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Survival of *Agrobacterium radiobacter* K84 on Various Carriers for Crown Gall Control

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Screening was performed on nine carriers to find an improved formulation for *Agrobacterium radiobacter* K84 cells. The survival data showed that it is possible to preserve *A. radiobacter* cells on dry solid supports for a long time provided that the storage temperature is 4°C and that the inoculation volume for 4×10^9 CFU g^{-1} is not less than 0.15 ml g of carrier^{-1}. On the other hand, a substantial carrier water content was necessary for room temperature storage. Many materials proved to be suitable as microbial carriers; in some cases, vermiculite allowed long storage times comparable to those reported for peat or carboxymethyl cellulose, which are already employed in some commercial *A. radiobacter* K84 products. Furthermore, vermiculite assured full and immediate biological activity in the prevention of crown gall, showing that it is suitable for a new formulation of strain K84. A hypothesis to explain the different survival abilities in wet and dry conditions is presented.

Crown gall, which is caused by the soilborne bacterium *Agrobacterium tumefaciens*, is a serious disease affecting dicotyledonous plants in many parts of the world (18). Among the economically important hosts are members of the family Rosaceae (almond, apple, stone fruits, raspberry, blackberry, and rose), chrysanthemums, grape vines, pecan trees, walnut trees, and willows (4). The pathogen infects through wounds frequently inflicted during transplantation and other agricultural operations. So far, chemical control of the disease has been mostly ineffective (7, 18). On the other hand, the biological control of crown gall by the bacterium *Agrobacterium radiobacter* K84 is effective and economic, and the strain is commercialized and used in agriculture. As a matter of fact, the use of strain K84 is considered an outstanding example of effective microbial biological control (17). Several reviews (8, 9, 11, 12) describe the intensive work done by many groups on the strain isolated by New and Kerr (13).

Strain K84 is commercially supplied as a culture in agar plates (Galltroll; AgBioChem, Inc., Orinda, Calif.), in a formulation containing carboxymethyl cellulose (CMC) (Norbac 84-C; Nortell Laboratories, Inc., Corvallis, Oreg.), and in a finely ground peat preparation similar to *Rhizobium* inoculum (several companies) (11). Furthermore, a method of producing freeze-dried inocula has been proposed (3). To be successful, a microbial product, besides being effective, should have the requisites normally found in the traditional synthetic products: commercially acceptable storage time (at least 1 year) at room temperature, simple use, and low production costs.

The agar plates present many drawbacks: short storage time at low temperatures (maximum of 120 days at 4°C), the need to manipulate an aesthetically unpleasant bacterial mass, and a laborious production process with high worker costs.

The products based on CMC are reported to offer good viability at low temperatures (a drop in viable count of a factor of <10 in 11 months at 4°C) (11). However, it seems that K84 cells stored in CMC, although viable, are retarded physiologically because colonies take about 5 days to appear on nutrient agar (11). Good results have been obtained with peat; K84 can be stored in peat for at least 4 months at room temperature before its titer is reduced 10-fold (21). However, peat, being an organic material extracted from natural deposits, shows great variability. Samples coming from different deposits, as well as batches from the same source, affect microbial survival differently. Furthermore, peat presents sterilization problems because it produces toxic substances when treated with heat, gamma radiation, or gaseous chemical agents (6, 20).

With the purpose of finding a suitable carrier for *A. radiobacter* with improved features (acceptable storage time at room temperature and substrates of constant quality that are easy to sterilize), we determined whether preserving cells on dry solid supports was possible. Usually, dry formulations are used for dormant microbes, namely, spore-forming gram-positive bacteria (14). However, the use of dehydrating carriers, such as anhydrous silica gel, is successfully applied for the preservation of filamentous fungi—not only conidia (dormant cells) but also mycelia, i.e., fragile, vegetative cells (15). We therefore wanted to test whether *A. radiobacter* cultures dehydrated without a freezing step (as in lyophilization) on dry supports could be successfully preserved. For this purpose, nine materials were screened to select those with the best qualities.

MATERIALS AND METHODS

Bacterial strain. *A. radiobacter* K84 (NCPPB 2407) and a pathogenic *A. tumefaciens* strain (NCPPB 1651) were supplied by the National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom. The pathogenicity of *A. tumefaciens* was assessed by tests on carrot discs (1). The sensitivity of the *A. tumefaciens* strain to agrocin 84 was verified by the Stonier method (19).

Media. The media used for growing *A. radiobacter* K84 were nutrient broth medium and YDPC medium. YDPC medium contained the following ingredients (per liter): yeast extract, 4 g; peptone, 4 g; $(NH_4)_2SO_4$, 5 g; $CaCO_3$, 10 g; and glucose, 20 g. The final pH was 7. Glucose was added as a separately sterilized solution. Nutrient broth medium, pep-

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tone, and yeast extract were from Difco (Detroit, Mich.); all of the other reagents were from Farmitalia Carlo Erba (Milan, Italy).

Growth conditions. The strain was precultured in nutrient broth medium for 1 day at 24°C. Next, a 25-ml aliquot of this culture was transferred into 800 ml of YDPC medium in a 2-liter Erlenmeyer flask and cultured on a rotary shaker at 250 rpm for 2 days at 24°C. The culture was harvested by centrifugation and resuspended with protective solution.

Carrier materials. Nine materials were screened as carriers for *A. radiobacter*: kaolin (Caolino Summano; Posina, Vicenza, Italy); silica gel (Carlo Erba); vermiculite (expanded type) and agriperlite (Vic Italiana, Milan, Italy); expanded clay (Ares 4; Trino, Vercelli, Italy); and four types of diatomaceous products, Celite and Micro-cel (Johns-Manville Products Co., Lompoc, Calif.), Diatom (Europe Sud S.p.A., Milan-Corsico, Italy), and Porosil MP (Ceca, Milan, Italy). The materials were washed (except for the powders [kaolin, Celite, Porosil MP, Micro-cel, and Diatom]) with distilled water and dried by heating at 80°C; the weight variation was monitored with a precision balance. The materials were considered dry when no further weight loss was detectable. The carriers (3 g) were dispensed into screw-cap borosilicate glass tubes (16 by 150 mm; Carlo Erba). The tubes were then autoclaved at 121°C for 1 h and dried by heating at 150°C until their weights were constant. In a second experiment, the carriers were sterilized by autoclaving twice at 121°C for 1 h, with an interval of 24 h after the first treatment, dried by heating at 80°C until a constant weight was reached, and aseptically dispensed (10 g) into polyethylene bags (bag size, 100 by 150 mm; Carlo Erba), which are commercially more suitable containers than glass tubes.

The carrier water content was determined as the difference between the weight of wet material and the weight of dry material.

Preparation of inoculant. A 5% (wt/vol) sucrose solution in sterile skim milk was used as a protective solution. Each glass tube was inoculated with 0.5 ml of the bacterial suspension, shaken thoroughly to mix its contents, and stored at 4°C or 21°C. The final moisture content of each tube was about 14% (wt/wt). In a second experiment, the culture was resuspended with a solution of 10% (wt/vol) skim milk powder (Mag-ist; Gespal, Milan, Italy) and 7% (wt/vol) sucrose. Two bacterial suspensions were prepared, with one three times more concentrated than the other. The carriers, contained in plastic bags, were inoculated either with 0.5-ml aliquots of the concentrated suspension (moisture, 4.7%) or with 1.5-ml aliquots of the diluted suspension (moisture, 13%). The bags were then heat sealed and stored at 4 or 24°C.

Viable-cell counts. To determine the survival rate of the bacteria, 20 ml of normal saline (0.88% [wt/vol] NaCl) was added to each sample. The suspension was shaken for 2 min with a wrist-action shaker, and then serial dilutions were spread on nutrient agar plates. The plates were incubated at 24°C and scored after 2 to 3 days. Colonies appearing in less than 2 days were not agrobacteria and thus were noted as contaminants. The number of CFU in the suspension was expressed as CFU per gram of carrier.

Biological effectiveness of inoculants. The inoculants were tested for effectiveness in the prevention of gall formation on 3-week-old tomato plants (*Lycopersicum esculentum* Mill. cv. S. Pierre). The method was similar to that described by Du Plessis et al. (5). The inoculum of the pathogen was prepared by washing cells from nutrient agar plates with normal saline, counting the cells, and diluting the suspension to the desired concentration (about 4×10^8 cells ml^{-1}). An *A. radiobacter* suspension was drawn from the carrier as described above for viable counts. The two suspensions were mixed in different ratios, and 25-μl aliquots of these mixtures were injected into stems at the first internode with a sterile 1-ml syringe. At least five plants were used for each determination. Tumor production was scored 4 weeks after inoculation. The gall size was determined by subtracting the stem diameter of a healthy plant from the stem diameter of a plant with a gall.

Statistical analysis. To compare the different formulations, degradation rates (regression coefficients) were calculated by regression analysis using the simple regression procedure of Statgraphics software (STSC, Inc., Rockville, Md.), with the base 10 logarithm of the cell concentration (in CFU per gram) as the dependent variable and the time (in days) as the independent variable.

The 95% confidence limits were calculated by multiplying the regression coefficient's standard error by Student's t value. R^2 (coefficient of determination) measures the contribution of the linear function of the independent variables to the variation in the dependent variable.

RESULTS

A. radiobacter K84 cells were grown with nine different carriers. The data for silica gel are not reported because this material was very toxic to *A. radiobacter* K84. The survival of the bacteria was monitored by determining the number of CFU per gram of carrier at different times after inoculation. Two independent samples were assayed for each determination. Some samples (10 of 208) were lightly contaminated and thus were discarded. The contaminated samples were found more frequently at 21°C (7 of 96 samples) than at 4°C (3 of 112) and more frequently in granular materials, such as agriperlite (3 of 26), expanded clay (2 of 26), and vermiculite (2 of 26), than in powdered carriers (0 or 1 of 26 samples for each material). This could be a result of a better heat exchange in the powdered carriers. However, the powdered carriers were difficult to wash because of the formation of thick suspensions. The results are shown in Table 1 as the base 10 logarithm of the mean number of CFU per gram. The regression coefficients with the 95% probability confidence limits, the R^2 values, and the maximum storage times for the carriers stored at 21 and 4°C are shown in Tables 2 and 3, respectively. The maximum storage time is the number of days needed to have a drop in the viable-cell count of a factor of 100 and is calculated as $-\log 100 \times$ regression coefficient^{-1}. The viability decrease in the storage time definition (100 times) is not an arbitrary value, but it delimits the minimum acceptable titer, considering that (i) the initial cell concentration in our formulations is about 4×10^9 cells g^{-1}, (ii) the final cell concentration in the solution at the moment of inoculation of plants has to be at least 10^6 cells ml^{-1} (13), and (iii) the treatment solution is obtained by diluting a gram of bacterial formulation in 30 to 40 ml of water. Therefore, the storage time is a real expiration time that represents the threshold after which the vital cell concentration is too low for effective K84 protection.

The regression coefficients at 4°C (Table 3) were lower for each carrier than the corresponding values at 21°C (Table 2), indicating better survival at a low temperature, regardless of the carrier used in the formulation.

At 21°C, the differences between most carriers were small; Porosil MP, expanded clay, and kaolin showed the best

TABLE 1. Survival of *A. radiobacter* K84 in different carriers at 4 and 21°C

Carrier	Temp (°C)	Survival (log_{10} mean viable titer [CFU g^{-1}]) at indicated time (days)							
		0	1	45	80	175	244	314	393
Agriperlite	21	9.59	9.78	7.68	6.96	4.46[a]	1.78[a]	ND[b]	ND
Expanded clay	21	9.15	9.23	7.40[a]	6.81[a]	6.66	4.39	ND	ND
Kaolin	21	8.72	8.11	6.56	5.48	3.89	3.74	ND	ND
Celite	21	9.30	9.31	8.79[a]	6.79	3.89	3.40	ND	ND
Diatom	21	9.16	8.19	6.77	6.80	3.05	3.23	ND	ND
Porosil MP	21	9.29	9.56	9.10	7.22	5.95	6.30	ND	ND
Micro-cel	21	9.54	8.46	3.84	1.73	1.73[a]	1.73	ND	ND
Vermiculite	21	9.33	9.33	8.81	7.97	6.11[a]	1.52	ND	ND
Agriperlite	4	9.59	9.78	9.80	9.68	8.21	ND	6.20[a]	6.59
Expanded clay	4	9.15	9.23	8.89	9.20	8.05	ND	7.08	4.65
Kaolin	4	8.72	8.11	7.77	7.64	5.55	ND	5.41	3.91
Celite	4	9.30	9.31	9.24	8.91	8.38	ND	7.29	6.72
Diatom	4	9.16	8.19	8.07	7.47	7.24	ND	6.25	4.91
Porosil MP	4	9.29	9.56	9.64	9.26[a]	8.78	ND	7.42	6.43
Micro-cel	4	9.54	8.46	8.46	8.11	5.16	ND	1.73	2.13
Vermiculite	4	9.33	9.33	9.46[a]	9.37	9.35	ND	8.71	7.70

[a] Single sample.
[b] ND, not determined.

results, with storage times of greater than 3 months (Table 2). At 4°C, vermiculite provided the longest storage time (563 days) and Micro-cel provided the shortest (99 days) (Table 3).

Good carriers at 4°C, such as vermiculite and Celite, were poor substrates at 21°C; likewise, carriers with good viability at 21°C, such as Porosil MP and expanded clay, did not excel at 4°C. This low correlation suggests that accelerated stability methods are not easily applicable in screening experiments. In those procedures, degradation rates at a number of elevated temperatures are plotted according to the Arrhenius equation, and the resulting lines are extrapolated to give estimated values at lower temperatures (2).

The biological effectiveness of K84 cells stored in vermiculite at 4°C for 314 days was tested with tomato plants coinoculated with *A. radiobacter* suspensions (obtained from vermiculite samples) and fresh pathogen cultures. The gall size (mean ± standard deviation) was 8.02 ± 0.93 and 7.10 ± 0.48 mm when the plants were inoculated with 1.7×10^8 and 1.7×10^7 cells of *A. tumefaciens* ml^{-1}. On the other hand, gall formation was completely prevented when pathogen-*A. radiobacter* mixtures were inoculated not only at a 1:10 ratio but also at a 1:1 ratio, the minimum reported to be effective (13).

We wanted to confirm the results of the first experiment by retesting Porosil MP and expanded clay (the two best carriers at 21°C) and vermiculite (the best carrier at 4°C) in a second experiment. The higher storage temperature in this case was, for practical reasons, 24°C. In this experiment no sample was contaminated, probably as a consequence of the different sterilization procedure (see Materials and Methods).

To simplify a possibly large-scale production process, we determined whether it was possible to decrease the inoculum volume while keeping the total number of bacteria constant. Some samples (denominated H for high inoculation volume) were inoculated with 1.5 ml of bacterial suspension (inoculum/carrier ratio, = 0.15 ml g^{-1}, approximately equal to that in the first experiment) and others (L for low inoculation volume) were inoculated with 0.5 ml (inoculum/carrier ratio, = 0.05 ml g^{-1}) of a threefold-more-concentrated suspension, so as to maintain a constant total number of bacteria introduced into each sample.

We also wanted to test the effect of the carrier water content on *A. radiobacter* survival. Bacteria were inoculated as described above into dry and wet (water content = 75% [wt/wt]) vermiculite samples.

The survival data are reported in Table 4 as the base 10 logarithm of the number of CFU per gram. In Table 5 and

TABLE 2. Regression analysis of survival data for strain K84 stored at 21°C on different carriers

Carrier	Regression coefficient[a] ± C.L., 10^{-2}	R^2	Maximum storage time (days)
Micro-cel	−9.479 ± 5.223 A	96.8	21
Agriperlite	−3.115 ± 0.425 B	99.0	64
Vermiculite	−2.963 ± 1.145 BC	92.8	67
Celite	−2.667 ± 0.783 BC	95.7	75
Diatom	−2.445 ± 1.049 BC	91.3	81
Kaolin	−1.997 ± 0.965 BC	89.2	100
Expanded clay	−1.711 ± 0.862 C	88.4	116
Porosil MP	−1.491 ± 0.893 C	84.3	133

[a] Values, shown with the 95% confidence limits (C.L.), followed by the same letter do not differ significantly.

TABLE 3. Regression analysis of survival data for strain K84 stored at 4°C on different carriers

Carrier	Regression coefficient[a] ± C.L., 10^{-2}	R^2	Maximum storage time (days)
Micro-cel	−2.011 ± 0.535 A	94.9	99
Kaolin	−1.094 ± 0.336 B	93.3	182
Expanded clay	−1.017 ± 0.414 BC	88.8	196
Agriperlite	−0.966 ± 0.329 BC	91.9	207
Diatom	−0.856 ± 0.287 BCD	92.1	233
Porosil MP	−0.758 ± 0.223 BCD	93.8	263
Celite	−0.669 ± 0.075 C	99.0	299
Vermiculite	−0.355 ± 0.227 D	76.2	563

[a] Values, shown with the 95% confidence limits (C.L.), followed by the same letter do not differ significantly.

TABLE 4. Survival of *A. radiobacter* K84 in different carriers at 4 and 24°C

Carrier	Temp (°C)	Inoculum[a]	Survival (log_{10}) viable titer [CFU g^{-1}]) at indicated time (days)				
			0	4	56	120	200
Expanded clay	24	H	9.68	9.53	7.34	4.00	2.34
Expanded clay	24	L	9.81	9.70	5.60	5.28	2.88
Porosil MP	24	H	9.68	9.45	7.00	2.00	1.00
Porosil MP	24	L	9.81	9.48	4.00	1.30	1.00
Vermiculite (wet)	24	H	9.68	9.60	8.51	8.30	8.04
Vermiculite (wet)	24	L	9.81	9.82	8.20	8.08	7.83
Vermiculite (dry)	24	H	9.12	8.58	8.08	2.70	1.00
Vermiculite (dry)	24	L	9.24	8.75	7.15	2.70	1.00
Expanded clay	4	H	9.68	9.53	9.53	9.53	9.34
Expanded clay	4	L	9.81	9.70	9.60	8.53	6.60
Porosil MP	4	H	9.68	9.45	9.00	8.86	9.11
Porosil MP	4	L	9.81	9.48	7.88	5.89	4.38
Vermiculite (wet)	4	H	9.68	9.60	9.66	9.45	9.41
Vermiculite (wet)	4	L	9.81	9.82	9.76	9.08	8.64
Vermiculite (dry)	4	H	9.12	8.58	8.66	8.83	8.67
Vermiculite (dry)	4	L	9.24	8.75	8.67	8.67	8.54

[a] H, high inoculation volume; L, low inoculation volume.

Table 6, the regression data for the carriers stored at 24 and 4°C, respectively, are shown. Some carriers show a low R^2 because the datum points are scattered near the initial titer. The regression curve has a very low slope (roughly parallel to the x axis), and therefore the y values are poorly determined by a linear function with the x values.

The data confirm the properties of the carriers under study that, when inoculated with the appropriate inoculation volume (H samples) and stored at 4°C (Table 6), show even better results than those of the first experiment (Table 3). The decay rates at 24°C (Table 5) were higher than those at 21°C (Table 2), probably because of the higher storage temperature used in the second experiment.

The results also indicate that a threefold reduction of the inoculum volume, when the total number of vital bacteria was kept constant, did not cause any significant difference in the degradation rate at 24°C (Table 5). However, inoculation with a smaller volume detrimentally affected survival in all carriers when they were stored at 4°C (differences between H and L samples were significant except for dry vermiculite). Therefore, the inoculation volume is an important factor for survival at 4°C.

TABLE 5. Regression analysis of survival data for strain K84 stored at 24°C on different carriers

Carrier	Inoculum[a]	Regression coefficient[b] ± C.L., 10^{-2}	R^2	Maximum storage time (days)
Expanded clay	H	−3.845 ± 1.1536 A	97.4	52
Expanded clay	L	−3.370 ± 2.1410 AB	89.3	59
Porosil MP	H	−4.687 ± 2.2447 A	93.6	42
Porosil MP	L	−4.627 ± 3.8208 AB	83.2	43
Vermiculite (wet)	H	−0.819 ± 0.6839 B	82.9	244
Vermiculite (wet)	L	−1.012 ± 1.0455 B	76.0	197
Vermiculite (dry)	H	−4.288 ± 2.1177 A	93.3	46
Vermiculite (dry)	L	−4.298 ± 1.5441 A	96.3	46

[a] H, high inoculation volume; L, low inoculation volume.
[b] Values, shown with the 95% confidence limits (C.L.), followed by the same letter do not differ significantly.

TABLE 6. Regression analysis of survival data for strain K84 stored at 4°C on different carriers

Carrier	Inoculum[a]	Regression coefficient[b] ± C.L., 10^{-2}	R^2	Maximum storage time (days)
Expanded clay	H	−0.121 ± 0.1356 A	73.1	1,647
Expanded clay	L	−1.542 ± 0.8043 CD	92.5	130
Porosil MP	H	−0.267 ± 0.5420 ABD	45.0	753
Porosil MP	L	−2.732 ± 0.6131 C	98.5	73
Vermiculite (wet)	H	−0.130 ± 0.1173 A	80.7	1,540
Vermiculite (wet)	L	−0.617 ± 0.2655 BD	94.8	324
Vermiculite (dry)	H	−0.071 ± 0.4465 AB	7.9	2,835
Vermiculite (dry)	L	−0.227 ± 0.4140 AB	50.4	885

[a] H, high inoculation volume; L, low inoculation volume.
[b] Values, shown with the 95% confidence limits (C.L.), followed by the same letter do not differ significantly.

The data obtained in the study of carrier water content show that, at 4°C, bacteria inoculated into wet vermiculite had decay rates slightly higher than those of bacteria inoculated into dry vermiculite (Table 6). On the other hand, at 24°C the high water content remarkably and significantly improved the survival of strain K84 (Table 5).

The biological activity of a wet-vermiculite H sample stored at 24°C for 294 days was assayed with tomato plants as described above. The titer of this sample was 2.5×10^7 CFU g^{-1}. The control gall size (mean ± standard deviation) was 6.37 ± 0.73 mm (with an inoculum of 1.9×10^7 cells of *A. tumefaciens* ml^{-1}). Gall formation was completely prevented at a 1:1 *A. tumefaciens*/*A. radiobacter* ratio.

DISCUSSION

This work is part of a study which has been undertaken to find a microbial formulation suitable for the bacterium *A. radiobacter* K84, which is used in agriculture for the prevention of crown gall. The aim is to define a product of constant quality, long storage time at room temperature, simple use, and low production costs. Some of these features are realized in already commercialized products, but not together in one product, with each formulation having some drawback. In this work, we wanted in particular to determine the feasibility of preserving *A. radiobacter* cells by dehydration on dry solid supports, a method used with filamentous fungi. For this purpose, nine materials were screened for bacterial survival for at least 200 days. The toxic effect of silica gel was probably due to contamination with a few dye particles, which are not suitable for preservation purposes (15). Vermiculite was one of the best carriers for *A. radiobacter* K84 at 4°C. The vermiculite storage time is estimated to be very long, on the order of years. The storage time values are extrapolations and therefore are to be judged with great caution. Nevertheless, considering the experimental data instead of the regression estimates, we find that the viable-cell counts after 200 days are still about 40% of the initial cell concentration, i.e., 40 times higher than the expiration limit (1%). Therefore, vermiculite offers, at 4°C, viability levels comparable to those reported for CMC at the same temperature (11). Furthermore, cells maintained on vermiculite are not as physiologically retarded as those maintained on CMC (11) but show full and immediate biological activity in the prevention of crown gall. At room temperature, the bacterial

survival rate in dry carriers was generally low. However, good results, similar to those reported for peat (4 months of storage at room temperature with a 10-fold cell concentration reduction) (21), were obtained with wet vermiculite instead of dry vermiculite, with a viable-cell count reduction of 100-fold in 6 to 7 months (Table 4). Compared with peat, vermiculite has the advantage of being sterilizable without toxicity problems.

Our study of *Agrobacterium* formulation confirms the useful characteristics of vermiculite as a microbial carrier, which have already been shown for *Rhizobium* strains and other bacterial species by Graham-Weiss et al. (6). Graham-Weiss et al. used vermiculite as a support medium for the direct fermentation of bacterial cultures for the production of reliable bacterial inoculants. Vermiculite is believed to allow a high survival rate by buffering pH shifts, adsorbing suppressive metabolites, and physically sheltering organisms against harsh environmental stress (22).

The vermiculite water content has opposite effects on cell viability at high and low storage temperatures. At 4°C the degradation rates are slightly lower with dry vermiculite than with wet vermiculite, while at 24°C wet vermiculite yields better results. These findings indicate that the mechanisms of survival in the carrier in the presence and in the absence of water are probably different. Perhaps, in the presence of water, bacteria are physiologically still active and starved; under these conditions, the low temperature represents a further stress which is not well tolerated. It is known that protein synthesis during starvation is needed for survival via the development of resistant cells of *Escherichia coli*. *E. coli* cells starved for 5 h at 37°C survived much better when transferred to 4°C than cells starved only for 10 min, indicating that the immediate exposure to a low temperature impaired starvation protein synthesis (10). On the other hand, the low water availability may block or slow down the biochemical pathways, allowing a high level of bacterial survival; however, under these conditions some important cell component becomes particularly unstable and needs a low temperature to maintain its functions. Structural injury, defined as a transient and reversible susceptibility to agents that are innocuous in unstressed populations, has been observed in dried microorganisms (16). From these reports, we can say that it is probable that physiologically active *A. radiobacter* cells need to synthesize starvation proteins before exposure to a temperature of 4°C and that dried *A. radiobacter* cells have a structural injury which causes sensitivity to a temperature of 24°C.

Our data showed that the inoculation volume is an important factor for survival at 4°C but is negligible at 24°C. Decreasing the inoculation ratio from 0.15 to 0.05 ml g^{-1} while maintaining a constant total number of bacteria resulted in a dramatic decrease in cell viability at 4°C. In this experiment, by lowering the inoculum volume, we changed two variables, namely, the amount of milk-sucrose solution introduced into each sample and the bacterial concentration in the inoculum suspension. Therefore, the decrease in viability could be due to a detrimental effect of a too-high cell concentration or to a smaller amount of protective solution. Other experiments will be necessary to clarify which are the important parameters and to determine their optimum levels.

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