JOURNAL OF BACTERIOLOGY, July 1968, p. 39-42
Vol. 96, No. 1

Isolation of *Acinetobacter* from Soil and Water

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Received for publication 27 March 1968

An enrichment culture procedure for isolating members of the genus *Acinetobacter* from soil and water is described. It involves the use of vigorously aerated mineral media at relative low *p*H, supplemented with acetate or other suitable carbon source and nitrate as nitrogen source. With this method, virtually all samples of soil and water yielded representatives of this genus. Semiquantitative comparisons of the numbers of *Acinetobacter* and of all bacteria capable of aerobic growth in a complex medium revealed that *Acinetobacter* constituted no less than 0.001% of the total heterotrophic aerobic population in soil and water and was one of the predominant organisms in some water samples.

In previous papers, the properties and taxonomy of the oxidase-positive and oxidase-negative moraxellas were discussed (1, 2). My interest in these bacteria was originally aroused by the frequent predominance of the oxidase-negative members of the group, which have been assigned to the genus *Acinetobacter* (2), in many enrichment cultures that were originally designed for isolating pseudomonads from water and soil. The principal difference between the enrichments used in our laboratory and those usually carried out for the aerobic pseudomonads was the vigorous aeration of the medium. In this paper, the chemical and physical factors leading to the emergence of *Acinetobacter* in enrichment media are analyzed, and the ubiquitous occurrence of these bacteria in water and soil is demonstrated.

Beijerinck (3) was the first to observe the predominance of an organism which he named *Micrococcus calco-aceticus* in mineral media containing either calcium acetate or quinate and incubated aerobically. Den Dooren de Jong (6) obtained a similar organism, at least as one of the members of what he called *Pseudomonas* group B, in an enrichment with caprylate. During a search for bacteria that could grow with 2,3-butylene glycol as a carbon source and with $NaNO_3$ as a nitrogen source, Lemoigne, Girard, and Jacobelli (9), using the silica gel plate method of Winogradsky, found that 18 of 25 soil samples yielded a bacterium which they called *Neisseria winogradskyi*; this bacterium has been shown to be identical with or closely related to Beijerinck's organism (2). Using aerated liquid enrichment media containing various straight chain hydrocarbons and inoculated with soil, Finnerty, Hawtrey, and Kallio (7) were able to isolate a similar organism, which they named *Micrococcus cerificans*. All of the above bacteria can probably be regarded as members of a single species, *Acinetobacter calco-aceticus* (2).

Before the isolation of a large number of strains from soil and water in our laboratory, most of the available strains of *Acinetobacter* had been isolated from clinical specimens (eyes, ears, nasopharynx, abscesses, blood, wounds, genital samples, stools, urine, etc.), and had been assigned a variety of specific names (2). In recent times, and with increasing frequency, these bacteria have been implicated in cases of postoperative meningitis, respiratory tract infections, endocarditis, and septicemia, particularly after extensive antibiotic treatment (4, 8).

MATERIALS AND METHODS

All liquid enrichment cultures were started in 125-ml Erlenmeyer flasks containing 20 ml of media having the following composition: 0.2% (w/v or v/v) of the carbon source; 0.1% $(NH_4)_2SO_4$, or 0.2% KNO_3, or 0.1% KNO_2 as the source of nitrogen; 0.02 M or 0.04 M KH_2PO_4-Na_2HPO_4 buffer at the desired *p*H; and 20 ml of Hutner's mineral base per liter (5); 0.02% $MgSO_4 \cdot 7H_2O$ was added to media containing nitrite or nitrate in place of $(NH_4)_2SO_4$ in order to supplement the sulfur content. The inoculum consisted of a 5-ml sample of water or of soil suspension per flask. When soil was used, a 10% (w/v) soil suspension was made and shaken for 15 min on a rotary shaker. The suspension was then filtered through cotton and centrifuged very lightly to remove the larger particles. The cultures were examined microscopically after 24 and 48 hr. Acinetobacters were tentatively recognized by their morphology. Any small motile rods were arbitrarily designated as "pseudomonads." Each culture was streaked on either

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mineral-acetate or yeast extract agar (2), and acinetobacters were identified on the basis of their characteristic morphological and physiological properties.

RESULTS

Certain compounds were selected as potential carbon sources for the enrichment and isolation of acinetobacters. These compounds were chosen either because of previous experience in our own and other laboratories or because they were found to be readily utilized by many strains that had been studied (2). A series of flasks containing 10 different carbon sources were inoculated with equal portions of a given water or soil sample. Four different water samples and four soil samples were used to inoculate eight such series of flasks. The cultures were incubated on a rotary shaker at 30 C. The number of enrichments with each carbon source from which acinetobacters were isolated is shown in Table 1. In further studies, it was found that media with pyruvate, caprylate, quinate, and dimethyl malonate also enriched these bacteria, but in less than 25% of the cultures.

It was noted that vigorous aeration favored the emergence of *Acinetobacter* in enrichment cultures. To test this, two series of flasks containing acetate, ethyl alcohol, succinate, L-arginine, DL-lactate, benzoylalanine, and 2,3-butylene glycol as sole carbon sources were inoculated with equal portions of the same water sample. One series was placed on a shaker and the other was left stationary. All cultures were incubated at 30 C. This was repeated with three other water samples and four different soil samples. The results are shown in Table 2 (experiment 1). It is clear that aeration does favor the development of *Acinetobacter*.

TABLE 1. *Efficacy of different carbon and energy sources for the isolation of Acinetobacter calco-aceticus in enrichment cultures*[a]

Carbon source	No. of enrichment cultures from which *A. calco-aceticus* was isolated
Acetate	8
Adipate	7
Ethyl alcohol	6
Succinate	6
2,3-Butylene glycol	6
n-Tetradecane	6
L-Arginine	5
Benzoylalanine	5
DL-Lactate	3
D-Xylose	3
Butyrate	3
n-Tridecane	2
m-Hydroxybenzoate	2
n-Butyl alcohol	1
Benzoate	0
L-Tryptophan	0

[a] The medium contained 0.04 M phosphate buffer, *p*H 6.0; 0.2% KNO_3; 0.02% $MgSO_4 \cdot 7H_2O$; and Hutner's base. The medium was aerated and was constantly agitated at 30 C. Eight enrichment cultures were started with each carbon source.

An analogous experiment was performed with aerated cultures in which the *p*H and the nitrogen source were varied. Four soil and four water samples were used to inoculate each medium. The carbon sources used were: acetate, butyrate, ethyl alcohol, and *n*-butyl alcohol. The medium contained 0.04 M phosphate, adjusted to *p*H 5.5, 6.0, 6.8, and 8.0, and $(NH_4)_2SO_4$ as the nitrogen and sulfur source. In addition, one series of flasks at *p*H 5.5 contained KNO_3 and $MgSO_4 \cdot 7H_2O$ instead of $(NH_4)_2SO_4$. All cultures were incubated on a shaker at 30 C. Four soil and four water samples were used as inocula for each medium. The results are summarized in Table 2 (experiment 2). It can be seen that lowering the *p*H selectively favors the emergence of *Acinetobacter*. This was noted in enrichments with all the substrates used. At *p*H 5.5, KNO_3 is more selective than is $(NH_4)_2SO_4$ in favoring the predominance of these organisms. In a different experiment, in which $(NH_4)_2SO_4$, KNO_3, and KNO_2 were compared as nitrogen sources, at *p*H 6.8, with acetate, DL-lactate, and D-xylose as carbon sources, no selective advantage could be shown for either nitrate or nitrite over ammonium ion.

Acinetobacters could be isolated by enrichment almost as readily at 40 as at 30 C. In media containing $(NH_4)_2SO_4$ at *p*H 6.8 and with acetate,

TABLE 2. *Effect of environmental conditions on the emergence of Acinetobacter in enrichment cultures*[a]

Expt	Conditions of enrichment	Percentage Enrichment cultures	
		Acinetobacter could be isolated	*Acinetobacter* was predominant
1	*p*H 6.0, agitated	80	32
	*p*H 6.0, not agitated	29	2
2	*p*H 5.5, agitated	94	63
	*p*H 6.0, agitated	85	47
	*p*H 6.8, agitated	50	22
	*p*H 8.0, agitated	31	9
	*p*H 5.5, agitated, KNO_3	100	87

[a] Different carbon sources were used as described in the text. The nitrogen source was NH_4Cl, except where KNO_3 is indicated. Temperatures of incubation, 30 C.

DL-lactate, or D-xylose as carbon sources, 21 of 24 cultures at 30 C and 19 of 24 cultures at 40 C yielded these bacteria.

On the basis of the above observations, a method was designed for the successful enrichment and isolation of acinetobacters from nature, and this method was used in an attempt to enumerate these organisms and to determine their distribution in samples of soil and water. The medium consisted of 0.2% sodium acetate (trihydrate), 0.2% KNO_3, and 0.02% $MgSO_4 \cdot 7H_2O$ in 0.04 M KH_2PO_4-Na_2HPO_4 buffer (*p*H 6.0) containing 20 ml per liter of Hutner's mineral base (5). A 20-ml amount of the sterilized medium and 5 ml of inoculum were incubated in 125-ml Erlenmeyer flasks at 30 C on a rotary shaker for 48 hr.

In an attempt to determine the distribution of *Acinetobacter* in nature, 30 different water samples from ponds, lakes, creeks, and rivers and 30 different soil samples were used as inocula. The samples were collected from an area approximately 150 miles long and 60 miles wide along the coast of central California. Twenty-eight of the 30 soil samples and 29 of the 30 water samples contained the organism, as evidenced by its presence in enrichment cultures.

Eight samples of water and eight soil suspensions were used to inoculate two series of flasks; one series contained the mineral acetate medium and the other contained yeast extract broth (2). One flask of each series was inoculated with 10 ml of the sample, the second with 1 ml, and the remainder with 1 ml each of successive decimal dilutions prepared in sterile water. The soil suspensions were prepared as previously described. In Table 3, the approximate numbers of acinetobacters present in the soil and water samples are compared with the corresponding numbers of all bacteria growing in yeast extract broth. It can be seen that, of the total bacterial population capable of aerobic growth in the complex medium, *Acinetobacter* represented at least 0.001% in all samples tested and was one of the predominant bacteria in two of the water samples.

TABLE 3. *Relative abundance in water and soil samples of Acinetobacter calco-aceticus and of all bacteria capable of aerobic growth in yeast extract*

Sample	Source	Smallest wt or vol of each sample yielding	
		A. calco-aceticus in acetate enrichment medium	Bacterial growth in yeast extract broth
1	Soils	10^{-1} g	10^{-4} g
2	Soils	10^{-1} g	10^{-5} g
3	Soils	10^{-1} g	10^{-5} g
4	Soils	10^{-2} g	10^{-6} g
5	Soils	10^{-2} g	10^{-6} g
6	Soils	10^{-3} g	10^{-6} g
7	Soils	10^{-3} g	10^{-6} g
8	Soils	10^{-3} g	10^{-6} g
9	Creeks	10 ml	10^{-3} ml
10	Creeks	10 ml	10^{-4} ml
11	Creeks	10^{-2} ml	10^{-2} ml
12	Creeks	10^{-2} ml	10^{-3} ml
13	Ponds	10 ml	10^{-1} ml
14	Ponds	10 ml	10^{-1} ml
15	Ponds	10 ml	10^{-2} ml
16	Ponds	1 ml	10^{-3} ml

DISCUSSION

The nutritional properties of *Acinetobacter* (2) and their ubiquitous occurrence in soil and water suggest that these organisms may be very important agents in the aerobic mineralization of organic matter in nature. A striking property of these bacteria is their ability to grow at the expense of a great variety of organic compounds as sole sources of carbon and of nitrate as sole source of nitrogen. Another property is their relatively high rate of growth at 30 C. In well-aerated mineral acetate media, generation times of 40 to 55 min were observed with several strains. With respect to their nutritional properties, the *Acinetobacter* species resemble many species of the aerobic pseudomonads (11). As has been shown, they often emerge as the predominant organisms in vigorously aerated enrichment media. The same media exposed to air without agitation commonly yield pseudomonads as the predominant organisms. This is probably due to the fact that the pseudomonads are motile and rise to the surface as a result of aerotactic response. Growth of the pseudomonads at the surface diminishes the oxygen supply for nonmotile organisms and arrests their development. Under conditions of homogenous aeration, *Acinetobacter* overgrows the pseudomonads either because of its greater growth rate or because of its occurrence in greater numbers in water and soil. Of further ecological interest is the selective advantage of low *p*H. It appears that under natural acidic conditions acinetobacters may usurp the role of the pseudomonads in the decomposition of simple carbon compounds.

An attempt was made to correlate the physiological properties of the various strains of *Acinetobacter* that had been characterized in previous studies (2) with their source of isolation (i.e., from soil and water or from clinical specimens). Bacteria isolated from soil and water could be

allocated to both of the major groups and to six of the seven subgroups established on the basis of numerical analysis (2). A majority (71%) of these strains fell into group A, for which the specific name *A. calco-aceticus* has been proposed. Of the strains that had been obtained from clinical sources, on the other hand, a slight majority (56%) fell into group B. None of the 6 strains of subgroup B3, which had been designated as *A. hemolysans*, was clearly a soil or water inhabitant, but 3 of the 10 strains of subgroup B4, for which the designation *A. hemolysans* subspecies *haemolyticus* was proposed, were isolated from soil. Of all the water and soil bacteria analyzed, only these three strains gave a positive gelatinase reaction.

Nine soil and water strains selected at random from groups A and B were analyzed for the guanine plus cytosine (GC) content of their deoxyribonucleic acid by Manley Mandel. The GC content of these strains varied from ca. 39 to 43 moles %. The highest GC value was found in strain 21 belonging to phenotypic subgroup B2. This subgroup contains strain 19, which is believed to represent typical *A. lwoffi*, and related strains of clinical origin that have the highest GC contents (44 to 47 moles %) recorded in the entire genus (*personal communications* from Manley Mandel and John Johnson).

Although the above observations suggested that particular species or biotypes of *Acinetobacter* are more likely to be associated with the human or animal body than others, it is quite possible that with a larger sampling or with the use of appropriate enrichment procedures the occurrence of these species or biotypes could be demonstrated in soil and water.

In an experiment designed to test for marked differences in maximal temperature for growth, 15 strains of clinical origin and 15 isolates from soil and water were tested for growth at 37 and 41 C in yeast extract broth. Of the 15 clinical strains, 11 grew at 37 C and, of these, 9 grew at 41 C. Of the 15 soil and water strains, 9 grew at 37 C and, of these, 2 grew at 41 C. The ability of a greater number of the clinical strains to grow at 41 C may simply reflect the fact that bacteria capable of growing at higher temperatures had already been selected either in the animal environment or by the method used for their initial isolation. The four strains of clinical origin that did not grow at 37 C presumably had been originally isolated at this temperature. Their inability to grow at 37 C was either due to the use of a different medium or to a loss of tolerance to this temperature during prolonged cultivation at 30 C.

No record could be found of any member of the oxidase-positive moraxellas [genus *Moraxella* (1)] having been isolated from soil or water. These organisms appear to be adapted to an existence as animal commensals or parasites, especially on mucous membranes of the eyes, pharynx, and respiratory tract (10). Thus, the oxidase-negative members of this group appear to be ecologically distinct from the oxidase-positive organisms, being primarily inhabitants of soil and water and probably only casual inhabitants of animal tissues. This ecological distinction is supported by the nutritional versatility of the oxidase-negative organisms, a property not shared by the oxidase-positive species.

ACKNOWLEDGMENTS

I am indebted to M. Doudoroff and R. Y. Stanier, in whose laboratories this work was initiated and carried out. I also thank N. J. Palleroni and L. N. Ornston for their suggestions and help and Sharron Gentry for technical assistance.

This investigation was supported by Public Health Service grant AI-1808 from the National Institute of Allergy and Infectious Diseases, National Science Foundation grant GB-4112, and by Public Health Service training grant AI-120-07.

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