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Effect of Aerosolization on Culturability and Viability of Gram-Negative Bacteria

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Estimations of the bacterial content of air can be more easily made now than a decade ago, with colony formation the method of choice for enumeration of airborne bacteria. However, plate counts are subject to error because bacteria exposed to the air may remain viable yet lose the ability to form colonies, i.e., they become viable but nonculturable. If airborne bacteria exhibit this phenomenon, colony formation data will significantly underestimate the bacterial populations in air samples. The objective of the study reported here was to determine the effect of aerosolization on viability and colony-forming ability of *Serratia marcescens*, *Klebsiella planticola*, and *Cytophaga allerginae*. A collison nebulizer was used to spray bacterial suspensions into an aerosol chamber, after which duplicate samples were collected in all-glass impingers over a 4-h period. Humidity was maintained at ca. 20 to 25%, and temperature was maintained at 20 to 22°C for each of two replicate trials per microorganism. Viability was determined by using a modified direct viable count method, employing nalidixic acid or aztreonam and *p*-iodonitrotetrazolium violet (INT). Cells were stained with acridine orange and observed by epifluorescence microscopy to enumerate total and viable cells. Viable cells were defined as those elongating in the presence of antibiotic and/or reducing INT. CFU were determined by plating on tryptic soy agar and R2A agar. It was found that culture techniques did not provide an adequate description of the bacterial burdens of indoor air (i.e., less than 10% of the aerosolized bacteria were capable of forming visible colonies). It is concluded that total cell count procedures provide a better approximation of the number of bacterial cells in air and that procedures other than plate counting are needed to enumerate bacteria in aerosol samples, especially if the public health quality of indoor air is to be estimated.

Indoor air pollution, both bacterial and abiotic, has become a very serious concern (25). Increasingly, new buildings are constructed to achieve energy efficiency, with interior air being changed much less frequently. Thus, building air is recycled through buildings, resulting in increased numbers of bacteria in the air (26, 28), with the result that ca. 10 to 25 million workers in the United States show symptoms of "sick building syndrome" (SBS) annually. This has considerable economic impact, because symptomatic workers have reduced productivity and are subject to increased absence (32). Consequently, it is important to understand the causes and predict the effects of SBS (11, 13, 20).

The role of bacteria in SBS is still largely unknown, but it is generally accepted that microorganisms are directly or indirectly involved. Airborne bacteria have been shown to be associated with both acute and chronic respiratory disease that has an infectious or allergenic component. For many environments, it has been demonstrated that bacteria enter into a viable but nonculturable (VBNC) state (3, 25). Potentially, VBNC bacteria may play a role in SBS, and exposure to airborne bacteria that are VBNC may be associated with allergies or other respiratory problems (17).

Bacterial analysis of air samples is very difficult, particularly the collection of airborne bacteria for quantitative enumeration (18, 33). The VBNC phenomenon adds to this problem, since the most commonly used methods for quantifying airborne bacteria involve the capture of microorganisms directly on solid media, as with the Anderson sampler, or in liquid buffer, e.g., all-glass impingers (AGIs). Certain compounds have been incorporated into impinger buffers to improve the recovery of collected bacteria (16, 17, 19). In the end, these methods all depend on culturing microorganisms in order to enumerate them (6, 9, 15, 29, 31). It is assumed that each microorganism produces a recognizable colony on an artificial medium within a specific time period and under the given growth conditions (5, 12–14, 17, 30). However, airborne bacteria may not respond to traditional culture techniques.

To overcome this problem, molecular methods can be helpful in detecting and enumerating VBNC bacteria in air samples, since such methods do not require cell growth (1, 21). Several methods can be used to evaluate the viability of those bacterial cells which have lost the ability to produce colonies on standard microbiological media. For example, uptake and reduction of *p*-iodonitrotetrazolium violet (INT) has been used to determine the viability of bacteria in aquatic environmental samples (24). A procedure that permits differentiation of VBNC microorganisms employs acridine orange staining of samples to which nalidixic acid (NA) has been added (10). NA-sensitive bacteria show elongation. Other antibiotics can be used for NA-resistant bacteria, e.g., aztreonam or other β-lactam antibiotics (23).

The objective of this study was to determine if gram-negative bacteria aerosolized at ambient room temperature and humidity lost the capability to grow on standard media employed for enumerating aerosolized bacteria, i.e., if they became unable to grow under standard incubation conditions but remained viable, based on cell elongation and reduction of INT.

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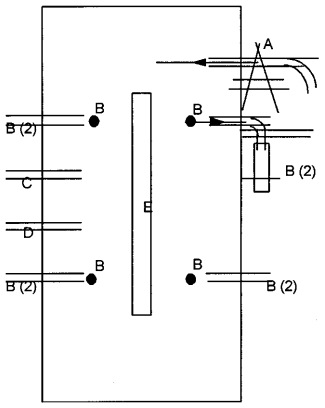

FIG. 1. Illustration of a bacterial aerosol chamber (view from front). A, collison nebulizer; B, impinger ports (12); C, hygrometer port; D, equal-pressure port; E, UV lamp (switch on back).

MATERIALS AND METHODS

Three strains of bacteria were used in this study, *Serratia marcescens* ATCC 14764, *Klebsiella planticola* ATCC 15050, and *Cytophaga allerginae* ATCC 35408, selected because they were originally isolated from air samples (2).

Aerosolization of bacteria. For each trial, bacterial cultures were prepared by inoculating cells thawed from storage at −70°C onto tryptic soy agar (TSA). *S. marcescens* and *K. planticola* were incubated overnight at 35°C, and *C. allerginae* was incubated at 25°C for 2 days. Subcultures were prepared on TSA plates and reincubated. For aerosolization, cells were collected and suspended in phosphate-buffered saline to a final concentration of ca. 10^9 ml^{-1} (optical density at 600 nm = 0.75 to 0.85). Ca. 60 ml of cell suspension was added to a sterile collison nebulizer (BGH, Inc., Cambridge, Mass.). An aerosol of this suspension was produced with medical-grade air (20 lb/in^2) for 3 min in an aerosol chamber with an interior volume of 550 liters (Fig. 1). After each trial, the chamber was sterilized with UV for 3 h followed by a 20-min exposure to aerosolized 70% ethanol. The chamber was not used for at least 48 h between trials to avoid any possibility of cross-contamination between experiments (27). All trials were conducted at room temperature (20 to 22°C) and 20 to 25% relative humidity (RH). Six trials were conducted: two each for *S. marcescens*, *C. allerginae*, and *K. planticola.*

Sampling. AGIs were used to collect airborne microorganisms. If a vacuum of at least 16 mm of Hg is achieved, an impinger can sample 12.5 liters of air min^{-1}. Air samples were collected for 3 min, simultaneously with aerosolization (time [t] = 0), at 20 min (t = 0.33), at 2 h (t = 2), and at 4 h (t = 4). Samples were collected in impinger buffer (100 mM K_2HPO_4, 30 mM KH_2PO_4, 0.4 mM $MgSO_4$) with and without 3.5 μM betaine (16). Duplicate impingers containing 40 ml of filtered (0.22-μm pore size) impinger buffer were used for each sampling.

Culture methods. The number of CFU in the suspension to be aerosolized and in the impinger fluid was determined at t = 0 by plating appropriate dilutions (e.g., 100 μl of a 1:10 dilution of impinger buffer at t = 0) onto five plates each of TSA and R2A media (Difco, Detroit, Mich.). At t = 0.33, 5 ml of impinger buffer was filtered through a 0.45-μm-pore-size membrane filter (type HA; Millipore) in triplicate, and the filters were incubated on TSA and R2A. At t = 2 and t = 4, 25 ml of impinger buffer was filtered through a 0.45-μm-pore-size filter, in duplicate, and incubated on TSA. The plates were incubated at 25°C for 1 week. A longer incubation period was needed to permit development of colonies after aerosolized bacteria were collected in impingers. Five plates were used to minimize error when few CFU were present (i.e., at $t > 0.33$). Data are expressed as CFU per milliliter.

Direct microscopy. Estimates of total cells in the impinger buffer were obtained by using the acridine orange direct count (AODC) procedure (7). Five-milliliter samples were treated with acridine orange (0.01% final concentration). The samples were incubated for 5 min in the dark and filtered with black polycarbonate filters (0.22-μm pore size). Viability of the aerosolized cells was determined by the method of Kogure et al. (10) with some modifications. NA (0.5 μg ml^{-1}) was used for *K. planticola*, and aztreonam (10 μg ml^{-1}) was used for *S. marcescens* and *C. allerginae*, which are resistant to NA. One milliliter of 1% yeast extract containing the appropriate antibiotic was added to 8.5 ml of impinger buffer and incubated at 25°C in an incubator shaker (Bellco Biotechnology, Vineland, N.J.) at 100 rpm for 1 week. INT (2 mg) was added to the samples on day 6 of the incubation, after which the samples were fixed with 2% formalin (day 7), stained with acridine orange, and observed with a Zeiss epifluorescent microscope (excitation wavelength, 490 nm).

RESULTS

The culturability of all three bacterial strains rapidly decreased following aerosolization. Four hours after aerosolization, there were no culturable cells on any of the media used in this study (Table 1), although viability was retained by the majority of the cells (Table 2).

There were no significant differences in the number of CFU per milliliter between TSA and R2A at t = 0 or t = 0.33 ($P <$

TABLE 1. Effect of time of aerosolization on culturability and viability of gram-negative bacteria

Time (h)	Type of count	No. of[a]:					
		S. marcescens		*K. planticola*		*C. allerginae*	
		No betaine	Betaine	No betaine	Betaine	No betaine	Betaine
0	AODC	38,675	70,525	43,750	70,350	45,675	60,550
	AODVC	24,850	41,650	35,350	59,850	38,325	52,850
	On TSA	1,740	1,740	680	3,480	246	1,350
	On R2A[b]	1,660	1,460	760	3,040	288	1,676
0.33	AODC	73,150	96,600	82,075	97,650	67,900	71,050
	AODVC	44,800	54,950	68,600	78,225	59,850	62,300
	On TSA	7	7	5	9	0[c]	0
	On R2A	5	4	6	6	0	0
2	AODC	38,325	45,500	43,750	57,750	42,875	59,850
	AODVC	21,350	19,250	34,475	40,075	35,350	51,625
	On TSA	0	0	0	0	0.02	0
4	AODC	21,875	34,300	30,975	67,725	33,425	41,825
	AODVC	11,900	14,175	25,725	51,625	25,200	29,575
	On TSA	0	0	0	0	0	0

[a] Bacteria were captured in the AGI. The impinger buffer contained 30 mM K_2PO_4 and 0.4 mM $MgSO_4$, with and without 3.5 μM betaine. Total cells (AODC), total viable cells (AODVC), and CFU were determined at each sampling time. Results are presented as both total cells and total viable cells per milliliter measured by direct microscopy and as CFU per milliliter, i.e., the number of colonies growing on TSA and R2A.

[b] R2A medium was not used at t = 2 and t = 4 because of insufficient buffer. Earlier times of sampling showed no significant differences for these media.

[c] Below the detection limit.

TABLE 2. Effect of betaine on viability with time

Time (h)	Viability[a]					
	S. marcescens		*K. planticola*		*C. allerginae*	
	No betaine[b]	Betaine	No betaine	Betaine	No betaine	Betaine
0	64.25	59.06	80.80	85.07	83.91	87.30
0.33	61.24	56.88	83.58	80.11	88.14	87.68
2	55.71	42.31	78.80	69.39	82.45	86.26
4	54.40	41.33	83.05	76.23	75.39	70.71

[a] Results are expressed as (AODVC/AODC) × 100.
[b] Impinger buffer with and without 3.5 μM betaine.

0.05). Addition of betaine to the impinger buffer yielded significantly increased colony formation in the case of *K. planticola* (fourfold increase) and *C. allerginae* (fivefold increase). No effect was observed on the recovery of *S. marcescens* (Table 1).

The culturability of *S. marcescens* decreased rapidly with time following aerosolization (Table 1). After aerosolization for 20 min, the number of CFU per milliliter decreased by about 2 logs, and after 2 h, no CFU were recovered. Results for *K. planticola* and *C. allerginae* were similar except that the initial decline was much greater, i.e., *K. planticola* decreased by 2.5 logs in the first 20 min and no CFU of *C. allerginae* were recovered after 20 min (except for a single colony, at $t = 2$, when betaine was added to the impinger buffer) (Table 1).

Viability. The percentage of viable cells, enumerated by direct microscopy after the addition of antibiotics, did not decrease over the 4-h sampling time (Table 2). However, percent viable cells varied with the organism being tested. Addition of betaine to the impinger buffer did not change significantly the percentage of total cells remaining viable.

DISCUSSION

From these results, it is concluded that the bacterial strains included in this study did not respond to standard culture methods after aerosolization. The plate count (CFU) provides an estimate of only those few cells least affected by exposure to air, e.g., by exposure to desiccation, sudden changes in temperature, and/or RH. The direct counts and direct viable counts indicate that most of the aerosolized bacteria remained viable for at least 4 h after aerosolization and probably longer, even though they did not form colonies on TSA or R2A. There is an indication (Table 1) that the presence of betaine increased the total (AODC) and viable (acridine orange direct viable count [AODVC]) counts. This apparent increase may be due to greater dispersion of clumps or protection of the cell wall by betaine, resulting in intact but nonviable cells.

These data demonstrate that a significant portion of airborne microbial populations, namely, cells that are viable but nonculturable, have heretofore been ignored in studies of the effect of aerosolization on microorganisms. These cells constitute differing percentages of the total population, depending on both the species and the extent and harshness of environmental exposure. It has been shown in human volunteer experiments that ingestion of VBNC *Vibrio cholerae* will result in diarrhea (4), but such studies have not been done with the VBNC bacteria inhaled rather than ingested. It can be hypothesized that cells in the VBNC state may cause SBS symptoms in human populations (6), but the appropriate studies to confirm this hypothesis remain to be done. The presence of large numbers of bacterial cells may be expected to cause an allergic response if inhaled (18). In any case, it is clear from the results presented here that plate count data will significantly underestimate the total bacterial burden, i.e., by ca. 100- to 1,000-fold. A 100- to 1,000-fold-greater exposure factor can explain allergic responses in individuals simply from exposure to a large amount of cell protein (16).

Impinger buffer amended with protective compounds, e.g., betaine, acted as a protective agent, maintaining the culturability of *K. planticola* and *C. allerginae*; however, it acted in the opposite way in the case of *S. marcescens* (Table 1), suggesting action at the cell envelope, with variation in the cell envelope being a factor. Because of strain variability, the use of protective agents in routine monitoring may introduce a bias, i.e., they may act as selective agents, protecting the culturability of only a portion of the microbial population. This study was conducted at an RH and temperature approximating normal building conditions. The effect of varying environmental conditions, e.g., RH, temperature, etc., on conversion of cells to the VBNC state was not determined but will be the objective of future studies.

Recently, direct detection of airborne bacteria by employing gene probes, PCR, and other molecular genetic methods has been proposed (1, 19). This approach offers both precision and accuracy, since the microorganisms need not be grown in culture. However, these methods do not differentiate between the total number of bacteria and numbers of VBNC or colony-forming bacteria, but they do offer a highly precise enumeration of specific microorganisms, for example, *Legionella pneumophila* (8, 22). The ultimate objective is to be able to predict infection by exposure to airborne pathogens. Simple enumeration is helpful but not sufficient; i.e., the relative pathogenicity of VBNC and colony-forming bacteria will need to be assessed. Whether they are equally infective or whether the VNBC bacteria are slower acting remains to be determined.

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REFERENCES

1. **Alvarez, A. J., M. P. Buttner, G. A. Toranzos, E. A. Dvorsky, A. Toro, T. B. Heikes, L. E. Mertikas-Pifer, and L. D. Stetzenbach.** 1994. Use of solid-phase PCR for enhanced detection of airborne microorganisms. Appl. Environ. Microbiol. **60:**374–376.
2. **American Type Culture Collection.** 1991. Catalogue of bacteria, bacteriophages and media. American Type Culture Collection, Rockville, Md.
3. **Colwell, R. R., P. R. Brayton, D. J. Grimes, D. B. Roszak, S. A. Huq, and L. M. Palmer.** 1985. Viable but non-culturable *Vibrio cholerae* and related pathogens in the environment: implications for release of genetically engineered microorganisms. Bio/Technology **3:**817–820.
4. **Colwell, R. R., P. Brayton, A. Huq, B. Tall, P. Harrington, and M. Levine.** 1996. Viable but nonculturable Vibrio cholerae O1 revert to a cultiviable state in the humann intestine. World J. Microbiol. Biotechnol. **12:**28–31.
5. **Crawford, G. C., and J. P. H. Jones.** 1979. Sampling and differentiation techniques for airborne organisms emitted from wastewater. Water Res. **13:**393–399.
6. **Heidelberg, J. F., M. Shahamat, M. Levin, I. Rahman, and R. R. Colwell.** 1994. Effect of aerosolization on the viability of *Serratia marcescens*, abstr. Q-239, p. 430. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
7. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of nuclepore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33:**1225–1228.
8. **Hussong, D., R. R. Colwell, M. O'Brien, A. D. Weiss, A. D. Pearson, R. M. Weiner, and W. D. Burge.** 1987. Viable *L. pneumophila* not detectable by culture on agar media. Bio/Technology **5:**947–950.
9. **Kingston, D.** 1971. Selective media in air sampling: a review. J. Appl. Bacteriol. **34:**221–232.
10. **Kogure, K., U. Simidu, and N. Taga.** 1979. A tentative direct microscopic method for counting living marine bacteria. Can. J. Microbiol. **24:**415–420.

11. **Kreiss, K.** 1993. The sick building syndrome in office buildings—a breath of fresh air. N. Engl. J. Med. **328:**877–878.
12. **Levin, M. A., M. Shahamat, I. Rahman, J. Heidelberg, and R. R. Colwell.** 1994. Comparison of media for recovery of aerosolized bacteria, abstr. Q-238, p. 430. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
13. **Levin, M. A., M. Shahamat, I. Rahman, J. Heidelberg, and K. Osmond.** 1994. Development of media for use in aerobiology. Am. Environ. Lab. **2:**40–42.
14. **Lighthart, B., and A. J. Mohr.** 1994. Atmospheric microbial aerosols. Chapman and Hall, New York, N.Y.
15. **Lundholm, I. M.** 1982. Comparison of methods for quantitative determination of airborne bacteria and evaluation of total viable counts. Appl. Environ. Microbiol. **44:**179–183.
16. **Marthi, B., and B. Lighthart.** 1990. Effects of betaine on enumeration of airborne bacteria. Appl. Environ. Microbiol. **56:**1286–1289.
17. **Marthi, B., B. T. Shaffer, B. Lighthart, and L. Ganno.** 1991. Resuscitation effects of catalase on airborne bacteria. Appl. Environ. Microbiol. **57:**1775–1776.
18. **Marthi, B.** 1994. Resuscitation of bioaerosols. *In* B. Lighthart and A. J. Mohr (ed.), Atmospheric microbial aerosols. Chapman and Hall, New York, N.Y.
19. **McElroy, G.** 1993. Development of collection fluids for use in the AGI30 sampler for recovery of selected bacteria commonly found in indoor air. M.S. thesis. University of Cincinnati, Cincinnati, Ohio.
20. **Menzies, R., J. P. Farant, J. Hanley, F. Nunes, and R. Tamblyn.** 1993. The effects of varying levels of outdoor-air supply on the symptoms of sick building syndrome. N. Engl. J. Med. **328:**821–827.
21. **Neef, A., R. Amann, and K. H. Schleifer.** 1995. Detection of microbial cells in aerosols using nucleic acid probes. Syst. Appl. Microbiol. **18:**113–122.
22. **Paszko-Kolva, C., M. Shahamat and R. R. Colwell.** 1993. Effect of temperature on survival of *Legionella pneumophila* in the aquatic environment. Microbial Releases **2:**73–79.
23. **Peterson, P. K., and J. Heroth.** 1988. The antimicrobial agent annual 3, p. 51–61. Elsevier Press, Amsterdam, The Netherlands.
24. **Rodriguez, G. G., D. Phipps, K. Ishiguro, and H. F. Ridgway.** 1992. Use of a fluorescent redox probe for the direct visualization of actively respiring bacteria. Appl. Environ. Microbiol. **58:**1801–1808.
25. **Roszak, D. B., and R. R. Colwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. **51:**365–379.
26. **Salem, H., and D. E. Garner.** 1994. Health aspects of microbial bioaerosols, p. 304–331. *In* B. Lighthart and A. J. Mohr (ed.), Atmospheric microbial aerosoles. Chapman and Hall, New York, N.Y.
27. **Shahamat, Y., M. A. Levin, M. Shahamat, and R. R. Colwell.** 1994. Sterilization efficacy of ultraviolet light, abstr. Q-237, p. 430. *In* Abstracts of the 94th General Meeting of the American Society for Microbiology 1994. American Society for Microbiology, Washington, D.C.
28. **Shelley, A.** 1990. The prevention of microbial contamination in air handling systems. Aust. Refrig. Air Cond. Heating **44:**30–36.
29. **Stetzenbach, L. D.** 1992. Airborne microorganisms, p. 53–66. *In* J. Lederberg (ed.), Encyclopedia of microbiology. Academic Press, San Diego, Calif.
30. **Thom, S. M., R. W. Horobin, E. Seidler, and M. R. Barer.** 1993. Factors affecting the selection and use of tetrazolium salts as cytochemical indicators of microbial viability and activity. J. Appl. Bacteriol. **74:**433–443.
31. **Thorne, P. S., M. S. Kiekhaefer, P. Whitten, and K. J. Donham.** 1992. Comparison of bioaerosol sampling methods in barns housing swine. Appl. Environ. Microbiol. **58:**2543–2551.
32. **Woods, J. E.** 1989. Cost avoidance and productivity in owning and operating buildings. Occup. Med. **4:**753–770.
33. **Zimmerman, N. J., P. C. Reist, and A. G. Turner.** 1987. Comparison of two biological aerosol sampling methods. Appl. Environ. Microbiol. **53:**99–104.