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Fate of *Enterobacter cloacae* JP120 and *Alcaligenes eutrophus* AEO106(pRO101) in Soil during Water Stress: Effects on Culturability and Viability

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A sandy loam soil near field capacity moisture content (ψ = −0.050 MPa) or air dried (ψ = −300 MPa) was inoculated with about 3 × 10^7 CFU of *Enterobacter cloacae* JP120 and *Alcaligenes eutrophus* AEO106(pRO101) per g and incubated in 40-g portions at 17°C in closed or open Erlenmeyer flasks. In the field-moist soil, selective plating, direct viable counts, and DNA hybridization showed only minor changes in the numbers of *E. cloacae* and *A. eutrophus* cells with time (14 days), and the results obtained with the three detection methods generally agreed. In the air-dried soil, the majority of both bacteria were found as intact DNA-carrying cells that were neither culturable nor viable by the methods employed in this study. The numbers of culturable *E. cloacae* and *A. eutrophus* cells dropped to 10^5 and 10^2 CFU/g, respectively, 2 h after inoculation. Direct viable counts showed that only about 1% of the cells detected by immunofluorescence microscopy were viable, but a fraction of viable nonculturable cells of both bacteria was present. *A. eutrophus* did not tolerate desiccation as well as *E. cloacae*. Only a minor fraction of the two test organisms regained their culturability or viability after rewetting of the air-dried soil; the number of total heterotrophic culturable bacteria, however, increased more than 10-fold and reached 73% of the level found in the field-moist soil at day 14.

Desiccation is a well known stress agent in the soil environment (4, 9, 16, 24, 28), but the responses of individual bacteria to changes in matric potential are poorly understood. Bacterial strains have been shown to lose culturability when introduced into natural environments in most (2, 3, 12, 21, 29) although not all (13, 23) cases. Further, it is generally found that only about 1% of the total bacteria in soil are culturable (5). Therefore, direct detection methods are advantageous for monitoring the fate of introduced bacteria (25). More complex questions can be addressed by the combined use of several detection methods than with any single method because of the different end-points.

DNA hybridization between probes and DNA extracted directly from bacteria separated from soil by a cell extraction method (11, 13) enables the specific detection of all intact bacteria regardless of their activity. By using the nalidixic acid method (3), immunofluorescence microscopy (IF) (1) offers a determination of cell viability (3). IF can also provide information on the number, as well as the size and morphology, of intact cells. Finally, selective plating (SP) is used to determine the number of culturable cells.

Experiments in our laboratory with *Enterobacter cloacae* introduced to top soil have indicated that SP, unlike IF, shows very low numbers of *E. cloacae* in dry soil (ψ [soil matric potential] < −100 MPa) but not in moist soil (ψ = −0.002 to −0.20 MPa) in which IF and SP results generally are similar (20, 21). Further, the presence of viable nonculturable cells of *E. cloacae* JP120 was demonstrated (21).

The objectives of the present study were (i) to compare the influence of water stress on the fate of two soil bacteria, *E. cloacae* JP120 and the 2,4-dichlorophenoxyacetic acid-degrading *Alcaligenes eutrophus* AEO106(pRO101) (8) and (ii) to investigate the presence of nonculturable cells by comparing the results of two direct detection methods, IF and DNA hybridization, with SP.

MATERIALS AND METHODS

Bacterial strains and culture conditions. *E. cloacae* JP120 is a spontaneous mutant resistant to rifampin (21). *A. eutrophus* AEO106(pRO101) (pRO101 is pJP4::Tn*1721*) (8) (obtained from R. H. Olsen, University of Michigan) is resistant to tetracycline because of the presence of Tn*1721* on plasmid pRO101. *E. cloacae* was grown for 20 h in Luria-Bertani (LB) medium (17) amended with 100 μg of rifampin (Sigma Chemical Co., St. Louis, Mo.) per ml, while *A. eutrophus* was grown for 24 h in LB amended with 30 μg of tetracycline (Sigma) per ml. Both strains were propagated at 30°C with shaking, washed twice with Winogradsky's salt solution (22) diluted 1:20, and mixed 1:1 for use as a spray inoculum.

Soil system. Sandy loam soil from a former garden area (7.4% clay, 8.1% silt, 31.7% coarse silt, 21.8% fine sand, 31% coarse sand, and 4.8% organic matter [pH 7.4]) (20) was passed through a 4-mm-pore-size sieve and stored at 4°C. At 14 days before inoculation, 1,100 g (dry weight) of the soil was distributed in a flat open tray and left at 17°C to air dry. Another 800 g of soil was incubated in a closed container at 17°C. The bacteria were inoculated into soil kept in flat trays in a laminar airflow bench. The soil was sprayed with a misting bottle until wet on the surface and was tilted. This procedure was continued until the soil had received a total of 1 g of spray solution per 100 g of soil (about 3 × 10^7 CFU of each bacterium per g). The soil was then transferred to sterile 250-ml Erlenmeyer flasks with 40 g per flask and incubated in a climate chamber at 17°C, 70% relative humidity, with (field-moist soil) or without (air-dried soil) rubber stoppers. After 9 days, half of the flasks with air-dried soil

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were rewetted by the addition of 8.3 ml of sterile-filtered, deionized water to each flask and closed with rubber stoppers. All rubber stoppers were removed briefly every 2 or 3 days to aerate the soil. Inoculation increased the water content of the air-dried soil by 1.07%, but most of this moisture (0.88%) had evaporated by day 2. During the study, the water potential (ψ) was -0.050 ± 0.004 MPa in the field-moist soil, -345 ± 228 MPa in the air-dried soil, and -0.019 ± 0.002 MPa in the rewetted soil, determined by the filter paper method (6). The corresponding water content averages were 15.5, 2.0, and 18.1% (dry weight), respectively.

Sampling. Sampling was done in triplicate. Samples were taken 2 h, 2 days, 9 days (2 h after rewetting), and 14 days after inoculation. Each sample consisting of the contents of an Erlenmeyer flask was homogenized with 100 ml of sterile-filtered Winogradsky's salt solution diluted 1:20 with a Virtis 23 homogenizer (Gardiner, New York City, N.Y.) (20) and kept on ice until further analysis.

Plating. For enumeration of specific bacteria, sample homogenates were diluted in Winogradsky's salt solution (diluted 1:20) and spread plated in duplicate. *E. cloacae* JP120 was enumerated after 1 day at 30°C on LB agar with 100 μg of rifampin per ml. *A. eutrophus* AEO106(pRO101) was enumerated after 2 days at 30°C on LB agar with 30 μg of tetracycline per ml. For enumeration of total CFU, samples were plated in duplicate as described previously (13) and enumerated after 7 days at 22°C. Natamycin (25 μg/ml; Merck, Schuchardt, Germany) was added to all agar media to control fungi (19).

Replica plating from LB agar was performed on days 0 and 14 to investigate the plating efficiency. The number of colony-forming *E. cloacae* cells obtained by SP averaged 69% of the number obtained by replica plating (significant difference, $P < 0.05$). Because of overgrowth by other bacteria, replica plating did not work with *A. eutrophus*.

IF. Soil homogenate (20 ml) was flocculated with $CaCl_2$, incubated with nalidixic acid (Sigma), preserved with 2% formaldehyde, and stored refrigerated until analysis (<7 months) as described previously (21). Samples were double stained (10) with 4'-6-diamidino-2-phenylindole (DAPI) and polyclonal rabbit antibodies specific against *E. cloacae* (21) or *A. eutrophus* (14) and enumerated microscopically as described previously (21). The recovery of *E. cloacae* cells by the flocculation step was quantitative as determined by SP or IF, confirming previous findings (21). For *A. eutrophus*, however, enumeration was invalidated by a low recovery: only 5 to 30% of the cells or CFUs were recovered in the supernatant (data not shown).

Viable cells were distinguished morphologically as described previously (21). The different morphologies of viable and nonviable cells were established by comparing sterilized and nonsterilized samples (Table 1). The staining of DNA with DAPI was useful in cases of doubt, since many nonelongated cells did not stain with DAPI.

DNA hybridization. Soil DNA was extracted from 80 ml of soil homogenate by the repeated blending and centrifugation method described by Jacobsen and Rasmussen (13) and suspended with 100 μl of TE buffer (17) (pH 8). The average recovery of culturable cells of *E. cloacae* and *A. eutrophus* was 35%, similar to values previously reported with this method (11, 13). A 1-kb *Sma*I fragment of Tn*1721* was used as a specific probe for *A. eutrophus* containing pRO101 (13). A probe specific against *E. cloacae* was obtained by selecting a 330-bp fragment obtained by polymerase chain reaction amplification of total *E. cloacae* DNA, with primers directed against the *phoE* gene (encodes an outer membrane protein) (27): 5'-CTGGGCGTAAGCAGCGATG-3' and 5'-GGTCAG AGAGGTACCGAAACC-3'. Both probes were labelled with ^{32}P by using a random priming kit (Boehringer Mannheim, Mannheim, Germany) and separated from unincorporated nucleotides on NucTrap columns (Stratagene, La Jolla, Calif.).

TABLE 1. Size of untreated or formaldehyde-treated cells of *E. cloacae* JP120 and *A. eutrophus* AEO106(pRO101) incubated with nalidixic acid and yeast extract

Organism and sample source[a]	Formaldehyde treatment[b]	Size (μm)[c]	
		Length	Width
E. cloacae JP120			
Inoculum	+	2.58 ± 0.10 b	1.52 ± 0.05 b
Inoculum	−	8.70 ± 0.86 a	1.77 ± 0.05 a
Soil	+	2.53 ± 0.11 b	1.47 ± 0.05 b
Soil	−	7.42 ± 0.91 a	1.53 ± 0.05 b
A. eutrophus			
Inoculum	+	2.48 ± 0.11 c	1.15 ± 0.04 b
Inoculum	−	9.87 ± 1.13 a	1.48 ± 0.07 a
Soil	+	1.90 ± 0.07 c	1.22 ± 0.05 b
Soil	−	6.80 ± 0.59 b	1.45 ± 0.06 a

[a] Soil, sample from field-moist soil at day 14.
[b] Treatment with 2% formaldehyde before incubation with nalidixic acid.
[c] Determined by IF using an eyepiece micrometer (average of 30 cells ± standard error of the mean). Within columns and for each species, numbers with the same letter are not significantly different at the 5% level (Duncan's multiple-range test).

Dot blots were carried out in duplicate experiments with loading of 2 μl of soil DNA directly onto Hybond-N hybridization transfer membranes (Amersham, Amersham, Buckinghamshire, United Kingdom). To quantitate DNA, a 2-fold dilution series of standards (total *E. cloacae* and pCJ17 [harboring Tn*1721*] [13]) were included. The DNA concentration of the two standard solutions was determined by measuring A_{260}. Filter-bound DNA was denatured in NaOH. After neutralization, nucleic acids were UV cross-linked to the filter paper in a Stratalinker 1800 oven (Stratagene). Prehybridization, hybridization, and washes were carried out at 65°C (13). The amount of ^{32}P-labelled probe was approximately 10 ng/ml of hybridization buffer. Filters were placed on X-ray film (Fuji Photo Film Co., Tokyo, Japan) for up to 56 h.

Autoradiograms (Fig. 1) were analyzed with the Cream program (7). Dot intensities (pixels) were converted to the numbers of *E. cloacae* genomes or plasmids (*A. eutrophus*) by using the two standard curves. No background hybridization was observed in the field-moist or air-dried soil (Fig. 1).

Statistics. Colony and cell counts as well as DNA determinations were averaged per gram of dry soil and logarithmically transformed. The log-transformed data were analyzed with analysis of variance procedures by using SAS/STAT, version 6.04 (Statistical Analysis Systems, SAS Institute, Cary, N.C.). Residual variances were calculated by using a model that included time, experiment, and the interaction between these two variables. Residual variances were compared by using the F test.

Reproducibility and precision. The experiments were performed in duplicate; the results of a representative experiment are shown. The precision of the analytical methods was compared by computing residual variances. The largest variability was found with the viable counts of *E. cloacae*

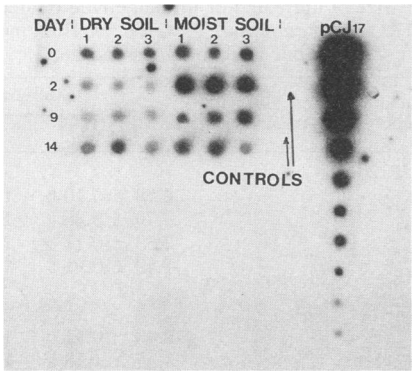

FIG. 1. Autoradiogram showing the result of hybridization between DNA extracted from air-dried (lanes labelled DRY SOIL) and field-moist (lanes labelled MOIST SOIL) soil and the probe against *A. eutrophus* AEO106(pRO101). Plasmid pCJ17, containing the fragment used as probe, was added to the filter in a twofold dilution series. Controls were DNA extracted from the unseeded soils.

(0.12) and total counts of *A. eutrophus* (0.050). Otherwise, residual variances ranged from 0.01 to 0.03.

RESULTS

E. cloacae. The fates of *E. cloacae* JP120 and *A. eutrophus* AEO106(pRO101) as determined with the different detection methods in field-moist, air-dried, and rewetted soil are shown in Fig. 2. For the field-moist soil, SP, IF, and hybridization results generally agreed. An average of 92% of the cells detected by IF was determined to be viable by the nalidixic acid method. SP showed a decrease ($P < 0.01$) through the observation period from 3.1×10^7 to 8.3×10^6 CFU/g. Furthermore, the concentration of bacterial DNA hybridizing with the *E. cloacae* probe was similar in the field-moist and air-dried soil and did not show any variation with time. Hybridization results and IF total cell counts were similar and of the same magnitude as the inoculum.

In the air-dried soil however, the fractions of culturable and viable cells (Fig. 2) were much lower than those in the field-moist soil. This difference was observed 2 h after inoculation, when only 1.1×10^5 CFU/g was detected in the air-dried soil, and was maintained throughout the experiment. On the average, 4.0% of the IF-detected cells were viable as determined by the nalidixic acid method.

An increase ($P < 0.05$) in the number of culturable *E. cloacae* cells (Fig. 2) was observed by rewetting the air-dried soil, but the number was still much lower in the rewetted soil than in the field-moist soil. The fraction of viable cells also increased ($P < 0.05$).

A. eutrophus. In the field-moist soil, 2.5×10^7 CFU of *A. eutrophus* per g was observed on day 0. On day 14, SP results (1.0×10^7 CFU/g) were significantly lower than those days 0 to 9 ($P < 0.05$). On the average, 73% of the IF-detected cells were determined to be viable.

The average concentration of cellular DNA in the field-most soil (5×10^7 plasmids/g) was higher than that in the air-dried soil (1×10^7 plasmids/g) ($P < 0.05$) and did not show any variation with time.

In the air-dried soil, the numbers of *A. eutrophus* cells that were detected by SP (Fig. 2) were several orders of magnitude lower than those in the field-moist soil; with SP, *A. eutrophus* cells could be quantified on only days 0 and 9. As with *E. cloacae*, the difference between the soils was established 2 h after inoculation and maintained throughout the observation period. An average of 1.4% of the IF-detected cells were viable. In the rewetted soil, increases in the total number of *A. eutrophus* cells detected by IF and in those that were viable were observed, but these numbers in the rewetted soil were several orders of magnitude lower than those in the field-moist soil.

Total CFU. A significant decrease in the numbers of total heterotrophic culturable bacteria with time was observed in the field-moist and air-dried soil (Table 2). In the rewetted soil, a significant increase in these numbers with time and compared with the numbers in the air-dried soil was observed. Although total number of CFUs increased in the rewetted soil, they did not reach the number found in the field-moist soil. At day 14, 2.6×10^7 CFU/g was detected in the rewetted soil. This was 73% of the number in the field-moist soil, but 14 times the number in the air-dried soil.

DISCUSSION

Comparison of detection methods. This study showed the presence of nonviable and viable nonculturable cells of *E. cloacae* and *A. eutrophus* in air-dried soil ($\psi = -300$ MPa), whereas in the field-moist soil ($\psi = -0.050$ MPa) the numbers of culturable, viable, and total cells were the same order of magnitude. SP and viable cell counts showed that *A. eutrophus* did not tolerate desiccation as well as *E. cloacae*. Further, hybridization, like IF, showed in air-dried soil the presence of cells that were not detected by SP.

The finding that more than 70% of the *A. eutrophus* cells were lost during flocculation is an indication that *A. eutrophus* is more closely associated with soil particles than is *E. cloacae* and should warrant that flocculation procedures be optimized according to not only soil type (23) but also the species of bacterium.

Although extraction problems also exist with DNA, as indicated by our recovery of culturable cells (35%) as well as the results of other studies (11, 13, 26), our results show that hybridization to DNA extracted directly from the soil bacterial fraction can give reproducible results.

Effects of water stress. It is well known that microorganisms vary in their tolerance of decreasing water potential and that gram-negative rods (such as *E. cloacae* and *A. eutrophus*) are among the least tolerant (4, 9). Most of the information on the relative tolerance of microorganisms to water stress has resulted from in vitro studies employing only osmotic potential (9, 24). Studies of varying matric potentials, however, have confirmed that respiration ceases when ψ is less than about -1.5 MPa (*Escherichia coli* [24]) to -5 MPa (fungi [28]).

TABLE 2. Effect of soil treatments and time on total CFU

Day	Log CFU/g of soil ± SEM[a]		
	Field moist	Air dried	Rewetted
0	8.06 ± 0.09 a	6.80 ± 0.19 a	ND
2	7.72 ± 0.09 b	6.93 ± 0.09 a	ND
9	7.71 ± 0.04 b	6.59 ± 0.03 ab	6.57 ± 0.02 c
10	ND	ND	6.92 ± 0.01 b
14	7.56 ± 0.01 b	6.27 ± 0.05 b	7.42 ± 0.04 a

[a] Results are the averages for three samples. Values that are underlined are not significantly different ($P > 0.05$, Duncan's multiple-range test). Within columns, values with the same letter are not significantly different ($P > 0.05$, Duncan's multiple-range test). SEM, standard error of the mean; ND, not determined.

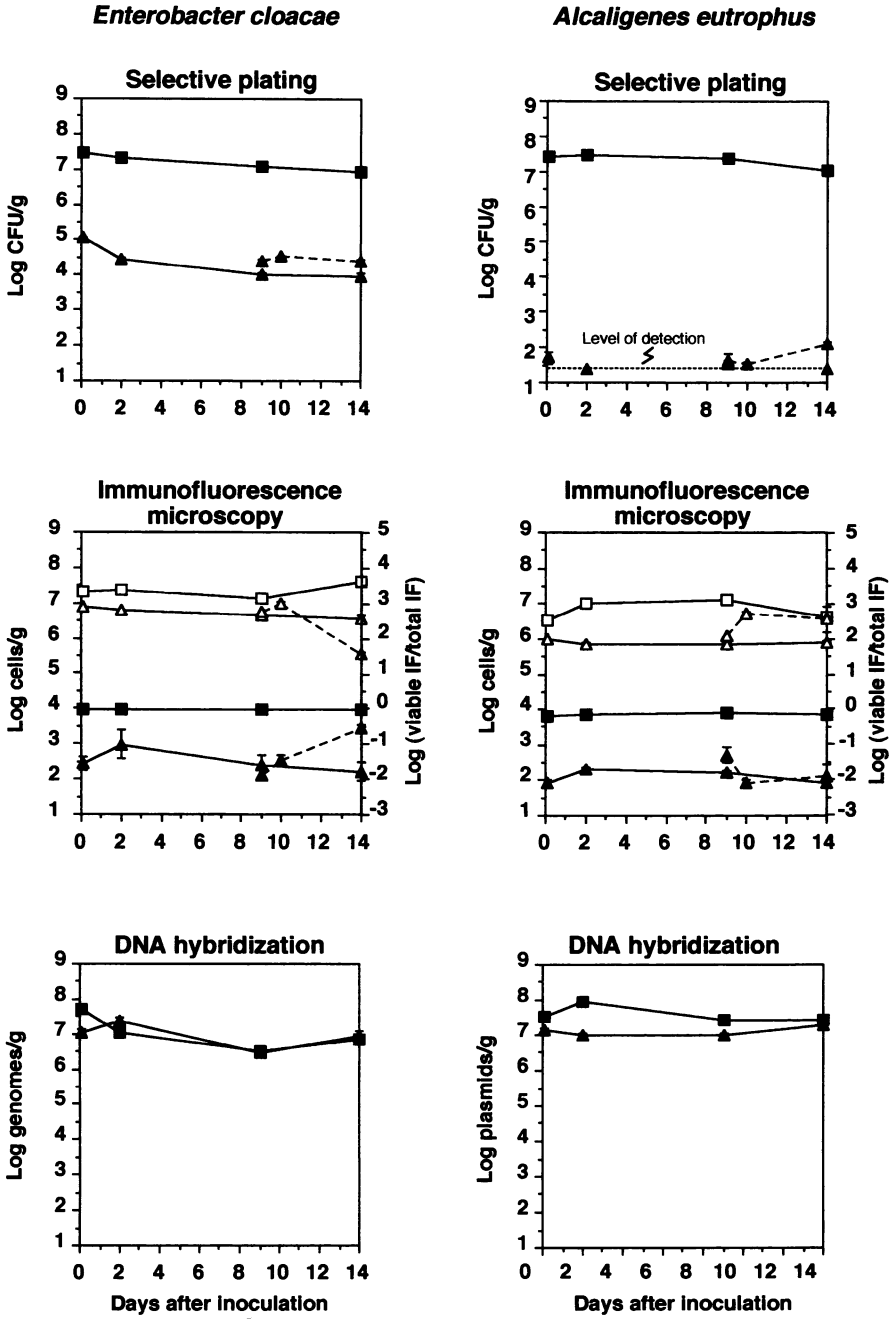

FIG. 2. Fate of *E. cloacae* JP120 and *A. eutrophus* AEO106(pRO101) in field-moist (squares), air-dried (triangles), and rewetted air-dried (dotted lines) soil detected by SP, IF after incubation with nalidixic acid (closed symbols, fraction of viable cells; open symbols, total cells), and DNA hybridization. Error bars represent the standard errors of the means (three samples).

The results of this study show that viable cells of the two gram-negative bacteria released into air-dried soil ($\psi = -300$ MPa) persisted for at least 2 weeks. A fraction of *E. cloacae* cells remained culturable even for this period, as did many of the total plate count bacteria. The presence of viable nonculturable cells of *E. cloacae* JP120 in the air-dried system is in accordance with the findings of Pedersen and Leser (21), who demonstrated the viability of nonculturable cells of this organism introduced onto bean leaves. The findings with *E. cloacae* in response to water stress are also in accordance with field observations (21) that the numbers of *E. cloacae* cells detected by IF and SP generally were similar in top soil, except during periods of extremely low water potential ($\psi < -100$ MPa).

Although another study (4) has suggested that with time gram-negative rods will lose their viability and culturability, the ability to withstand a short-term desiccation can be an important parameter governing field performance of introduced bacteria (18).

Moreover, our study showed an almost quantitative per-

sistence of cells and DNA in the air-dried soil. Only a minor fraction of these cells regained their culturability or viability when the soil was rewetted. Although it is generally believed that dead cells do not remain in the environment (1, 23), it might be that in special environments, like extremely dry soil (this study) and leaf surfaces (21), conditions exist which allow dead cells with intact cell walls and DNA to remain—at least for a period.

Rewetting of dry soil has been associated with a release of microbial biomass and a resulting respiratory burst by which about 17 to 70% of the total biomass is metabolized (15, 16), a possible fate for our nonculturable cells. When 1-month-stored air-dried soil was rewetted, Lund and Goksøyr (16) observed, besides the respiratory burst, an accompanying increase in plate counts from 1.6×10^6 to 4×10^8 CFU/g within 4 days. In this study, rewetting led to a smaller increase (from 4.0×10^6 to 2.6×10^7 CFU/g).

Conclusions. Plate counts and viable direct counts showed that *A. eutrophus* did not tolerate desiccation as well as *E. cloacae*. Although the different detection methods gave approximately the same results for the field-moist soil, paramount differences were observed in the air-dried soil. Here, a fraction of viable but nonculturable cells of *E. cloacae* JP120 and *A. eutrophus* AEO106(pRO101) was demonstrated. Most cells of both species, however, were found as intact DNA-carrying cells that were neither culturable nor viable by the methods employed in this study. Whether these cells can regain their culturability by other methods or they are dead remains to be shown.

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