APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1991, p. 2420–2425
0099-2240/91/082420-06$02.00/0

Vol. 57, No. 8

Distribution, Clearance, and Mortality of Environmental Pseudomonads in Mice upon Intranasal Exposure

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Received 25 March 1991/Accepted 20 May 1991

When introduced intranasally, *P. cepacia* AC1100 (~10^8 CFU/animal) and *P. aeruginosa* AC869 (~10^3 CFU/animal) were readily cleared from the mouse. However, a ~10^7-CFU dose of AC869 persisted for 14 days. Strain AC869 had a 50% lethal dose of 2.7×10^7 CFU. Slight morbidity occurred in animals treated with ~10^7 CFU of AC869 or ~10^8 CFU of AC1100.

The environmental release of engineered microorganisms has prompted an investigation of health effects potentially associated with exposure to these organisms. Members of the genus *Pseudomonas* have many applications, including hazardous chemical degradation (for reviews, see references 12 and 15), pesticides (23), and prevention of ice nucleation on plants (17). Human exposure to these microorganisms or their products may occur in the agricultural or industrial setting during production or application (11, 24). Because a high concentration of the microbial product may be found in the air, water, or soil, exposure may occur through inhalation, ingestion, or skin penetration (16).

This study describes health effects associated with intranasal (i.n.) exposure of mice to two pseudomonads that have potential for environmental application. The distribution of microorganisms in the lungs and gastrointestinal (GI) tract was investigated due to the role these two systems have in disease. Because microorganisms can colonize the nasopharyngeal area and cause prolonged infection (25), survival of the dosed strains was monitored from this region as well.

Pseudomonas aeruginosa AC869, a 3,5-dichlorobenzoate degrader (5) (resistant to ampicillin, chloramphenicol, kanamycin, naladixic acid, penicillin, streptomycin, tetracycline, and mercuric chloride; sensitive to gentamicin [determined by antibiotic disks; BBL Microbiology Systems, Cockeysville, Md.]), was a gift from A. M. Chakrabarty, University of Illinois College of Medicine, Chicago. *P. cepacia* AC1100, a 2,4,5-trichlorophenoxyacetic acid degrader (14) (resistant to naladixic acid; sensitive to ampicillin, chloramphenicol, gentamicin, kanamycin, penicillin, streptomycin, tetracycline, and mercuric chloride [determined by antibiotic disks]), was obtained from A. M. Chakrabarty through the U.S. Environmental Protection Agency, Gulf Breeze, Fla. The pseudomonads were grown on *Pseudomonas* Isolation Agar (Difco Laboratories, Detroit, Mich.) prepared according to the manufacturer's direction. *Pseudomonas* Isolation Agar was supplemented with 50 μg of kanamycin sulfate per ml when indicated. Yeast extract-tryptone broth without added NaCl was used for strain growth (19). All incubations were done at 30°C.

Bacterial strains were concentrated in Dulbecco phosphate-buffered saline (PBS; GIBCO Laboratories, Grand Island, N.Y.) prior to i.n. exposure. The nasal cavity was washed with PBS, and the lungs were homogenized in PBS. The intestines were homogenized in reduced buffer (13).

Thirty-day-old male CD-1 mice (Crl:CD-1[ICR]BR, COBS; Charles River Portage, Portage, Mich.) were used in this study. Animals were quarantined for 2 weeks prior to treatment. Animals were provided deionized water and food (Purina Rodent Chow 5001; Purina Mills Inc., St. Louis, Mo.) ad libitum and housed in polycarbonate cages (five per cage) with pine shavings. They were weighed the day prior to treatment and subsequently thereafter at sacrifice. PBS-concentrated overnight yeast extract-tryptone cultures of the bacterial strains (CFU per milliliter determined by serial dilution) were diluted in PBS to the final concentrations indicated. Food was withheld 16 h prior to treatment. Mice were anesthetized in a desiccator jar (100-mm inside diameter) with methoxyfluorane. A 0.05-ml volume of the cell suspension was instilled unilaterally into the nostril cavity with a 1-in. (2.54-cm), 22-gauge feeding needle. Control animals received 0.05 ml of PBS.

Animals were sacrificed by CO_2 asphyxiation at 3 h and 1, 2, 5, 7, 10, and 14 days after i.n. challenge. Mice were dissected aseptically, and the trachea was exposed. Following 70% ethanol sterilization, a small incision was made in the trachea and a 1-in., 22-gauge feeding needle was inserted into the airway. The needle was secured with heavy thread, and a 10-ml syringe containing PBS was attached. A 5-ml volume of PBS was then injected into the tracheal airway and collected from the nostrils. Following the tracheal-nasal washing procedure, the lungs were removed and placed into preweighed sterile 50-ml tubes containing 5 ml of PBS. Next, the small intestine, cecum, and large intestine were removed and placed into preweighed sterile 50-ml tubes containing 5 ml of buffer. The tissues were homogenized with a Polytron tissue homogenizer (Brinkmann Instruments, Westbury, N.Y.) and plated on *Pseudomonas* Isolation Agar plates with (strain AC869) or without (strain AC1100) kanamycin. Dilutions of the homogenate were also plated to ensure countable plates. To confirm the source of the enumerated colonies, a representative colony was removed from the plate and antibiotic sensitivity was determined with antibiotic disks. Three animals per treatment were sacrificed at each time point. Each experiment was repeated for a total of six animals per time per treatment unless otherwise indicated. To determine what effect the treatment had on animal and lung weights, a two-way analysis of variance was performed

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TABLE 1. Animal and lung weights in *P. aeruginosa* AC869- and *P. cepacia* AC1100-treated mice[a]

Treatment	Time (days)	Body wt (g)	Lung wt (g)	Lung/body (%)
None[b]	0.13	27.4 ± 0.8	0.30 ± 0.04	1.08 ± 0.11
	1.0	28.1 ± 1.1	0.29 ± 0.03	1.02 ± 0.10
	2.0	30.1 ± 0.6	0.28 ± 0.02	0.93 ± 0.05
	5.0	30.6 ± 1.0	0.27 ± 0.02	0.85 ± 0.06
	7.0	31.2 ± 0.8	0.28 ± 0.02	0.90 ± 0.05
	10.0	31.1 ± 1.1	0.28 ± 0.03	0.89 ± 0.09
	14.0	32.8 ± 0.8	0.29 ± 0.01	0.88 ± 0.04
P. aeruginosa AC869 (1.61×10^3)	0.13	29.3 ± 1.1	0.44 ± 0.02	1.51 ± 0.04
	1.0	33.8 ± 0.6	0.31 ± 0.02	0.94 ± 0.06
	2.0	33.8 ± 1.2	0.28 ± 0.03	0.81 ± 0.05
	5.0	32.6 ± 1.2	0.31 ± 0.02	0.94 ± 0.06
	7.0	33.7 ± 0.8	0.30 ± 0.02	0.88 ± 0.04
	10.0	32.7 ± 0.7	0.35 ± 0.05	1.06 ± 0.13
	14.0	34.3 ± 1.3	0.33 ± 0.05	0.95 ± 0.11
P. aeruginosa AC869 (1.61×10^7)	0.13	28.6 ± 1.1	0.35 ± 0.04	1.21 ± 0.11
	1.0	27.5 ± 1.2[c]	0.36 ± 0.03	1.36 ± 0.14[c]
	2.0	29.3 ± 1.6[c]	0.42 ± 0.03[c]	1.47 ± 0.14[c]
	5.0	30.6 ± 1.5	0.39 ± 0.03[c]	1.32 ± 0.20[c]
	7.0	33.2 ± 0.6	0.37 ± 0.03	1.12 ± 0.09[c]
	10.0	30.1 ± 0.5[c]	0.37 ± 0.04	1.22 ± 0.13
	14.0	33.3 ± 1.0	0.43 ± 0.05	1.29 ± 0.13[c]
P. aeruginosa AC869 (2.17×10^9)	0.13	26.2 ± 1.5	0.29 ± 0.05	1.06 ± 0.16
	1.0	26.8 ± 0.8	0.40 ± 0.06	1.50 ± 0.23[c]
P. cepacia AC1100 (5.3×10^8)	0.13	27.4 ± 0.4	0.27 ± 0.02	0.97 ± 0.07
	1.0	26.1 ± 0.8	0.29 ± 0.03	1.09 ± 0.13
	2.0	25.3 ± 0.9[c]	0.37 ± 0.04[c]	1.53 ± 0.19[c]
	5.0	27.7 ± 0.6[c]	0.45 ± 0.04[c]	1.67 ± 0.16[c]
	7.0	28.2 ± 1.0	0.44 ± 0.03[c]	1.54 ± 0.10[c]
	10.0	28.0 ± 1.0	0.45 ± 0.03[c]	1.61 ± 0.08[c]
	14.0	29.5 ± 1.1[c]	0.38 ± 0.04	1.30 ± 0.14[c]

[a] Male CD-1 mice were treated i.n. with *P. aeruginosa* AC869 or *P. cepacia* AC1100 as described. Animals were weighed and sacrificed at the indicated time intervals. The lung weight was determined, and the percentage of body weight was calculated. Values are reported as means ± standard errors.
[b] Representative control data.
[c] Significantly different from untreated control at $P < 0.05$.

(27). Values are considered significantly different at $P < 0.05$.

P. aeruginosa AC869 was lethal for mice at the higher doses administered. After treatment i.n. with 2.17×10^9 CFU of strain AC869, lethality occurred within 24 to 36 h. For strain AC869, the 50% lethal dose was 2.7×10^7 CFU. At lower doses, the effect was less dramatic. Treatment with 1.61×10^7 CFU resulted in slight morbidity and some mortality, generally within 3 to 4 days after the microbial dose. Even at this lower dose (1.61×10^7 CFU), mice were lethargic, had ruffled fur, and suffered from weight loss. Evidence of lung hemorrhaging was observed upon dissection. A statistically significant increase in lung weights accompanied by a decrease in body weights was observed in animals treated with $\sim 10^7$ CFU (Table 1). No mortality and no observable signs of morbidity were evident in mice administered 1.61×10^3 CFU of strain AC869.

Mice administered 5.3×10^8 CFU of *P. cepacia* AC1100 showed signs of slight morbidity (ruffled fur, weight loss, and inactivity) 2 days after treatment, but recovered quickly from the symptoms. However, a statistically significant increase in lung weight was observed on day 2 after treatment and continued throughout the experimental time (Table 1). Body weights of treated mice were significantly less than those of control animals at 2, 5, and 14 days posttreatment. No mortality was observed at 5.3×10^8 CFU/animal, so effects at lower doses were not determined. The presence or absence of extracellular virulence factors such as elastase (20, 22), collagenase (4), exotoxin A (3, 18), phospholipase C, alkaline protease (22), or pyochelin production (6) may cause some of these intra- and interspecies differences.

The morbidity or mortality observed in mice treated with both pseudomonads was most likely due to lipopolysaccharide (endotoxin) toxicity. Because of the lipopolysaccharide composition of the cell wall, all gram-negative microorganisms contain endotoxin. Weight loss, ruffled fur, and lethargic behavior have been linked to endotoxin toxicity (7). Endotoxin induces macrophage production of tumor necrosis factor, which is responsible for the signs of morbidity and is a mediator of endotoxin-induced shock which can result in animal death (31). It has been demonstrated that endotoxin increases pulmonary capillary permeability (1, 2); promotes translocation of microorganisms from the GI tract to the mesenteric lymph nodes, spleen, and liver (7); activates macrophages and monocytes (21), including their production of collagenase (33); and causes fever and bronchoconstriction (26). In this study, the endotoxin levels at the higher dose may have overwhelmed the immune system and led to animal death. Endotoxin may have also enhanced the ability of strain AC869 to translocate from the intestinal tract and

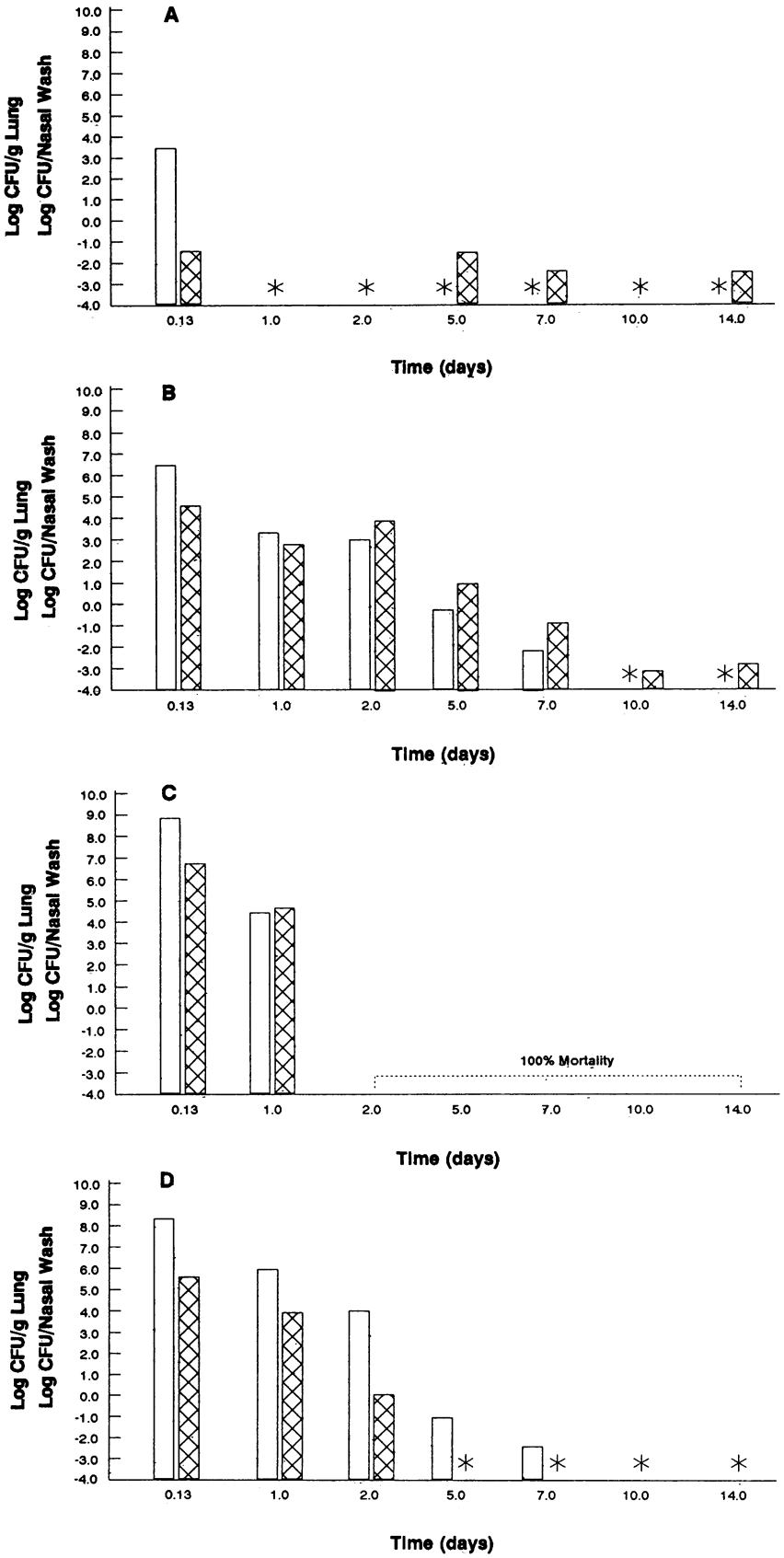

FIG. 1. Enumeration of *P. aeruginosa* AC869 and *P. cepacia* AC1100 from the lungs and nasal cavity of male CD-1 mice. Mice were dosed i.n. with 1.61×10^3 (A), 1.61×10^7 (B), or 2.17×10^9 (C) CFU of strain AC869 or 5.3×10^8 CFU (D) of strain AC1100. Recovery of the dosed strains was determined in the lungs (□) and nasal cavity (⊠) as described. Each bar represents the average recovery of organisms from six animals. An asterisk (*) indicates that the dosed strain was not detectable. None of the dosed strains were recovered from control animals that received PBS only.

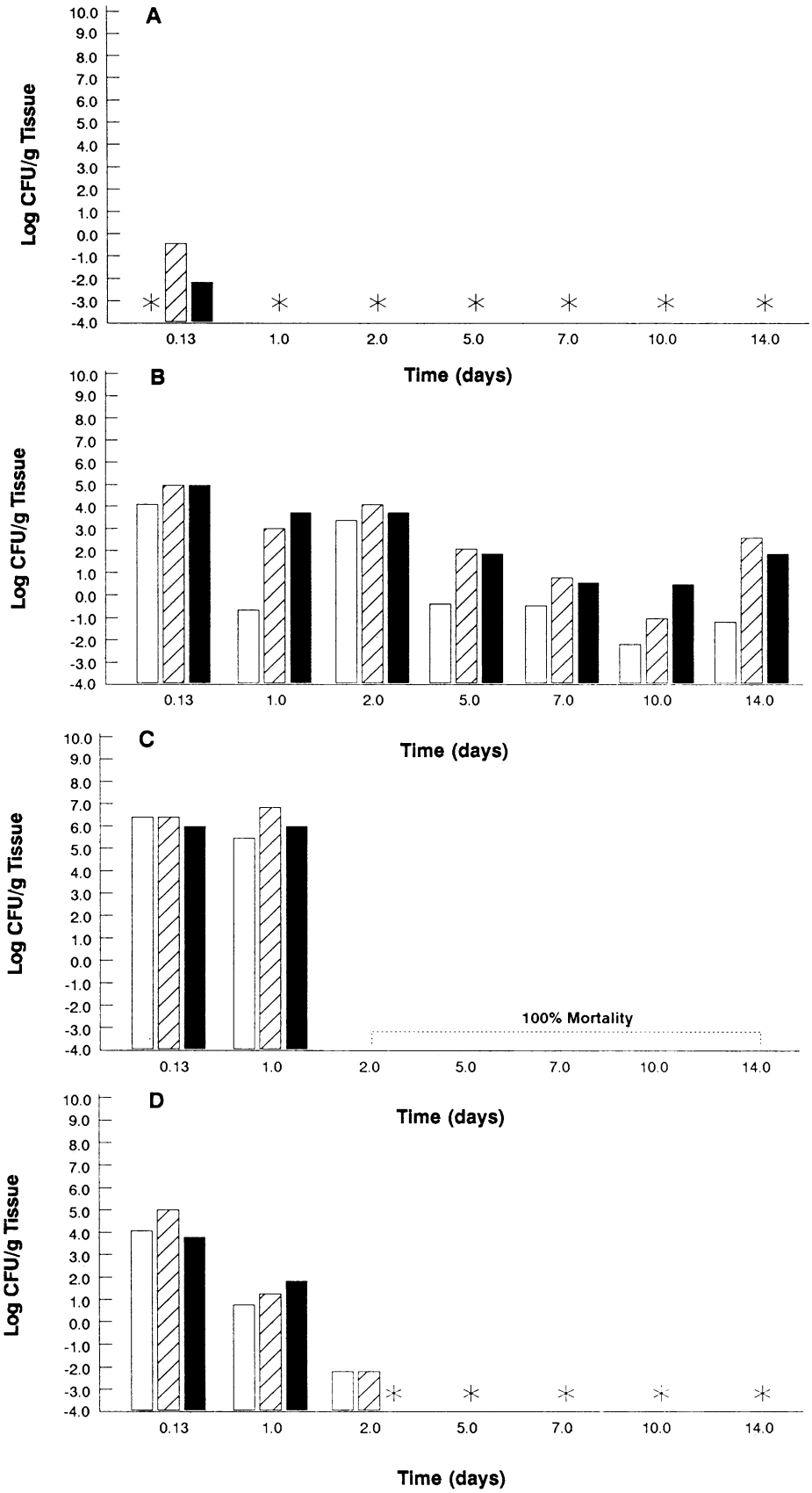

FIG. 2. Enumeration of *P. aeruginosa* AC869 and *P. cepacia* AC1100 from the small intestine, cecum, and large intestine of male CD-1 mice. Mice were dosed i.n. with 1.61×10^3 (A), 1.61×10^7 (B), or 2.17×10^9 (C) CFU of strain AC869 or 5.3×10^8 CFU (D) of strain AC1100. Recovery of the dosed strains was determined in the small intestine (□), cecum (▨), and large intestine (■) as described. Each bar represents the average recovery of organisms from six animals. An asterisk (*) indicates that the dosed strain was not detectable. None of the dosed strains were recovered from control animals that received PBS only.

caused a lethal systemic infection. The increase in lung weights observed in *P. cepacia*- and *P. aeruginosa*-treated mice may be due to an influx of macrophages and endotoxin-associated pulmonary edema (8) coupled with lung hemorrhaging (strain AC869). A concentration-dependent lung weight elevation also has been reported in rats treated with *P. syringae* aerosols (10).

Both strains administered were detectable at all doses 3 h after treatment (Fig. 1). Strain AC869 was cleared from the lungs of mice in a dose-related fashion. When inoculated at the low dose (1.61×10^3), strain AC869 was rapidly cleared from the lungs within 24 h but was detectable periodically in the nasal washes throughout the course of the experiment in low numbers (Fig. 1). Even though strain AC869 was cleared from the lungs by 10 days after treatment with 1.61×10^7 CFU, it was present in low numbers in the nasal cavity as evidenced by recoverable organisms in the nasal washing. *P. cepacia* AC1100 was no longer detectable in the nasal cavity after day 2 and was cleared from the lungs by day 7 posttreatment.

Pulmonary clearance of microbes is strain, dose, and host related, and therefore no clear consensus is observed in results from other laboratories. In general, clearance of *P. aeruginosa* from the lungs has been shown to fall into one of three classes: (i) multiplication followed by clearance, (ii) no clearance at 4 h after treatment, or (iii) steady clearance upon introduction (28). In one study, only 13% of the initial *P. aeruginosa* (strain 16) dose remained in the lungs 4 h following aerosol treatment of mice with 1.85×10^5 CFU. However, when mice were treated with a different *P. aeruginosa* strain (strain 22; 2.65×10^5 CFU), 97% of the initial aerosol dose remained in the lungs. In contrast, 95% of each strain was cleared from rats by 4 h (29). Toews et al. (30) demonstrated that a *P. aeruginosa* aerosol dose of 8×10^4 CFU in mice was rapidly cleared within 4 h but a higher dose of 6×10^5 caused multiplication in the lungs by 4 h. Multiplication in the lungs has been associated with elevated lecithinase activity (28).

Three hours after dosing, *P. aeruginosa* AC869 and *P. cepacia* AC1100 were detectable in the GI tract (Fig. 2). Strain AC1100 was cleared from the large intestine after day 1 and was eliminated from the small intestine and cecum 2 days posttreatment (Fig. 2). These findings differ from results of our previous study in which strain AC1100 was administered by gavage ($\sim 10^9$ CFU). In that study, the microorganism was not detectable in the GI tract by 3 h after dosing (15). Antibiotic treatment did not enhance survival, and no translocation to the liver or spleen was observed. The nasal cavity, through microbial colonization, may serve as a reservoir for intestinal tract exposure. Bacteria may enter the lung and be cleared by mucociliary transport action to the mouth where they may be protected by a mucous coating, which would allow for their survival in the stomach and transport into the small and large intestines and cecum (Fig. 2).

After treatment with 1.61×10^7 CFU of strain AC869, strain AC869 was detectable in the small and large intestine and in the cecum 14 days after dosing. This may occur because strain AC869 survives in the GI tract better than strain AC1100 when introduced by gavage (9). GI tract survival of opportunistic pathogens, such as *P. aeruginosa*, is dependent on the ecological balance in the intestinal tract and the immune status of the host (32). Also, because mice are coprophagic, repeated inoculation promotes intestinal tract survival. The harboring of strain AC869 in the intestinal tract may enhance its potential as an opportunistic pathogen, especially under stressful conditions.

In humans, adverse health effects have occurred from pulmonary exposure to microorganisms and their endotoxins in agriculture and industry (11, 24). Potential occupational exposures in a nut-processing factory are reported at 3.5×10^5 CFU/8 h (11). Because biotechnological applications require production and environmental application of high concentrations of microorganisms, it is important to determine whether adverse health effects may occur upon pulmonary exposure to these strains. For example, snow gun dispersions contain 0.8×10^9 to 1.6×10^9 CFU of viable and nonviable *P. syringae* per liter, but particles are generally regarded as nonrespirable (10). It is possible that strain engineering may alter expression of pathogenic factors and, depending on the exposure route, may cause a detrimental effect. Likewise, engineering could reduce the potential for pathogenic effects. In this study, pulmonary exposure to *P. aeruginosa* AC869 had a more dramatic negative effect on animal health than exposure to *P. cepacia* AC1100, and strain AC869 remained in the mouse 14 days after treatment.

We thank Melanie Gardner and Lan Tran for technical assistance. We are grateful to Maryjane Selgrade and Gail M. Nelson for helpful comments and review of the manuscript. We also thank A. Chakrabarty for the gift of strains AC869 and AC1100.

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