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Separation and Purification of Bacteria from Soil

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Bacteria were released and separated from soil by a simple blending-centrifugation procedure. The percent yield of bacterial cells (microscopic counts) in the supernatants varied over a wide range depending on the soil type. The superantants contained large amounts of noncellular organic material and clay particles. Further purification of the bacterial cells was obtained by centrifugation in density gradients, whereby the clay particles and part of the organic materials sedimented. A large proportion of the bacteria also sedimented through the density gradient, showing that they had a buoyant density above 1.2 g/ml. Attachment to clay minerals and humic material may account for this apparently high buoyant density. The percent yield of cells was negatively correlated with the clay content of the soils, whereas the purity was positively correlated with it. The cell size distribution and the relative frequency of colony-forming cells were similar in the soil homogenate, the supernatants after blending-centrifugation, and the purified bacterial fraction. In purified bacterial fraction from a clay loam, the microscopically measured biomass could account for 20 to 25% of the total C and 30 to 40% of the total N as cellular C and N. The amount of cellular C and N may be higher, however, owing to an underestimation of the cell diameter during fluorescence. A part of the contamination could be ascribed to extracellular structures as well as partly decayed cells, which were not revealed by fluorescence microscopy.

Separation of bacteria from soil has been used previously in investigations of bacterial respiratory activity (10), electron microscopic studies (3, 5, 14), DNA studies (18), and fluorescent antibody studies (21). However, the purity of these bacterial fractions was not investigated quantitatively. The aim of the work described in this paper was to investigate the possibility of separating a representative and essentially pure fraction of bacterial cells from a soil sample, thus enabling us to perform experiments with indigenous soil bacteria which would otherwise be precluded by the presence of soil particles. As such, the method has been applied to electron microscopic studies and experiments on the separation of soil bacteria according to cell diameter by filtration through polycarbonate membranes (R. A. Olsen and L. R. Bakken, Abstr. Third Int. Symp. Microb. Ecol., p. 64 and 81, 1983). It has also been used to obtain rough estimates of bacterial uptake of labeled C and N added to soil (L. R. Bakken, Ph.D. thesis, Agricultural University of Norway, Aas, Norway, 1983).

MATERIALS AND METHODS

Three different soils were collected from two cultivated fields and a spruce forest near the Agricultural University at Aas, Norway. The soil samples were maintained at field moisture content, crushed to pass through a 2-mm-mesh-size screen, and stored at 5°C. The soil characteristics are shown in Table 1. The clay loam (CL) was used in all the separation experiments. The other soils were included in some of the experiments to obtain information about the general applicability of the results.

Cells were released and separated from larger soil particles by repeated blending-centrifugation steps as described by Faegri et al. (10), with small modifications (Fig. 1). Three different homogenizers were tried during the development of the method: the Waring blender (Waring, New Hartford, Conn.) the Braun Melsungen cell homogenizer (no. 853032), and the Ilado X 10/20 homogenizer (In. Labor Gmbh, Ballrechten-Dottingen, Federal Republic of Germany). The soil samples were homogenized in a dilution medium (water, detergent, or buffer solutions at 10 to 15 ml/g of soil). The detergents and buffer-salt solutions used were 0.22% sodium hexametaphosphate buffered to pH 8.5 with Na_2CO_3 (Calgon) (17), 0.3% sodium pyrophosphate (5), Winogradsky salt solution (15), 0.2% bromhexinchloride (Bisolvon; Nyco, Oslo, Norway), and 0.5% Tween 80 (6). In one experiment, acidification to pH 3 (with acetic acid and H_2SO_4) and addition of $CaCl_2$ (16) were tried as methods to selectively flocculate clay minerals before sedimentation of the coarse particles.

The soil homogenate was centrifuged for 15 min to sediment large particles. A swing-out rotor was used, and the centrifugal force was 630 to 1,060 × *g* at the top and the bottom of the liquid (depth, 10 cm), respectively. The temperature was 10 to 15°C during homogenization and centrifugation. The first supernatant (S1) was decanted, and the residue (RS1) was subjected to repeated blending-centrifugation steps, resulting in a series of supernatants (S2,S3,. . .,S*n*) and a final residue (RS*n*). The cells in the combined supernatants were concentrated by centrifugation (10,000 × *g* for 20 min), resuspended in a small volume, and saved for density gradient centrifugation.

The density gradients were normally prepared from Ludox HS 40 (Du Pont Co., Wilmington, Del.). A similar gradient medium, Percoll (Pharmacia Fine Chemicals, Uppsala, Sweden), was used in experiments which involved plate counting

TABLE 1. Characteristics of the soils

Soil type and plant cover	Amt of clay (% [dry wt])	Amt of organic C (%[dry wt])	C/N ratio	pH_{H_2O}
CL from field with barley	23	3.0	10:1	5.5
SL from brown earth under spruce	9	5.5	20:1	4.5
OS from field with wheat	1–2	39	23:1	5.0

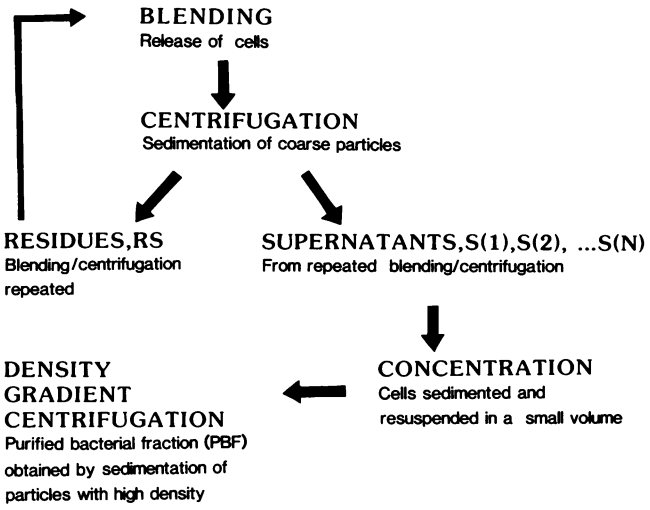

FIG. 1. General procedure for the release of cells by repeated blending-centrifugation steps and purification by density gradient centrifugation (see text for further explanations).

of bacteria. Both are colloidal silica solutions, but the silica particles in Percoll are covered by polyvinylpyrrolidone to remove their alleged toxicity (20). The pH of the silica solutions was reduced to 7.0 by adding 1 N HCl. Vigorous stirring was necessary during the addition of HCl to avoid precipitation of the silica particles. The density of the solutions was regulated to 1.16 g/ml by adding filter-sterilized distilled water (filtered through a 0.2-μm-pore-size filter), and the gradients were formed by centrifugation for 30 to 40 min with a centrifugal force of 27,000 × *g*. These gradients were rather steep (from 1.0 to 1.14 g/ml) (Fig. 2). When loaded with bacterial suspension and centrifuged for 1 h at 10,000 × *g*, the cells formed a narrow band in this region (1.0 to 1.14 g/ml), which was easily removed with a pipette. To observe the buoyant density of the cells accurately, gradients were formed from silica solutions with a density of 1.07 g/ml, resulting in a broad bacterial band. Comparison with parallel gradients loaded with density marker beads (Pharmacia) yielded fairly accurate information about the buoyant density of the cells (4).

As an alternative to the colloidal silica solutions, metrizamide (Nyco) was tried as a density medium. The pH of the metrizamide solution was regulated to 7.0 by adding small amounts of 0.1 N NaOH. The tubes were half filled with metrizamide solution (density, 1.28 g/ml), loaded with soil bacterial suspension, and centrifuged for 1 h at 10,000 × *g*, resulting in a sharp band of bacterial cells at the meniscus between the metrizamide and the overlying liquid. To investigate the buoyant density of bacterial cells from pure cultures, stepwise gradients were prepared with densities ranging from 1.05 to 1.30 g/ml.

The purified bacterial suspensions recovered from the density gradients contained density gradient material (silica particles or metrizamide) which was removed by dilution 1:20 in filter-sterilized (0.2-μm-pore-size filter) distilled water followed by sedimentation of the cells by centrifugation for 20 min at 10,000 × *g*. The silica particles were only partly removed by this washing procedure, but the concentration was low enough to permit fluorescence microscopic counting (high concentration of silica particles caused clogging of filters and high background fluorescence). Chemical analysis was only done on bacterial suspensions from Ludox gradients, because Ludox contained neglegible amounts of organic materials.

The yield of cells by the different procedures was investigated by fluorescence microscopic counting (11) and size measurements (13). The yield was also studied with respect to viable cells, counted as CFU on soil extract agar (64 mg of K_2HPO_4, 36 mg of NaH_2PO_4, 10 mg of glucose, 10 mg of xylose, 10 mg of peptone, 10 mg of yeast extract, 20 g of agar, 400 ml of soil extract, 600 ml of distilled water).

Total C in bacterial suspensions was measured as CO_2 with an infrared CO_2 analyzer (A. D. C., Hoddesdon, England) after wet-oxidation by the method of Allison et al. (1). Before oxidation, any CO_2 present was removed by heating with 2 N H_2SO_4 containing 5% $FeSO_4$ (2). Total N was measured as NH_4^+ after digestion in H_2SO_4 (8).

Pure cultures of soil bacteria were used to obtain data on the C and N content of bacterial cells. They were cultivated in glucose-yeast extract broth, harvested, washed in distilled water (4), dried at 105°C, and analyzed for total C and N. Protein content was measured as free α-amino acids after acid hydrolysis. Free α-amino acids were measured as CO_2 evolution during the ninhydrin reaction.

RESULTS

Release of cells by blending-centrifugation. The different homogenizers were compared in preliminary blending-centrifugation experiments with CL. The Ilado homogenizer was unsuitable because the bearings were destroyed by the coarse mineral particles. The Braun Melsungen cell disrupter, when operated for 3 min at half speed, gave the same percent yield of cells in the supernatant as did the Waring blender when run for 3 min intervals. The Waring blender was chosen for the rest of the experiments. It was run for three 1-min intervals with intermittent cooling in an ice bath as described by Faegri et al. (10). The Ilado homogenizer was used for the resuspension of the bacterial pellet before

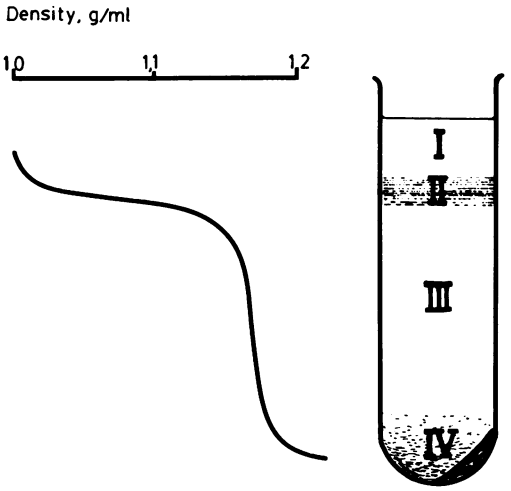

FIG. 2. Appearance of the Ludox gradient after centrifugation with a suspension of bacteria released from soil by blending-centrifugation. Layers: (I) clear top layer, light brown color; (II) sharp band of bacterial cells, Yellow-brown color with SL and CL and dark brown with OS; (III) clear layer, no particles observed except from SL, which gave some aggregates of dark particles in this layer; (IV) bottom layer, containing pellet consisting of clay and dark humic material. Above the pellet humic material was floating as large aggregates. The aggregates were readily dispersed by stirring.

density gradient centrifugation, since the Waring blender was not sufficiently effective for this purpose.

The different dilution media were compared for blending-centrifugation of CL. They gave nearly identical numbers of cells in the supernatant (S1), i.e., 12 to 15% of the total number of cells in the soil sample. Distilled water was chosen for further experiments. Flocculation of clay minerals by acetic acid, H_2SO_4, or $CaCl_2$ invariably resulted in a very low yield of cells in the supernatants (0.5 to 1.5% of total), and the flocculating agents were therefore not used in further experiments. The amount of water per gram of soil in the soil homogenate was varied from 10 to 50 ml/g in a blending-centrifugation experiment with CL. The yield in S1 was somewhat higher with 50 than with 10 ml/g (17 and 14%, respectively), but the difference was not statistically significant. Thus, very little was gained by adding more water than 10 ml/g.

The different soils gave a significantly different percent yield of cells in the supernatants (Table 2), and the yield was lower in S2 than in S1 for all soils.

A sample of CL was subjected to eight repeated blending-centrifugation steps (Table 3). A rough estimate of the size distribution of the cells was made by separately counting the number of bacteria within three different volume groups. The yield of cells gradually decreased from 1.6×10^9 in S1 to 0.4×10^9 per g of soil (dry wt) in the last supernatant (S8). The residue contained 23% of the total number of cells in the soil sample. The yield, if expressed as a percentage of the number of cells actually present in the homogenate before each centrifugation, was remarkably constant (16, 12, 15, 17, 14, 13, 15, and 13% in S1 through S8, respectively).

The percentage of the smallest cells (<0.5 μm) was slightly higher in S1 through S4 than in S5 through S8 and RS8. An opposite trend was observed for the medium-sized cells (0.5 to 0.8 μm). The largest cells were counted within the same area as the others, and their estimated frequency was therefore based on the observation of very few cells. Consequently, the estimates are inaccurate and should be treated with reservation.

The relative frequency of larger cells was studied in more detail in another experiment with CL. Seven volume groups were included, and the counting area was regulated for each group to obtain more reliable estimates of the frequency of the largest cells (13). Rods were included in the same size groups as cocci by volume. Only one blending-centrifugation step was run. The previously observed tendency to an increased frequency of the smallest cells in the supernatant was not observed in this experiment (Table 4). The relative frequency of the size groups 1 and 2 was roughly the same in the supernatant as in the soil homogenate, whereas the relative frequency of size groups 3 and 4 in the supernatant was about two-thirds that in the soil homogenate.

The frequency of cells with diameters between 1.4 and 1.9 μm was 0.3% in the soil homogenate and 0.03% in the supernatant. No cells with diameters larger than 1.9 μm were observed in the supernatants. These cells seem to be completely sedimented by the centrifugation. A majority of the cells with diameters between 1.4 and 1.9 μm was also sedimented.

The removal of all the cells with diameters larger than 1.9 μm indicated that both hyphae and spores of fungi were removed by the centrifugation. This was confirmed by counting the number of viable fungal units on soil extract agar plates with 30 ppm (30 μg/g) of streptomycin and tetracycline as bacterial inhibitors. Of the viable fungi, 90% were recovered by resuspending the sediment, whereas fewer than 0.4% were recovered in the supernatant.

Distribution of bacterial cells in the density gradients. In an experiment with all three soil types, the distribution of cells in the gradient was investigated. The gradients were partitioned as shown in Fig. 2, and the bacteria were counted microscopically after removal of the Ludox particles.

The top layer contained practically no bacterial cells. The bacterial layer (II) had a very high concentration of bacterial cells: 30, 26, and 70% of the total number of cells were recovered in this layer from CL, sandy loam (SL), and organic soil (OS), respectively. The next layer (III), which covered a narrow range of buoyant densities, contained very few cells; only 4 and 8% of the total were recovered in this layer from CL and SL, respectively. Of the bacterial cells

TABLE 2. Yield of bacteria (microscopic counts) in the supernatants from repeated blending-centrifugation steps

Soil type	Total bacterial no. ($\times 10^9$ per g [dry wt] of soil) (%) in supernatant[a]	
	S1	S2
CL	1.5 (15)	1.1 (10)
SL	3.6 (36)	2.4 (23)
OS	11.8 (28)	7.0 (17)

[a] The standard deviation of each estimate was 10 to 15% of the value.

TABLE 3. Release of cells from CL by repeated blending-centrifugation steps: yield and size distribution (microscopic count) of cells in the supernatants

Supernatant and residue	Total no. of cells in each supernatant ($\times 10^9$ per g [dry wt] of soil)[a]	% of total in soil sample	Size distribution (%) among cocci of diam[b]:		
			<0.5 μm	0.5–0.8 μm	>0.8 μm
S1	1.6	16	68	28	4
S2	1.0	10	71	27	2
S3	1.1	11	71	26	3
S4	1.1	11	72	26	1
S5	0.7	7	61	32	6
S6	0.6	6	53	36	10
S7	0.6	6	60	35	6
S8	0.4	4	58	41	1
RS8	2.3	23	59	35	6

[a] Expressed as numbers per gram (dry weight) of soil applied.
[b] Rods were included by volume.

TABLE 4. Size distribution of cells in the soil homogenate and supernatant after centrifugation

Size group	Diam limits for cocci (μm)	Calculated mean cell vol (μm^3)	No. of cells per ml (% of total) in:	
			Soil homogenate	S1
1	<0.5	0.034	6.5×10^8 (65)	1.0×10^8 (66)
2	0.5–0.7	0.11	2.5×10^8 (25)	0.42×10^8 (28)
3	0.7–1.0	0.32	0.7×10^8 (7)	0.08×10^8 (5)
4	1.0–1.4	0.90	0.13×10^8 (1.3)	1.4×10^6 (0.9)
5	1.4–1.9	2.35	0.03×10^8 (0.3)	0.05×10^6 (0.03)
6	1.9–2.6	5.96	0.01×10^8 (0.1)	$<0.01 \times 10^6$ (<0.01)[a]
7	2.6–3.7	16.40	0.6×10^6 (0.06)	$<0.01 \times 10^6$ (<0.01)

[a] No cells were observed. The upper confidence limit (5%), assuming binomial distribution, is shown.

TABLE 5. Size distribution of bacteria in the PBF compared with that in supernatants from blending-centrifugation (S1 + S2)

Soil type	Suspension	% of total no. within vol groups[a]: Cocci 1[b]	Cocci 2	Cocci 3	Cocci 4	Cocci 5[c]	Rods 2	Rods 3	Rods 4
CL	S1 + S2	66	14	3	0.3	0.03	12	3	0.6
CL	PBF	57	14	4	0.7	0.04	21	2	0.2
SL	S1 + S2	77	13	4	0.3	0.03	3	1	ND[d]
SL	PBF	77	14	2	0.2	0.1	6	1	ND

[a] Identical to the groups defined in Table 4.
[b] Rods with equivalent volumes included.
[c] The estimated frequency is based on the observation of three to six cells, and the standard error of the estimate is 40 to 60%.
[d] ND, Not detected.

from CL, 60% were recovered in the bottom layer. Very little material from the OS was recovered in the bottom layer.

With CL, several experiments were done by using density gradients of Ludox and Percoll. The percent recovery in the bacterial layer (II) varied between 30 and 40% with both Ludox and Percoll. The number of blending-centrifugation steps was varied from one to five without affecting the distribution of the cells in the density gradients significantly.

Metrizamide could replace collodial silica as a density medium. A high density of the metrizamide solution was necessary, because the buoyant density of bacterial cells (pure cultures) was found to be higher in metrizamide (1.15 to 1.20 g/ml) than in Ludox (1.04 to 1.12 g/ml). The percent recovery of soil bacteria at the meniscus between the metrizamide solution and the overlying liquid varied over the same range as the recovery in the bacterial layer from colloidal silica gradients, i.e., 30 to 40%.

The amount of suspension loaded on the gradient could be varied within a wide range without affecting the percent recovery of cells in the bacterial layer. In an experiment with 15 ml of Ludox per tube, various amounts of soil-bacterial suspension (containing 1.2×10^9 cells per ml) was loaded on the gradients. The yield of cells in the bacterial layer of the gradient was 40, 29, 33, 38, and 42%, with a load of 1, 5, 10, 15, and 20 ml, respectively.

The size distribution of cells in the density gradient. The purified bacterial fractions (PBFs) from CL and SL were compared with the supernatant after blending-centrifugation (S1 + S2) with respect to size distribution of rods and cocci (Table 5). In general, the size distribution was little affected by the density gradient centrifugation. With both soils, an increased frequency of the smallest rods was observed in the PBF. The frequency of the smallest cocci was reduced accordingly for CL, whereas the frequency of this group was not changed for SL. Minor differences in frequency can be observed for the other size groups as well, but they are within the experimental error.

Comparison of cell dimensions in phase contrast and fluorescence microscopy. A comparison between cell size observed in phase contrast and fluorescence microscopy was made with cells from the bacterial layer and the bottom pellet of a Ludox gradient (CL). The cells were stained with acridine orange (final concentration, 0.1 g/liter), sedimented by centrifugation, and mounted in 2% agar for immediate observation, both in phase contrast and during fluorescence. Only cells with diameters larger than 0.4 μm were recorded, owing to difficulties in observing the smaller cells by phase contrast microscopy.

Of the cells in the bottom pellet, 50% were larger when observed under phase contrast than with fluorescence, and the calculated average cell volume based on phase contrast observations was about twice that based on fluorescence observations. The cells from the bacterial layer gave a better agreement between the two microscopic methods: only 30% of the cells were larger in phase contrast than during fluorescence and the observed differences were smaller, resulting in an average cell volume which was only 25% higher with phase contrast than with fluorescence microscopy.

Frequency of viable cells in soil homogenate and bacterial fractions. The number of viable cells, measured as CFU (platable cells) on the soil extract agar, was found to represent 2% of the microscopic counts in the homogenate of CL. The percentage of platable cells measured in the supernatants from five repeated blending-centrifugation steps varied between 2.5 and 5%. About 3% of the total number of cells in the final residue were platable. PBFs (CL) after centrifugation on Percoll gradients have also been investigated (R. A. Olsen and L. R. Bakken, Abstr. Third Int. Symp. Microb. Ecol., p. 64, 1983). In these suspensions, the platable cells varied between 2 and 5% of the total number of cells.

Chemical analysis of cells from pure culture: estimates of cellular C and N in the bacterial fractions. The cells from eight different pure cultures previously used in a study of the buoyant density and dry weight of cells (4) were analyzed for total C, total N, and α-amino acid N after acid hydrolysis.

The average C and N content of the cellular dry matter was found to be 44 ± 2 and 12 ± 2%, respectively. These values were used to convert the microscopically determined biomass into estimates of cell C and cell N. The average content of α-amino acid N after hydrolysis was found to be 40% of the total N in the cells. The average cell volume of the PBF was calculated from the size distribution of the cells (Table 6). The average cell volume was 0.11 μm^3 in the soil homogenate and 0.086 μm^3 in the bacterial fraction. The difference was due to removal of the largest cells by the blending-centrifugation. The density and dry weight of bacterial cells have been estimated to be 1.07 g/ml and 30% (wt/wt), respectively (4). Thus, the average dry weight per cell in the bacterial fraction would be 2.8×10^{-14} g. With 44% C and 12% N in the dry matter, the average C and N content per cell was 1.22×10^{-14} and 0.34×10^{-14} g, respectively. Different values were obtained for the other

TABLE 6. Average volume of the cells in the soil homogenate and the PBF from CL, as calculated from the relative frequency (X_i) and cell volume of each size group

Size group (i)	Mean cell vol (V_i) (μm^3)	Soil homogenate X_i	Soil homogenate $X_i \times V_i$	PBF X_i	PBF $X_i \times V_i$
1	0.034	0.65	0.022	0.57	0.019
2	0.113	0.25	0.028	0.35	0.040
3	0.322	0.07	0.023	0.06	0.019
4	0.905	0.013	0.012	0.009	0.008
5	2.35	0.003	0.007	ND[a]	ND
6	5.96	0.001	0.006	ND	ND
7	16.37	0.0006	0.010	ND	ND
Avg cell vol ($\Sigma X_i \times V_i$)			0.11 μm^3		0.086 μm^3

[a] ND, Not detected.

two soils owing to a different size distribution of the cells (data not shown).

Purity of bacterial fractions. Preliminary experiments had shown that the purity of the supernatants after blending-centrifugation was very low. Large amounts of clay as well as noncellular organic material were present. Most of the clay and a large part of the noncellular organic material were removed by density gradient centrifugation.

Table 7 shows the results of an experiment with CL, SL, and OS. The purity of the suspensions was investigated by total cell counts and total N analysis of the suspensions. A considerably increased purity was observed after blending-centrifugation compared with that of intact soil, and further purification was obtained by density gradient centrifugation. Thus, the purity of the bacterial fraction from CL, SL, and OS was 32, 24, and 19% respectively. Prewashing the cells in 1% Calgon before density gradient centrifugation did not significantly improve the purity.

Four experiments were run with CL to investigate the purity of the bacterial fraction with respect to organic C. The percentage of the total organic C which was accounted for as cellular C ranged from 16 to 24% in different experiments; the average was 20%. Prewashing in 2% sodium hexametaphosphate did not significantly increase the purity.

Protein content of the bacterial fraction. The PBF was analyzed for protein N. Of the total N in the fraction, 40% was accounted for as protein N. The same value was found for bacterial cells from pure cultures. Protein N accounted for 27% of the total N in the intact soil (CL).

DISCUSSION

The percent yield of cells in the supernatants after blending-centrifugation was much lower in the CL than in the OS and SL (Table 2). This reflects large differences between the soil types as regards attachment between bacterial cells and soil particles. Similar observations were made by Drazkiewicz and Hattori (9). Although both investigations indicate that high clay content results in more extensive attachment, other factors seem to be important as well.

TABLE 7. Purity with respect to cellular N: comparison of intact soil, supernatants after homogenization-centrifugation (S1 + S2), and PBFs

Soil and fraction	Purity (estimated cellular N as % of measured total N)
CL	
Intact soil	1.2
S1 + S2	7.3
PBF	32.0
PBF (prewashed)[a]	36.0
SL	
Intact soil	0.9
S1 + S2	9.9
PBF	24.0
PFB (prewashed)[a]	23.0
OS	
Intact soil	0.8
S1 + S2	8.2
PBF	19.0
PBF (prewashed)[a]	7.3

[a] Cells were washed three times in 1% Calgon before density gradient centrifugation.

Balkwill et al. (5) applied a similar blending-centrifugation technique on a silty CL soil. By using repeated blending-centrifugations, they observed that the yield of viable cells (plate counts) in the supernatants leveled off, leaving a large fraction (>50%) of the cells essentially nonreleasable. The release of cells from CL did not follow a similar pattern. The yield of cells, if expressed as a percentage of total cell numbers present in the soil homogenate before each centrifugation, did not decrease appreciably through eight repetitions. Thus, the number of cells remaining attached to soil particles should approach zero when the number of blending-centrifugation steps in increased. A large number of repetitions would be necessary to test whether a small proportion was essentially nonreleasable.

Cell size distribution. The loss of cells with diameters larger than 1.4 to 1.9 μm during centrifugation (Table 4) is in general agreement with sedimentation velocities of spheres as calculated from Stokes' law (19). The size distribution of the smaller cells (representing 98% of the total) was very similar in the soil homogenate, in the supernatants after blending-centrifugation (Table 3 and 4), and in the PBF (Tables 5 and 6). Thus, attachment to soil particles seems to occur with the same frequency within these groups. The only exceptions were small rods, which occurred more frequently in the PBF than in the supernatants (Table 5).

Purity of the bacterial fractions. The purity of the suspension after blending-centrifugation was found to be 5 to 10% with respect to nitrogen. This is considerably higher than in intact soil, in which bacterial N represented about 1% of the total N. The density gradient centrifugation gave a still higher purity of the bacterial suspension. However, more than 50% of the cells were lost by sedimentation through the gradient (CL). Bacterial endospores may have a density of 1.29 g/ml (unpublished data), but it is unlikely that vegetative bacterial cells should have such a high buoyant density (4). It must therefore be assumed that the great number of cells which sediment through the density gradients are attached to or covered by clay and humic material. The combined fluorescence and phase contrast observation of cells from the Ludox gradient is in general agreement with this assumption; the large difference between phase contrast and fluorescence measurements of the diameters of cells from the bottom pellet may be ascribed to soil material covering the cells, resulting in either a shading effect during fluorescence observation or enlargement artifacts during phase contrast observation. Electron microscopic studies of bacterial cells from soil have shown that a substantial part of the cells may be covered by soil materials (3).

The results with SL and OS indicate that the amount of clay minerals is important for the density gradient work: the yield of cells during gradient centrifugation was highest with OS, whereas the purity obtained was lower with OS than SL and CL (Table 7). The yield is therefore negatively correlated with the clay content of the soil, whereas the purity is positively correlated with it. This seems to indicate that the clay minerals help to separate the relatively pure cells from soil particles and cells covered by soil materials by increasing the buoyant density of the latter. A part of the soil organic matter may have a buoyant density too close to that of the bacterial cells to permit a separation based on buoyant density.

The results with density gradients are in general agreement with the observations of Martin and MacDonald (14), who found that about 70% of the cells from CL would sediment through a Percoll gradient. They also observed that sophistication of the homogenization procedure before den-

sity gradient centrifugation did not significantly reduce the loss of cells through the gradient.

The purity of the bacterial fraction has been estimated by converting the biovolume, as estimated from fluorescence microscopic counting and size measurement, into biomass C and N. However, combined fluorescence and phase contrast observations indicated that the estimated biovolume may be 25% higher as a result of incomplete staining of the cells. Thus, the purity of the bacterial fraction from CL may be 25% with respect to C and 40 to 45% with respect to N.

The purified bacterial fraction from CL has been studied by transmission electron microscopy of thin sections (R. A. Olsen and L. R. Bakken, Abstr. Third Int. Symp. Microb. Ecol., p. 81, 1983). In addition to the apparently intact cells, the fraction contained partly decayed cells with very little cytoplasmic material. A large number of the cells were surrounded by extracellular material, sometimes with a structure like fimbria (12). In general, the electron microscopic study confirmed the measurement of the purity of the bacterial fraction, although some of the contamination could be ascribed to extracellular structures and to partly decayed cells which probably could not be recognized during the fluorescence microscopic counting.

Protein content of the bacterial fraction. The protein N represented 27% of the total N in CL. This was increased to 40% in the bacterial fraction. A high proportion of protein N in the bacterial fraction would be expected, since cellular N was found to represent a very large proportion of the total N (30 to 40%). The experimental error was very large owing to the small amounts of material, and the close agreement with the average value of the bacterial pure cultures may be an arbitrary result.

Frequency of viable cells. If the bacterial fraction is representative of the total population in the soil, the frequency of viable cells as obtained on agar plates (platable cells) should be the same as in soil.

The results indicate that the frequency of platable cells may be somewhat higher in the purified bacterial suspensions than in the soil homogenate. However, the plate counts from the soil homogenate may be considerably lower than the real number of viable cells, owing to the fact that several cells stick together, giving rise to only one colony (5). The presence of such cell aggregates could easily be observed during fluorescence microscopic counting of the soil homogenate, whereas in the bacterial fraction, adherence between cells was more rare. Thus, the apparent difference in the precentage of viable cells between the bacterial fraction and soil homogenate may for a large part be attributed to this difference in aggregation.

Conclusion. The general aim of this study was only partly achieved, since the biomass C and N can only account for a fraction of the total C and N in the PBF. Further studies are needed to elucidate the origin of the contaminating materials. As such, the method has proved useful for a series of filtration experiments, electron microscopic studies, and DNA determinations in soil bacteria (R. A. Olsen and L. R. Bakken, Abstr. Third Int. Symp. Microb. Ecol., p. 64 and 81, 1983). New applications will probably be found. It should be stressed, however, that the representativity of the bacterial fraction remains a problem for every new application, and it should be checked by other methods if possible.

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