks, $P = 4 \times 10^{-8}$). Counts with 0.1× TSA reached their maximum after 2 weeks of incubation (Fig. 4), and beyond 1 week, the increase in viable counts with time was not statistically significant ($P = 0.75$ by repeated-measures ANOVA). The use of CSEA and WSA resulted in increasing colony counts with increasing incubation times ($P = 3 \times 10^{-3}$ and 7×10^{-4}, respectively, by repeated-measures ANOVA for weeks 1 to 12), and the final counts were higher than those obtained with 0.1× TSA ($P = 2 \times 10^{-3}$ and 0.016, respectively, by Student's t test). The media based on VL55 with xylan as the growth substrate (VXylA and VXylG) and DNBG resulted in

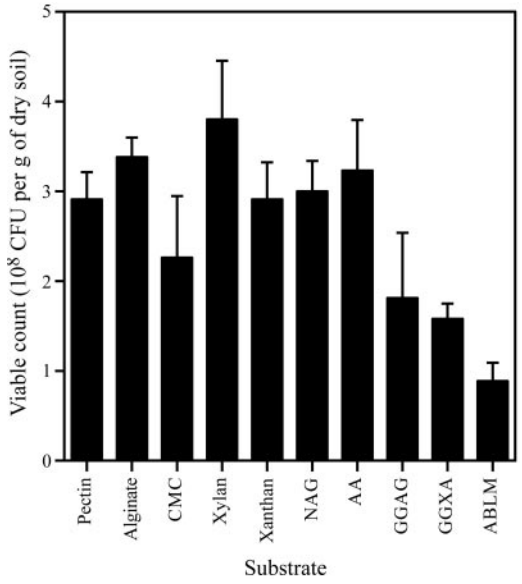

FIG. 3. Mean viable counts after 12 weeks of growth on VL55 medium with different growth substrates. Each solid bar represents the mean of three counting sets prepared from the March 2001 soil sample and three counting sets prepared from the April 2001 soil sample. The error bars indicate standard errors. Abbreviations: CMC, carboxymethylcellulose; NAG, *N*-acetyl-glucosamine; AA, amino acid mix; GGAG, mix of D-galacturonate, D-glucuronate acid, L-ascorbate, and D-gluconate; GGXA, mix of D-glucose, D-galactose, D-xylose, and L-arabinose; ABLM, mix of acetate, benzoate, L-lactate, and methanol.

even higher colony counts (Fig. 4), and these continued to increase over the 12-week incubation period ($P < 6 \times 10^{-5}$ by repeated-measures ANOVA). Even after 12 weeks, the numbers of colonies on the two media based on VL55 medium continued to increase. The analyses that follow were performed with colonies appearing in the first 12 weeks. There was no statistical support for differences between the counts obtained on DNBG, VXylA, and VXylG ($P > 0.48$ by Student's *t* test). However, VXylG (solidified with gellan) resulted in counts that were 15% higher than when the same medium was used with agar as the solidifying agent (VXylA). Culturability after incubation for 12 weeks ranged from a mean of 1.5% of the microscopically determined total cell count on 0.1× TSA to a mean of 15% on DNBG or VXylG. The media DNBG, VXylA, and VXylG all resulted in higher viable counts than did the media 0.1× TSA, CSEA, and WSA ($P = 3 \times 10^{-4}$ to 0.027 by *t*-test comparisons between counts on each medium). The continued increase in colony numbers with extended incubation times over the 12-week incubation period was also observed with all of the media shown in Fig. 3 that were based on medium VL55 with gellan as the solidifying agent but with different growth substrates (data not shown).

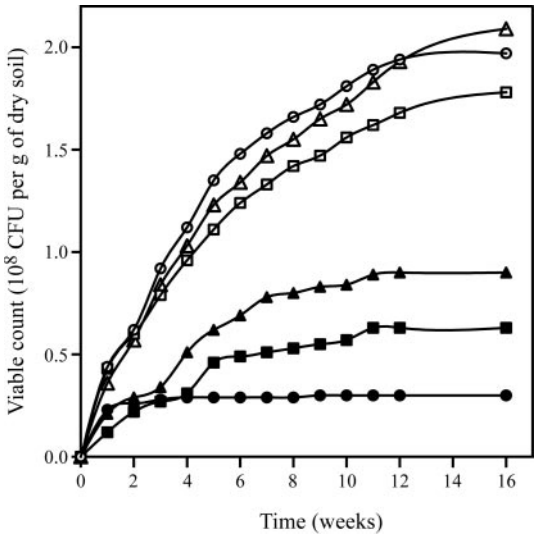

FIG. 4. Increases in viable counts on different media with increasing incubation times. Symbols: ●, 0.1× TSA; ■, WSA; ▲, CSEA; △, VXylG; □, VXylA; ○, DNBG. Each point represents the mean of seven experiments, each of which included five replicate plates. For clarity, the standard errors are not shown; the mean standard error of all the points was 14.5% of the values plotted.

Identities of isolates. A total of 250 colony-forming bacteria that grew on plates inoculated with the soil sample from May 2003 were identified on the basis of their 16S rRNA gene sequences. Of these, 212 appeared during the first week, the fourth and fifth weeks, and the eighth week or later on plates that received an expected inoculum of 17.8 or 178 cells each. Twelve of these were derived from six mixed colonies that were successfully separated into two pure cultures each. The other 38 colonies were randomly selected from those that appeared during week 8 or later on plates receiving an expected inoculum of 1,780 cells each. Of these 250 isolates, 249 were affiliated with eight bacterial phyla (Table 3). The remaining isolate, which was phylogenetically affiliated with the eukaryotic algal family *Trebouxiophyceae*, was identified on the basis of the sequence of a 16S rRNA gene from its plastids. This isolate was not included in subsequent analyses. A list of all of the isolates obtained in this study detailing their phylogenetic affiliations, GenBank accession numbers of their 16S rRNA gene sequences, isolation media, and times of colony appearance is available upon request.

Some colonies were observed to spread rapidly over the entire plate within 1 week of inoculation with diluted aliquots of soil. These occurred mainly on plates containing 0.1× TSA (on 29 of 105 plates) but were also observed on DNBG (on 2 of 105 plates) and VXylG (on 1 of 105 plates). Two of these colonies were part of the collection of 250 isolates, and both were members of the family *Bacillaceae*. A further eight spreading colonies that were not part of the main isolate collection were selected from the plates. Five of these were members of the family *Bacillaceae*, two were members of the family *Flexibacteriaceae*, and one was a member of the family *Paenibacillaceae*. These 10 spreading isolates consistently displayed this phenotype when subcultured on 0.1× TSA (all 10), DNBA (8 of 10), CSEA (5 of 10), or WSA (4 of 9; one could not grow on this medium). However, only two of nine displayed a spreading phenotype when grown on a medium (VGluA) based on VL55 (one isolate could not grow on this medium).

Effect of incubation time and medium on cultured groups. We compared the appearances of isolates from different phylogenetic groups at different time points. To do this comparison, we divided the 212 isolates from the terminal growth-positive plates of counting sets into the following two categories: (i) isolates affiliated with commonly isolated groups

TABLE 3. Phylogenetic affiliations of 250 isolates cultured from soil for this study

Phylum	Class, subclass, or subdivision	No. of isolates (on all six media)	No. of isolates appearing on indicated medium		
			DNBG	VXylA	VXylG
Acidobacteria[a]	Subdivision 1	9	1	5	3
	Subdivision 2	1		1	
	Subdivision 3	5		4	1
	Subdivision 4	1			1
Actinobacteria	*Acidimicrobidae*[a]	2	2		
	Actinobacteridae	119	19	16	22
	Rubrobacteridae[a]	7		6	1
Bacteroidetes	*Flavobacteria*	1			
	Sphingobacteria	5	2		3
Chloroflexi[a]	Ellin7237 lineage	1			1
Firmicutes	"*Bacilli*"	7			
Gemmatimonadetes[a]	Subdivision 1	1	1		
Planctomycetes[a]	"*Gemmatae*"	1			1
	"*Isosphaerae*"	1	1		
	WPS-1	2			2
Proteobacteria	*Alphaproteobacteria*	64	17	15	12
	Betaproteobacteria	19	3	1	3
	Gammaproteobacteria	3		1	1
Viridiplantae	*Chlorophyta*	1			

[a] Rarely isolated group.

that are well represented by cultured representatives, i.e., members of the subclass *Actinobacteridae* of the phylum *Actinobacteria* and members of the phyla *Proteobacteria* (classes *Alphaproteobacteria*, *Betaproteobacteria*, and *Gammaproteobacteria*), *Bacteroidetes*, and *Firmicutes*, and (ii) isolates affiliated with other groups that are only rarely isolated from soil (Table 3). This was a very conservative separation, as many members of the commonly isolated category were affiliated with poorly studied families with few reported isolates. For example, 14 (12%) of the 119 members of the *Actinobacteridae* and 11 (13%) of the 86 members of the *Proteobacteria* were members of as yet undescribed families (three and four families in the *Actinobacteridae* and *Proteobacteria*, respectively).

The number of isolates that belonged to rarely isolated groups increased from 0 to 16% of the isolates with increasing incubation times (Table 4), and a χ^2 test with the pooled data from all six media suggested that incubation time was a significant factor for obtaining isolates from these groups ($P = 7 \times 10^{-3}$ by the χ^2 test). The medium also influenced the numbers of members of rarely isolated groups that were isolated (Table 5) ($P = 2 \times 10^{-3}$ by the χ^2 test). None appeared on the 0.1× TSA, WSA, and CSEA media. When only the results from the three media on which members of rarely isolated groups appeared were analyzed, there was no observable effect of medium ($P = 0.19$ by the χ^2 test), but the incubation time still had a significant effect ($P = 5 \times 10^{-3}$ by the χ^2 test). Members of rarely isolated groups were isolated from all three of these media in the 4- to 5-week and 8- to 12-week periods (Tables 4 and 5). Ten of the 11 isolates of the phylum *Proteobacteria* that represented new families appeared at week 8 or later. This was not the case for the 14 isolates of the subclass *Actinobacteridae* that represented new families, with only one appearing after week 8.

Effect of inoculum size on phylogenetic groups. We compared the identities of late appearing colonies (appearing in week 8 or later) on plates with different inoculum sizes, i.e., different dilution levels (Table 2). Because some media did not yield any members of rarely isolated groups, this analysis was limited to counting sets created with DNBG, VXylA, and VXylG. Inoculum size did not appear to have an effect on the isolation of members of rarely isolated groups ($P = 0.56$ by the χ^2 test). Even on the plates receiving the largest inoculum of soil (an expected 1,780 cells/plate), we isolated members of the *Acidobacteria* (11 isolates in four subdivisions), *Acidimicrobi-*

TABLE 4. Isolates of bacteria that formed visible colonies at different times on terminal plates of counting sets inoculated with soil

Time of colony appearance [wk(s)]	No. of isolates identified		% of isolates affiliated with rarely isolated groups		Groups represented (no. of isolates on all media)
	All media	DNBG VXylA, and VXylG	All media	DNBG, VXylA, and VXylG	
1	71	36	0	0	*Actinobacteridae* (39), *Bacteroidetes* (1), *Firmicutes* (3), *Proteobacteria* (28)
4 and 5	77	41	6	12	*Acidobacteria* (3), *Actinobacteridae* (47), *Firmicutes* (1), *Planctomycetes* (2), *Proteobacteria* (24)
8 to 12	64	32	16	44	*Acidobacteria* (2), *Acidimicrobidae* (1), *Actinobacteridae* (31), *Chloroflexi* (1), *Firmicutes* (3), *Proteobacteria* (20), *Rubrobacteridae* (6)

TABLE 5. Isolates of bacteria that formed visible colonies on terminal plates of counting sets with different media inoculated with soil

Medium	No. of isolates identified	% of isolates affiliated with rarely isolated groups	Groups represented (no. of isolates)
0.1× TSA	33	0	*Actinobacteridae* (18), *Bacteroidetes* (1), *Firmicutes* (5), *Proteobacteria* (9)
WSA	37	0	*Actinobacteridae* (24), *Firmicutes* (2), *Proteobacteria* (11)
CSEA	33	0	*Actinobacteridae* (20), *Proteobacteria* (13)
DNBG	36	8	*Acidimicrobidae* (1), *Acidobacteria* (1), *Actinobacteridae* (19), *Planctomycetes* (1), *Proteobacteria* (14)
VXylA	33	21	*Acidobacteria* (2), *Actinobacteridae* (14), *Proteobacteria* (12), *Rubrobacteridae* (5)
VXylG	40	13	*Acidobacteria* (2), *Actinobacteridae* (22), *Chloroflexi* (1), *Planctomycetes* (1), *Proteobacteria* (13), *Rubrobacteridae* (1)

dae (1 isolate), *Rubrobacteridae* (1 isolate), *Gemmatimonadetes* (1 isolate), and *Planctomycetes* (2 isolates). In total, 36% of all isolates appearing on these media in week 8 or later belonged to rarely isolated groups.

Effect of medium type and incubation time on development of colonies by pure cultures. Fifty isolates were selected for comparisons of their ability to grow on different media. Thirty-two of these belonged to the broad category of commonly isolated groups, and the other 18 were members of rarely isolated groups (Table 1). These were selected to cover several different phyla so that no more than two members of any one family-level group were included. A larger proportion of bacteria affiliated with commonly isolated groups was able to grow on 0.1× TSA, WSA, and CSEA than that for members of rarely isolated groups (Table 6). Members of both groups grew well on VGluA and DNBA. However, members of rarely isolated groups grew significantly more slowly on all media than did members of commonly isolated groups (Table 6).

To test the quality of these media, we tested eight strains from the collection of the Department of Microbiology and Immunology, University of Melbourne, for the ability to grow on the five media used for these experiments. All eight isolates produced visible colonies on all five media after a mean 2.7 (SD = 1.9) days of incubation at 25°C.

DISCUSSION

Choice of media. A wide range of different media have been used to estimate the size of the bacterial community of soil and to isolate representatives of this community (2, 14, 21). However, it has been known for a long time that the number of bacteria that are able to form colonies on microbiological media is generally only a small part of the total number of bacteria in soil (5, 14, 28). In addition, the advent of molecular ecological technologies has revealed the presence of many novel groups of bacteria in soil, highlighting the inadequacy of cultivation methods for the general study of soil bacteria (3, 10, 23). Recently, the use of nontraditional media has allowed the isolation of members of some of these previously uncultured groups (12, 15, 24). One of these media, VL55 medium, was formulated to mimic the low concentrations of inorganic ions in soils, with increased concentrations of ammonium and phosphate ions to allow sufficient biomass formation to produce visible colonies. The pH was adjusted to the pH of the soil at the site being studied (27). For this study, we tested the effect of a range of growth substrates in this basal medium. Based on the results, we chose xylan as the growth substrate for further experiments, as it yielded the highest mean viable counts in this study. Xylan was also used successfully as a growth substrate for the isolation of representatives of poorly studied groups of bacteria in earlier studies (15, 24). We compared this medium and DNBG, which was also successfully used in an earlier study (12), with three media that are more commonly used to grow soil bacteria, i.e., CSEA, WSA, and 0.1× TSA.

TABLE 6. Ability of cultures to grow and time until visible colony appearance on different media for pure cultures of soil bacteria from commonly isolated and rarely isolated groups[a]

Medium	% of cultures with ability to grow		Mean time to colony appearance (days)		Statistical significance (P value in t test)[b]
	Members of commonly isolated groups	Members of rarely isolated groups	Members of commonly isolated groups	Members of rarely isolated groups	
0.1× TSA	97	33	5	23	0.03
WSA	100	44	15	43	0.02
CSEA	91	50	10	35	5×10^{-4}
DNBA	100	100	7	30	4×10^{-5}
VGluA	100	94	4	19	1×10^{-4}

[a] Thirty-two isolates from commonly isolated groups and 18 isolates from rarely isolated groups were tested.

[b] Statistical significance was determined for the differences between times to colony appearance.

Effect of inoculum size. Earlier studies have repeatedly reported that smaller inocula result in higher viable counts (4, 11, 13, 14, 21). In our experiments, diluting the inoculum resulted in a 2-fold increase in the final viable count for each 10-fold decrease in inoculum size. We observed an increase in the variability, measured as the coefficient of variation, as the inoculum size decreased, which is to be expected. Thus, the precision of the viable counts decreased as the number of colonies on each plate decreased. We believe, however, that the general increase in viable counts with decreasing inoculum sizes was real because it occurred consistently across a large number of counting sets. It is clear that the standard acceptable ranges of colony numbers on a plate that are generally used for determining viable counts (20 to 200, 25 to 250, or 30 to 300 [6, 16, 22]) are inappropriate for experiments with soil, as counts in these ranges are clearly underestimates due to the depressed counts obtained with large inocula. This is probably due to growth inhibition of some species by others when the colonies are too close together or the depletion of nutrients by fast-growing colonies so that slow-growing ones do not reach a

detectable size. It has been observed that the use of larger plates partly overcomes this crowding effect (4). The variability between replicates increased dramatically when smaller colony numbers were used to calculate viable counts. The choice of what number of colonies to use to determine the viable count became a compromise between increased reproducibility at >40 colonies per plate (90-mm diameter) and increased viable counts when plates with <40 colonies were used. We decided to calculate viable counts from plates at the smallest inoculum size that yielded a minimum of 10 colonies per plate, averaged over all replicate plates at that inoculum size (dilution level) within a counting set. To overcome the associated increase in variability, we repeated each counting experiment up to seven times (with three or five replicate plates for each of three inoculum sizes).

We had expected to see differences in the kinetics of colony formation for the different inoculum sizes, but this was not the case. It was anticipated that the rapid development of larger numbers of fast-growing colonies on plates with larger inocula would prevent slower growing colonies from appearing due to inhibitory or competitive effects. The crowding effect on plates with larger numbers of colonies also did not affect the proportion of members of rarely isolated groups. These organisms formed visible colonies late in the incubation period (see below). If they were prevented from forming visible colonies on crowded plates, then equally slow-growing members of commonly isolated groups were inhibited to a similar extent. This would result in members of both groups being isolated in similar ratios, regardless of the inoculum size. Indeed, there is no reason to assume that slow growth is restricted to members of the rarely isolated groups, and we found that members of new families of *Proteobacteria* are also slow growing (unpublished data).

Effect of medium. We found that 0.1× TSA was the poorest of the six media that we investigated in detail for obtaining rarely isolated bacterial groups. No isolates belonging to rarely isolated groups were obtained on this medium in this study, although a member of the phylum *Acidobacteria* has been isolated on this medium (18). This medium also allowed the expression of a spreading phenotype so that a few single colonies, mainly members of the family *Bacillaceae*, rapidly covered most or all of the surface area of the plate. CSEA and WSA were also poor medium choices, resulting in low culturabilities and no isolates affiliated with rarely isolated groups. The media 0.1× TSA, CSEA, and WSA also did not support the growth of as many pure culture isolates of members of rarely isolated groups as the other media did, but they were very good media for growing pure cultures of members of commonly isolated groups.

DNBG and the media based on VL55 medium resulted in higher viable counts and allowed the development of visible colonies of members of rarely isolated groups. Very few spreading colonies were noted on these plates, and the spreading phenotype was not expressed on media based on VL55 by most isolates that spread on other media. We found that the use of gellan as a solidifying agent with VL55 medium resulted in higher viable counts than did the use of agar as the solidifying agent, in agreement with an earlier finding comparing these two gelling agents in experiments with DNBG and DNBA (12). This finding was not supported by statistical tests of the data, but that may have been the result of our attempting to detect small differences in data sets with high variabilities. The use of agar as the solidifying agent did not appear to result in an inhibition of growth of members of rarely isolated groups, in agreement with findings of an earlier study (24).

Jensen (13) stated that for a medium to be suitable for plate counts, it must fulfill the following four requirements as much as possible. Firstly, its composition must be standardized so that it can be reproduced with sufficient accuracy anywhere and at any time. In this study, only CSEA did not fulfill this criterion, since it contained a site-derived soil extract. Olsen and Bakken (21), however, showed that CSEA media prepared with soil extracts from different soils gave practically identical colony counts, so this may not eliminate CSEA as a useful medium. Secondly, the medium must permit the development of as large a range as possible of the bacteria present, which was true for VXylA, VXylG, and DNBG. Thirdly, the medium must allow the least possible growth of unwanted nonbacterial microorganisms, such as fungi. The number of fungal colonies was low and about the same for all six media (data not shown). Finally, the growth of spreading colonies must be suppressed. Overall, media based on VL55 medium or DNB seem to best fulfill Jensen's criteria.

Effect of incubation time. It is well known that increasing the incubation time results in increased viable counts (12, 13, 14, 26, 28), particularly on media with low nutrient concentrations (14). However, incubation times on the order of months are only rarely used, and incubation times are generally in the range of 1 week to 1 month (14, 22, 28). Extended incubation periods seem to be important, as members of rarely isolated groups appeared predominantly after incubation for 2 months on suitable media inoculated with soil. This suggests that members of these groups are particularly slow growing or have very long lag periods. Pure cultures of members of these groups were similarly slow growing and took significantly longer to produce visible colonies than did pure cultures of members of commonly isolated groups. Members of rarely isolated groups may be able to grow more rapidly once media and growth conditions have been optimized, but we suggest that they will not, in general, be as rapid growing as commonly studied soil bacteria.

Identities of isolates. Many of the isolates from commonly isolated groups were affiliated with family-level groups that have few cultured representatives but have been detected in soils as 16S rRNA genes. In addition, it was possible to isolate members of bacterial groups that were previously labeled unculturable, as we found in earlier investigations (12, 15, 24). In this study, isolates of the phylum *Acidobacteria* representing four of the eight subdivisions defined by Hugenholtz et al. (10) were cultured. Some of these belong to new families distinct from those that were previously isolated (15). We also isolated members of the poorly studied subclasses *Acidimicrobidae* and *Rubrobacteridae* of the phylum *Actinobacteria*.

Three of the isolates of the phylum *Planctomycetes* were only distantly related to cultured representatives of this group. One was affiliated with the "*Gemmatae*" subdivision but was only distantly related to members of the genus *Gemmata*. Instead, it was related to a group of bacteria that were previously only known from 16S rRNA genes detected in soil (17). The other two were members of the WPS-1 lineage of the phylum *Planc-*

tomycetes, a class-level group that was previously only known from 16S rRNA genes from soil (20; L. Schoenborn and P. H. Janssen, unpublished data) and other habitats. These two isolates, together with a third from another study performed in our laboratory (P. Sangwan and P. H. Janssen, unpublished data), are the first known cultured representatives of this group.

One member of the phylum *Gemmatimonadetes* was cultured. To date, this phylum is represented by one isolate of a named species, *Gemmatimonas aurantiaca* (29), and by three isolates from the Ellinbank soil site (15). We also cultured an isolate that represents the first cultured member of a new subdivision of the phylum *Chloroflexi* which is unaffiliated with any of the recognized subdivisions (10; P. Hugenholtz, personal communication).

Conclusions. The system we have been studying is a krasnozem clay loam soil under a mixed rye grass and clover pasture which is managed under a fertilization and grazing regimen that can be considered to be close to the district norm (19). We attribute our success in isolating members of rarely isolated groups to the methods used rather than to any unusual properties of this soil system. The successful isolation of members of groups of bacteria that are widely distributed and common in soils worldwide seems to be a result of using appropriate media and extended incubation times. We have empirically used this approach in previous studies (12, 15, 24). This study demonstrates the significance of medium choice and incubation time on the successful isolation of representatives of groups of numerically abundant but rarely isolated soil bacteria.

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