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**ECBC-TR-606**

# INVESTIGATION OF VIABILITY OF *PANTOEA AGGLOMERANS* (FORMERLY *ERWINIA HERBICOLA*) AFTER AEROSOLIZATION FROM MEDIA CONTAINING ENRICHING AND COATING CHEMICALS


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Percent viability of the sensitive bacteria *Pantoea agglomerans* (ATCC_33243, formerly *Erwinia herbicola or Eh*), is an important issue in aerosol testing of vegetative cells, which might be found in the environment. The viability of this bacterial strain has usually been poor during aerosol testing. However, we have found a number of examples in the literature where outdoor testing with various strains of this bacterium apparently was improved with certain additives before or after bacteria growth We have been testing aerosol survival of this strain using various components found in published recipes to improve its effectiveness as a test organism. We report on tests performed with bacteria grown and aerosolized from Tryptic soy broth (TSB), TSB with sugar additives (sucrose and trehalose), and aerosolized from these growth media after centrifuge-washing with water. We also report experiments of aerosolized bacteria treated with glycerin and with glycerin plus Na-alginate (G+NA) under various conditions. We found improved survival for the bacteria dried on a surface, but were not able to obtain consistent results in the time allotted to the project when the bacteria were aerosolized.

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# INVESTIGATION OF VIABILITY OF *PANTOEA AGGLOMERANS* (FORMERLY *ERWINIA HERBICOLA*) AFTER AEROSOLIZATION FROM MEDIA CONTAINING ENRICHING AND COATING CHEMICALS

## INTRODUCTION

Research and development of aerosol sampling devices for vegetative bacteria requires the use of laboratory simulants for the assessment of sampler efficiency and sampling strategies. *Pantoea agglomerans* (ATCC_33243, formerly *Erwinia herbicola,* which will be referred to as *Eh* throughout this report) is a vegetative species, which has had wide use for outdoor testing with aerosols. This species is used as a model for assessing viability losses of airborne vegetative bacteria.

The *Eh* is a gram-negative, non-capsulated, non-spore-forming, motile rod, ~0.5 μm in diameter and ~1–2 μm in length (Arakawa et al., 2003). We have grown it in either nutrient agar or nutrient broth at 26 °C and found that it reaches stationary phase in ~8 hr. The species is a ubiquitous enteric bacterium found in plants and in the feces of humans and animals (De Champs et al., 2000). It is pathogenic to some varieties of vegetables (Manulis and Barash, 2003), but is often used outdoors as a bio-control agent in fruit crops for its antibiotic-secreting properties (Vanneste et al., 1992; Ishimaru et al., 1988) and as a simulant by the U.S. Department of Defense (DoD) (Winters et al., 1994; Forney et al., 1997). Although it is not classified as a known human pathogen, there have been reports of opportunistic and localized *Eh* bacteremias (Kratz et al., 2003). Preliminary laboratory work found *Eh* to have poor survival characteristics when aerosolized (Winters et al., 1997; Forney et al., 1997). This makes *Eh* a good model for studying frail vegetative bacteria and the effects of aerosolization on their viability.

A number of peer-reviewed articles in the literature have reported testing with various strains of *Eh* treated to optimize survival as an aerosol. Ozaktan et al. (2004) reported that a strain of *Eh* (Eh 24) showed increased survival when grown in a sucrose medium, suspended in a recipe of $MgSO_4$, glycerol, and sodium-alginate, and partially dried as a powder. They reported good bacteria survival after long periods on the shelf in this powdered form although they did not evaluate their results quantitatively. In addition, they report *Eh* growth on the foliage of pear trees, when the powder was sprayed to protect the orchard from fire blight.

Costa et al. (2002) tested the effects of spray-drying on *Eh* (CPA-2) viability. As carriers for the spray, they used a range of salts and dairy products of which $MgSO_4$, $K_2SO_4$, and nonfat skimmed milk (NFSM) showed the best results in terms of recovered live bacteria. Outlet temperature of the spray-dryer had more influence on bacteria death than inlet temperature. The highest recovery was obtained when NFSM was used as the rehydration medium. In a related study, Costa et al. (2002-b) tested the effect of several nitrogen and carbon sources on the growth of *Eh* (strain CPA-2). Synthetic yeast extract enhanced maximum growth and disaccharides (e.g., sucrose, lactose, and trehalose) improved this growth significantly from $3.2 \times 10^9$ to $5.5 \times 10^9$ colony forming units (cfu)/mL when the culture reached stationary phase.

Bonaterra et al. (2005) developed a system for osmoadaptation for another *Eh* strain (EPS125) by combining saline osmotic stress (NaCl) and osmolyte amendment [glycine-betaine (GB)] to the growth medium. Osmoadapted cells accumulated trehalose and GB intracellularly and showed a higher tolerance to desiccation than non-osmoadapted cells. Osmoadaptation in NaCl plus GB during inoculum preparation increased survival on the peel surface of apple fruits. This effect was significant under low relative humidity (RH) and fluctuating RH conditions, but was not significant at high RH. Growth of *Eh* (EPS125) with NaCl, without the addition of GB, was an effective osmoadaptation treatment. However, adding GB to the NaCl amended growth medium increased the growth rate of the cultures 4-5-fold. This is an advantage for mass production of *P. agglomerans* EPS125 in a NaCl amended growth medium.

Finally, Leslie et al. (1995) demonstrated that *Escherichia coli* (DH5a), also a vegetative gram-negative bacterium, showed increased survival when dried in the presence of 100 mM trehalose. The increased survival was attributed to the sugars' ability to lower the membrane phase transition temperature and to protect protein structure in the dry state. *E. coli* dried with trehalose and exposed to light and air for 4 hr had an increase in colony forming units between 2,000 and 4,000 times the number obtained with *E. coli* dried without trehalose.

To improve its effectiveness as an aerosol test organism, we tested aerosol survival of *Eh* using some of the components found in the above described published papers. We report on tests performed under various conditions with bacteria grown from Tryptic soy broth (TSB), TSB + 5% sucrose, TSB + 5% trehalose, as well as bacteria treated after growth in 5% sucrose TSB with either $MgSO_4$ + glycerin, or $MgSO_4$ + glycerin + Na-alginate (G+NA) as well as untreated bacteria.

## 2. EQUIPMENT AND FACILITIES

A series of experiments were conducted to test the effect of different treatments on viability of *Eh* after the bacteria was aerosolized or sprayed on a surface and allowed to dry for various time periods. All experiments were conducted at The Johns Hopkins University, School of Public Health Aerosol Laboratory.

### 2.1 General Setup.

Tests were conducted inside a chemical fume-hood, where a 50-$cm^3$ chamber was connected to the generation, sampling, and auxiliary ports as shown in Figure 1. All dilution and plating was conducted inside a level 2 Biosafety cabinet.

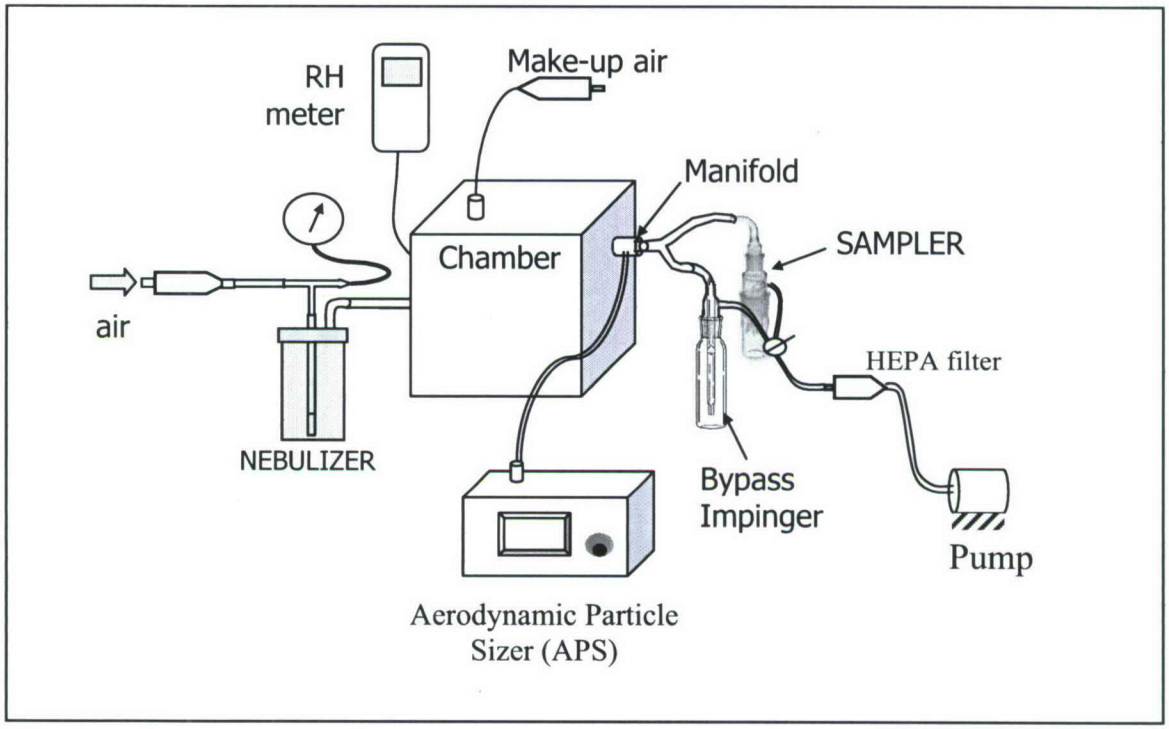


Figure 1. Experimental Chamber Setup (Not to Scale)

2.2 Generation.

Two nebulizers were used to aerosolize *Eh* for the experiments described below. A Collison 3-jet (BGI Inc., Minneapolis, MN) was used for all but experiment #5, where a DeVilbiss model 40 (De-Vilbiss, Somerset, PA) was used because of its small volume and ease of cleaning. Both nebulizers were connected to the house air through a drying cylinder and a HEPA filter to achieve low background particle concentrations in the chamber. House air pressure was monitored to insure consistency between generation runs. Air flow was initiated, and water was aerosolized for 5 minute before each run to establish background levels at the desired RH.

2.3 Sampling.

The sampling stage consists of a sampling manifold, the different samplers to be used (Biosampler ® or membrane filters), and an Aerodynamic Particle Sizer (APS) (Model 3320. TSI Inc., St Paul, MN). An isokinetic sampling manifold was used to ensure that representative samples of the aerosol inside the chamber (Hinds 1982) enter the inlets of the samplers and the APS. The sampling manifold consists of concentric stainless steel cylinders with connecting ports for the respective sampler flow rates of 5 Lpm and 12.5 Lpm. (Rule et al., 2005). The 5-Lpm port was used to monitor the particle concentration inside the chamber with an APS, and the 12.5-Lpm port was used for the Biosampler and membrane filters. A by-pass impinger was added to the sampling port in experiments #2, #3, and #6 to achieve steady-state conditions before sampling.

2.4 Auxiliary Ports.

Chamber conditioning and monitoring were achieved through three ports in addition to the sampling and generation ports. One of the ports was used to supply the chamber with HEPA-filtered make-up air, which was needed to prevent the chamber from collapsing in the event of a demand for air greater than the nebulizer port's supply. Temperature and RH were monitored using a Digital Thermo-Hygrometer (Control Company, China), and pressure was monitored using a Magnahelic (Dwyer Inst. Inc., Michigan City, IN).

## 3. TEST PROCEDURES AND ANALYSIS

### 3.1 Sample Preparation.

The different bacterial growth conditions and after-growth treatments used in the experiments described in this section were prepared as follows:

- *Eh* in TSB: An *Eh* colony from a streaked refrigerated plate was sub-cultured with a disposable loop into a flask containing 20 mL TSB, and left overnight (18 hr) in a shaking incubator at 200 rpm and 28 °C.

- *Eh* in 5% sucrose: Same procedure as above, except inoculum was transferred into 20 mL TSB enriched with 5% sucrose (5 gr sucrose per 100 mL TSB).

- *Eh* in 5% trehalose: Same procedure as above, except inoculum was transferred into 20 mL TSB enriched with 5% trehalose (5 gr trehalose per 100 mL TSB).

- *Eh* in 5% suc + Gly: After culturing overnight in 5% sucrose TSB, cells were centrifuged at 2,000 g for 20 minutes; and the bacterial pellet was resuspended in 0.1 M MgSO4 in a 1:1 (w/v) ratio, and then combined with 15% (v/v) glycerol.

- *Eh* in 5% suc + NaAlg: After combining with 15% (v/v) glycerol as described in the previous step, the suspension was mixed with an equal volume of autoclaved 1.5% Na-Alginate.

### 3.2 Sample Washing.

When the experiment required the suspensions described above to be aerosolized, to reduce broth and treatment residues, the suspensions were washed immediately after preparation by centrifuging 2 times with sterile de-ionized water at 2,000 g for 20 minutes. The aerosol lab at ECBC uses this method, or washing with phosphate-buffered-saline (PBS), or diluting directly from the broth. Water washing produces the lowest survival percentage. It was the only washing liquid used in these experiments due to limited time available.

### 3.3 Sample Storage.

The *Eh* used in the set of experiments described in this report was stored in TSB refrigerated at 4 °C, and streaked periodically to verify colony morphology. The *Eh* survives for several months this way. Survival is confirmed by the formation of uniform colonies with a distinctive bright yellow mucoid look 48 hr after streaking on a trypticase soy agar (TSA) plate.

## 4. RESULTS

### 4.1 Experiment #1: Powder Preparation.

Cultures of *Eh* were treated as described by Ozaktan[10] to obtain a coarse powder. After every stage of the powdering process, aliquots were plated in triptic soy agar (TSA) to determine viability losses attributable to each step. Culturable counts (colony forming units per milliliter) were compared to total counts (cells per milliliter), determined using a hemacytometer (Bright-Line, Hausser Scientific, Horsham, PA) and phase-contrast microscope (BH-2, Olympus America, Inc., Melville, NY).

Results for Experiment #1. We were not able to completely follow the published procedure during the powdering process, e.g., we did not find a way to sieve the final product without additional stress to the bacteria, nor did we store the final powder at >15% RH-controlled conditions. We did not observe any significant number of viable bacteria in a small sample of the powder produced with our modified procedure; therefore, we did not pursue this approach but attempted to analyze simpler methods as in the other experiments described below.

### 4.2 Experiment #2: Chamber Concentration Decay.

To estimate chamber suitability for long-term airborne tests, an experiment was conducted with aerosolized 0.8 μm PSL beads. Concentration inside the chamber was monitored every 30 minutes with an APS 3321. Experiments were conducted with and without a fan inside the chamber. Sampling and monitoring ports were clamped.

Result for Experiment #2. As observed in Figure 2, activating a fan inside the chamber increased losses over time, likely due to increased impaction on the walls of the chamber. A concentration high enough to yield culturable counts was measured inside the chamber after 2.5 hr without the fan. We did not use the fan for the rest of the chamber experiments for this reason.

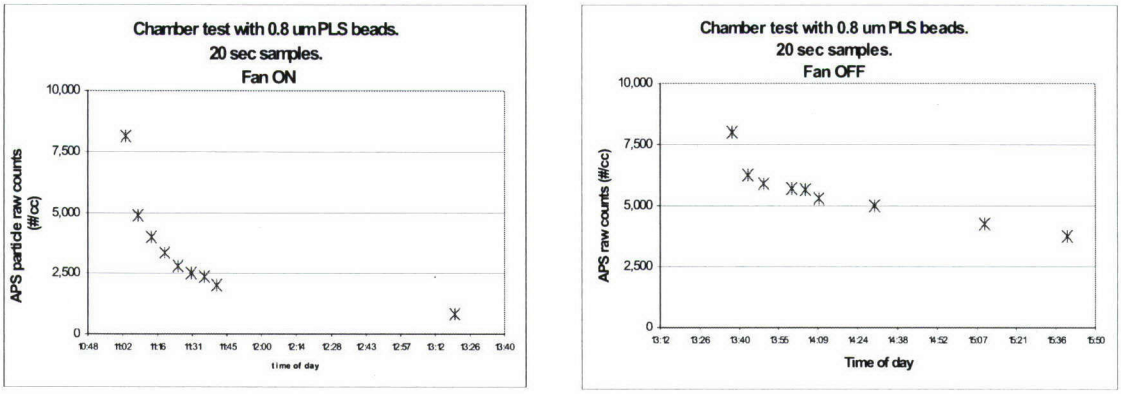


Figure 2. Concentration Decay over Time inside the 50-cm$^3$ Chamber;
( a) with a Fan, (b) without a Fan

4.3 Experiment #3: Chamber Residence of 2.5 hr.

In an initial exploratory phase to test treatment efficiency, aliquots of *Eh* in TSB, *Eh* in 5% sucrose, and *Eh* in 5% suc + Na-alginate were aerosolized with a 3-jet Collison nebulizer at 15 psig into the Plexiglas chamber with the fan off. Sampling was performed with a Biosampler while nebulizing (t = 0) and at t = 2.5 hr after the nebulizer was turned off. Tests were repeated 3 times. Viability at each time point [V(at hr sampled)] was assessed using the ratio of culturable (colony forming units per milliliter) to total hemacytometer counts (cells per milliliter). Viability (relative) after 2.5 hr was calculated dividing V(2.5) by V(0).

Results for Experiment #3. After 2.5 hr in the chamber, no conclusive results were observed between *Eh* suspensions nebulized from three different treatments (Figure 4). A decision was made to try some basic deposition experiments to test feasibility.

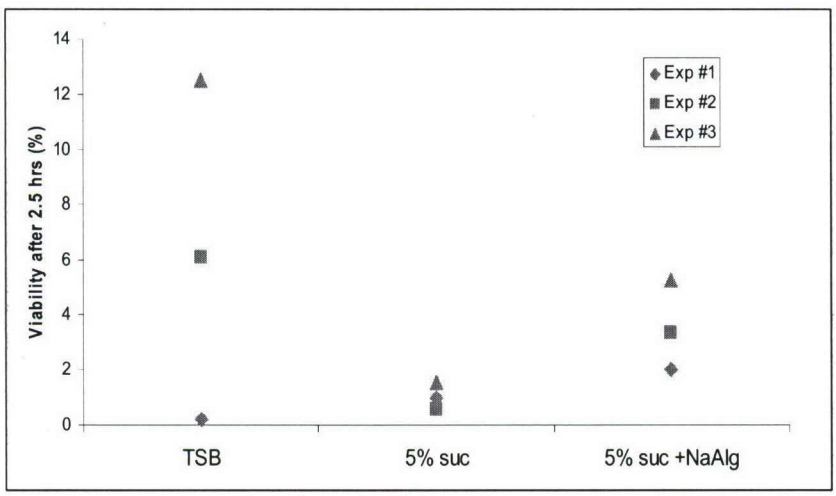


Figure 3. Relative Viability (Cf/Co) after Aerosolizing *Eh* into a 50-cm$^3$ Chamber.
Co = Counts at t = 0 and Cf = Counts at t = 2.5 hr. All data are shown.

4.4 Experiment # 4: Filter Sampling and Extraction.

To test feasibility of sampling on a filter, *Eh* in TSB was aerosolized with the Collison nebulizer and sampled for 10 min with 4 different membrane types [Millipore's ATTP 0.8 μm track etched polycarbonate, HAWP 0.45 μm mixed cellulose ester (MCE), AAWP 0.8 μm MCE, and Gelman's DM-800 0.8 μm metricel vinyl/acrylic]. Experiments were repeated 3 times. Filters were placed in sterile 25-mm closed-face cassette holders (#1107, Gelman Instruments, Ann Arbor, MI). After sampling, filters were placed in 50 mL centrifuge tubes containing 10 mL of recovery solution. To extract *Eh* from the filters, the centrifuge tubes were vortexed and shaken intermittently for 5 min before analyzing. Extraction efficiency and viability were compared for different filters using two PBS-based recovery solutions, one containing 0.01% Tween 20 and a second containing 0.01% Triton-X.

Results for Experiment #4. Percent recoveries of 70% or higher were achieved for all four membrane filter types tested for this experiment with at least one of the recovery solutions (Table). The recovery solution containing Tween 20 performed better in 3 of four tests. Viability was the lowest for the AAWP type filter.

Table. Tests of Recovery and Viability after Sampling with Different Filter Types and Extraction Solutions

| Filter type/extraction solution | Culturable counts (average) | tot counts (hemacyt) | APS counts ** | Flow rate Q | Sam-pling time t | Concentration based on APS counts & Flow rate*** | | Recover-ed particles | Viability |
|---|---|---|---|---|---|---|---|---|---|
| | cfu/mL | cnts/mL | part/cc | Lpm | min | part/filter | part/mL | % | % |
| **AAWP/** tween-20 | 6.0E+04 | 1.1E+07 | 1278.36 | 12.55 | 10 | 1.6E+08 | **1.6E+07** | 69 | 0.54 |
| /Triton-x | 3.8E+04 | 1.2E+07 | | | | | | 75 | 0.32 |
| **ATTP/** tween-20 | 6.4E+05 | 9.0E+06 | 1278.36 | 7.88 | 10 | 1.0E+08 | **1.0E+07** | 90 | 7.1 |
| /Triton-x | 1.3E+05 | 8.5E+06 | | | | | | 85 | 1.5 |

Table. Tests of Recovery and Viability after Sampling with Different Filter Types and Extraction Solutions (Cont'd.)

| Filter type/ extraction solution | culturable counts (average) | tot counts (hemacyt) | APS counts ** | Flow rate Q | Sam-pling time t | Concentration based on APS counts & Flow rate*** | | Recovered particles | Viability |
|---|---|---|---|---|---|---|---|---|---|
| | cfu/mL | cnts/mL | part/cc | Lpm | min | part/filter | part/mL | % | % |
| **DM-800/** tween-20 | 1.1E+06 | 8.0E+06 | 1278.36 | 8.5 | 10 | 1.1E+08 | **1.1E+07** | 73 | 14.0 |
| /Triton-x | 3.9E+05 | 4.8E+06 | | | | | | 44 | 8.1 |
| **HAWP/** tween-20 | 2.3E+05 | 2.8E+06 | 1278.36 | 2.75 | 10 | 3.5E+07 | **3.5E+06** | 80 | 8.2 |
| /Triton-x | 3.1E+05 | 2.1E+06 | | | | | | 60 | 14.8 |

** dN/dlogDp view based on the 0.7 μm size bin
*** average of 10 measurements over 10 min

| Filter | pore size (μm) | Membrane type |
|---|---|---|
| AAWP | 0.8 | Mixed cellulose ester |
| ATTP | 0.8 | Track-etched polycarbonate |
| DM-800 | 0.8 | Metricel vinyl/acrylic |
| HAWP | 0.45 | Mixed cellulose ester |

## 4.5 Experiment #5: Nebulization onto Glass Slides.

To improve quantification of sampled cells after very brief aerosolization, *Eh* was nebulized onto microscope glass slides. This was accomplished by aerosolizing treated *Eh* solutions using a sterile small-volume glass nebulizer (model 40 DeVilbiss) operated with HEPA-filtered air at a pressure of 5 psi. The nebulizer output was diverted into 3 streams via a modified Y (Figure 4). Aerosolized *Eh* was simultaneously collected onto 3 glass slides located inside the chamber (one for every drying time to be tested), located approximately 5 cm (2 in.) from the nebulizer jet stream. An APS was used to monitor concentration, to determine safe levels for opening the chamber's port to remove the slides.

The slides were left to dry inside a humidity-controlled environment at 21 °C and 40% RH (±5%) and rinsed with 990 μL PBS into a 1.5 mL centrifuge tube. One slide was analyzed after 4 hr, the second after 24 hr, and the third after 48 hr. Samples were serial-diluted, one aliquot was plated in TSA, and a second aliquot was counted with the hemacytometer to establish total cells. Each experiment was repeated 3 times.

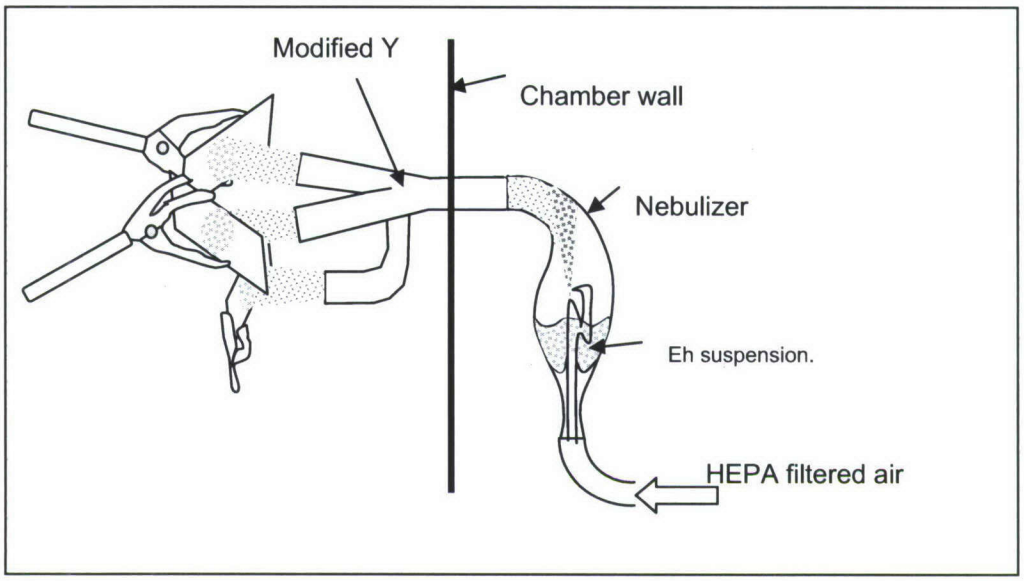


Figure 4. Experimental Setup for Deposition of Aerosolized *Eh* onto Glass Slides

Results for Experiment #5. The results of this experiment are interesting in that they suggest that these bacteria acquire a substantial increase in resistance to damage by drying after treatment by glycerol or Na-alginate. The viability at 24 and 48 hr is substantially increased compared to no treatment for both chemical treatments. Viability after 48 hr for the Na-alginate experiment was statistically significant ($p < 0.05$); whereas, that for the glycerol experiment was less significant ($p = 0.1$). It is not clear why this increased survivability for drying on a surface did not lead to similar results in our aerosolization experiments. However, the results suggest that more experiments to follow this lead would be worthwhile.

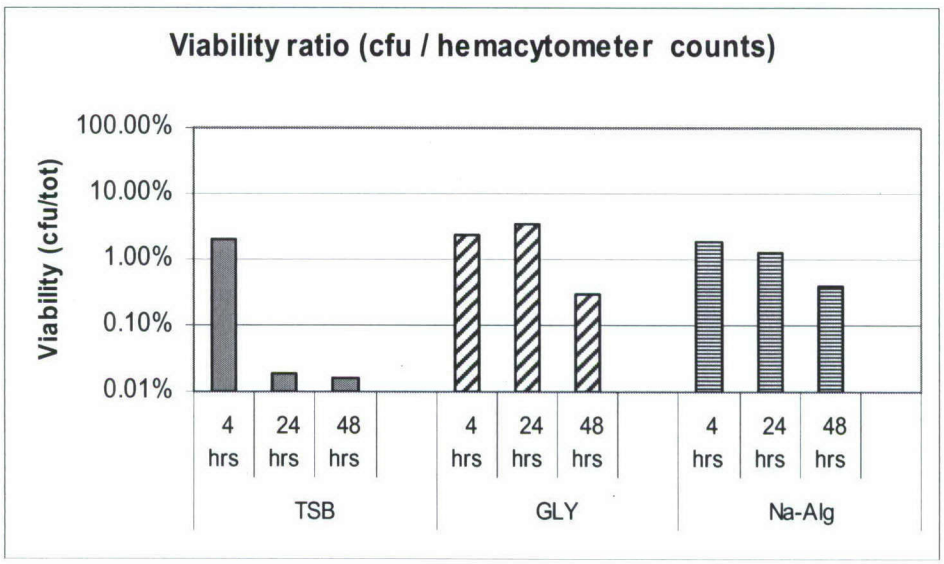


Figure 5. Average of Three Glass Slides for Each Drying Time and for Each Treatment Tested

4.6 Experiment #6: Chamber Residence of 24 hr.

For the last set of experiments, a longer retention time in the chamber was tested. The chamber's ports were sealed, and valves were added to each port to avoid leakage during waiting time. Tests were performed with *Eh*, and concentration decay was improved to a loss of 1 log after 19 hr (Figure 6) with decay described by Equation 1. All data are based on APS counts.

$$C_f = C_o[e^{-0.12\,x}] \quad (1)$$

Where $C_f$ = concentration at time x (total particles/cc)
$C_o$ = Initial concentration (total particles/cc)
x = elapsed time (hr)

*The Eh* was aerosolized into the chamber with a 3-jet Collison nebulizer at15 psig. Air was sampled from the chamber with a sterile SKC Biosampler® containing 10 mL PBS. A flow rate of 12 Lpm was achieved using a 40 Lpm pump (ABS-II-S, BGI, Waltham, MA). Sampling rate was calibrated before and after sampling with an electronic flow calibrator (DC 2M, BIOS International, Butler, NJ). The Collison nebulizer and the by-pass impinger were activated 5 minutes before sampling to achieve steady state inside the chamber. The nebulizer remained on for the 5-minute duration of the initial (t = 0) sample. Concentrations inside the chamber were monitored with an APS 3321. After sampling was completed, all valves were turned off (Nebulizer, sampler, APS, make-up air, and pressure gage), and the sampler's liquid volume was measured and transferred to a sterile centrifuge tube for analyzing. After 4 hr (t = 4), air was sampled from the chamber by opening the sampling, APS, and make-up air valves during 5 minutes. Twenty four hours (t = 24) after nebulizing, the valves were opened again, except this time air sampling time was increased to 20 minutes (data were normalized for the difference in sampling time) to obtain a more easily measurable number of colony forming units). Experiments were repeated 3 times for each treatment. A ratio of culturable over total counts (measured with the APS) was obtained for each time period ($t_i$) to assess viability [$V(t_i)$], and finally a ratio of $V(t_f)/V(t_o)$ was obtained to calculate viability losses over time. Statistical significance was determined using two-tailed t-tests with unequal variances.

Results for Experiment #6. After sealing the chamber, the new decay time (Figure 6) allowed for a 24-hr wait period between samples.

However, no colonies were observed for any of the treatments after 24 hr in the chamber. The 4-hr results are shown in Figure 7. No statistically significant difference was observed between any of the treatments.

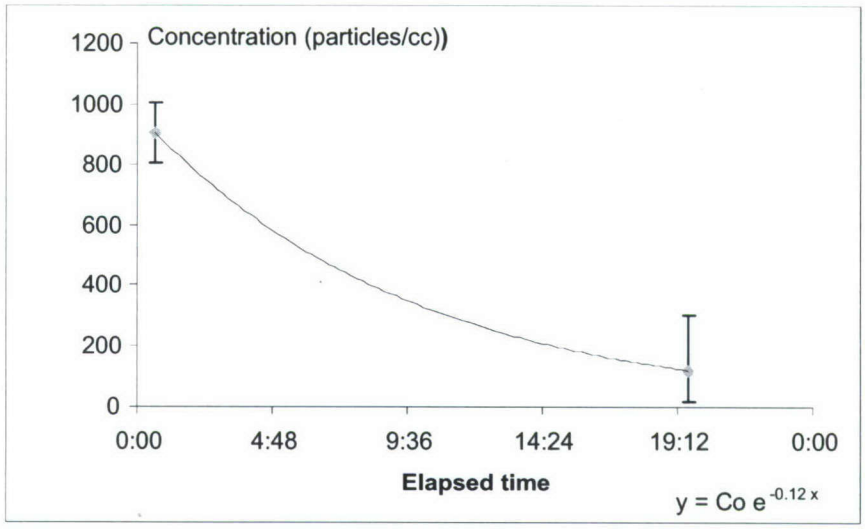


Figure 6. Concentration Decay Overnight inside the Modified Chamber. $Cf = Co[e^{-.12\,x}]$

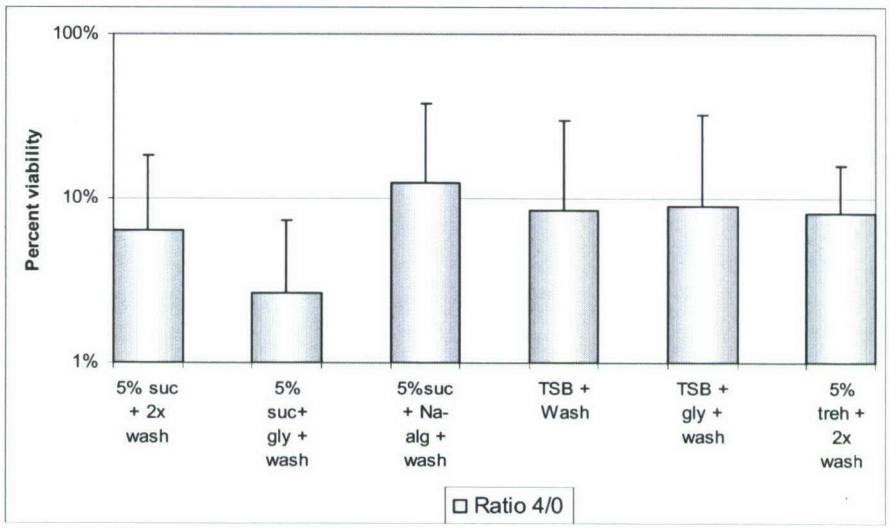


Figure 7. Viability of *P. agglomerans* by Treatment (Means of 3 Experiments) after 4 hr as Aerosol

## 5. DISCUSSION

We did not obtain viable bacteria after we prepared the bacteria in the manner suggested by experiments of Ozaktan et al.[10] We attributed this to the need to sieve the final product without additional stress to the bacteria, and the need to store the final powder at >15% RH-controlled conditions. Viability losses during the last stage of the powdering process (data not shown). In addition, given that: (1) *Eh* stays viable for several months when refrigerated in broth, (2) no field tests are presently planned that would require a powder preparation, and (3) aerosolizing from a liquid suspension is the currently accepted method, experiments were

conducted using aqueous solutions of *Eh,* and focused on viability differences using alternate growth conditions and after-growth treatments.

- Membrane filters may be an easy and inexpensive way to test viability after drying. Additional tests are needed to establish variability of the methods and determine the optimal combination of filter type and recovery solution.

- The experiments with glass slides seem to indicate that treating *Eh* with Na-Alginate improves viability. However, the results are not conclusive given the small number of experiments performed.

- Results of sampling aerosolized *Eh* after 4 hr in the chamber were not conclusive either, and no colonies were found after 24 hr. A residence time between 4 and 24 hr might be needed to see a statistically significant difference.

- A flaw in the experiments could be the use of too small a chamber, which could have affected experiments. An option was explored of nebulizing into a rotating drum chamber to minimize losses to the chamber, but such a chamber was not available during the time allocated for these tests.

## 6. CONCLUSIONS

Experiment #5 gave a clear indication that treatment with glycerol + Na-alginate (ATCC_33243) greatly increases the survival of the *Erwinia herbicola* bacterial strain presently used in testing at Dugway Proving Ground (Dugway, UT) and by many in the biodefense community when they are allowed to dry on a surface.

Our chamber experiments with these bacteria did not allow us to find a treatment, which comparably improved survival for this strain in aerosol form. Unfortunately, the time allotted to these experiments was not sufficient for us to determine the reason for this.

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