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Bacterial Plasmolysis as a Physical Indicator of Viability

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Bacterial plasmolytic response to osmotic stress was evaluated as a physical indicator of membrane integrity and hence cellular viability. Digital image analysis and either low-magnification dark-field, high-magnification phase-contrast, or confocal laser microscopy, in conjunction with pulse application of a 1.5 M NaCl solution, were used as a rapid, growth-independent method for quantifying the viability of attached biofilm bacteria. Bacteria were considered viable if they were capable of plasmolysis, as quantified by changes in cell area or light scattering. When viable *Salmonella enteritidis* biofilm cells were exposed to 1.5 M NaCl, an ~50% reduction in cell protoplast area (as determined by high-magnification phase-contrast microscopy) was observed. In contrast, heat- and formalin-killed *S. enteritidis* cells were unresponsive to NaCl treatment. Furthermore, the mean dark-field cell area of a viable, sessile population of *Pseudomonas fluorescens* cells (~1,100 cells) increased by 50% as a result of salt stress, from 1,035 ± 162 to 1,588 ± 284 μm^2, because of increased light scattering of the condensed, plasmolyzed cell protoplast. Light scattering of ethanol-killed control biofilm cells underwent little change following salt stress. When the results obtained with scanning confocal laser microscopy and a fluorescent viability probe were compared with the accuracy of plasmolysis as a viability indicator, it was found that the two methods were in close agreement. Used alone or in conjunction with fluorochemical probes, physical indicators of membrane integrity provided a rapid, direct, growth-independent method for determining the viability of biofilm bacteria known to undergo plasmolysis, and this method may have value during efficacy testing of biocides and other antimicrobial agents when nondestructive time course analyses are required.

Traditional methods for determining cellular viability rely upon the culture of organisms on agar surfaces, a method which may (i) bias results for organisms capable of growing on a specific substrate under a defined set of conditions and (ii) not always be sensitive to the condition of individual bacteria (13, 29, 31, 33). The limitations of using culture-based techniques to accurately enumerate living cells from environmental samples are now well documented and can easily be demonstrated by comparing direct microscopic counts, obtained by using 4′,6-diamidino-2-phenylindole or acridine orange, with the number of CFU which form on the surfaces of agar plates (1, 4, 23, 32, 42). Genetic techniques have similarly indicated a much lower diversity among culturable organisms than the diversity present in many natural systems. Ward et al. (41) determined that none of 15 unique 16S rRNA sequences found in a well-studied thermal microbial mat system matched the rRNA sequences of organisms cultivated and analyzed during previous studies, suggesting that a large number of unculturable organisms were present.

Recently, various methods for determining viability or cellular activity have experienced widespread application. These methods typically provide information concerning cells' internal chemistry or metabolic state. For example, detection of living cells has been based on the content of hydrolytic enzymes (39), cellular elongation (3), incorporation of radiolabelled substrates (35), electron transport chain activity (2, 34, 40, 42), the presence of internal reducing equivalents (30), membrane potential (15), or the amount of intracellular rRNA (8). Consequently, definitions of cell viability based on different methods understandably vary. Significantly, many viability indicators have not proven to be generally applicable to bacteria obtained from the environment or cultured in laboratories because of a wide range of growth requirements or the variability in the metabolic states of the bacteria present. From the large body of research conducted to date with chemical probes, it is apparent that a consensus regarding a universal chemical definition of cellular viability, as well as a universal method for determining this cell state, has not yet been reached.

Bacterial plasmolysis in response to osmotic stress was first demonstrated during the early 1900s (10). Hypertonic solutions have since been used to demonstrate gross morphological changes in a wide range of bacteria, with the cytoplasm shrinking and the plasma membrane contracting from the cell wall as a result of internal water loss (17, 27, 28, 37). Research has subsequently been conducted with sucrose and various salts in an attempt to understand membrane transport phenomena, stress resistance, activity, and cell surface physiology (12, 27, 36, 38).

While plasmolysis commonly occurs in gram-negative species, plasmolysis, as well as changes in cell area or volume, have also been documented in gram-positive bacteria (27, 36). The ability to undergo plasmolysis indicates that a semipermeable membrane is present and functioning to maintain protoplast integrity. For senescent or injured bacteria which may recover, the internal pools of metabolites (e.g., amino acids, ATP, nucleic acids) are theoretically retained if the cytoplasmic membrane remains intact. Consequently, the cells may survive and resume growth if favorable conditions are encountered. In the event of cell death, the cell membrane no longer functions as a selective barrier between the cell and the environment, which results in leakage of cytoplasmic materials into the surrounding milieu and a loss of cell metabolic function. Therefore, physical

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demonstration of a functional cell membrane may be used as a viability indicator for bacteria which are known to undergo plasmolysis.

Early research demonstrated the potential of osmotic challenge for viability determinations by spectroscopic methods. However, this batch approach required that the target cells be cultured and lacked the sensitivity to determine the responses of individual cells, attached cells, or cells from the environment (26). Because of advances in analytical imaging these physical responses can now be determined quantitatively. During the present study, the responses of biofilm bacteria (either a well-studied *Pseudomonas fluorescens* strain or the pathogen *Salmonella enteritidis*) inactivated with either ethanol, heat, formalin, or repeated osmotic challenge were compared with the responses of unstressed, viable (actively growing) cells. Three microscopic methods (low-magnification dark-field microscopy, high-magnification phase-contrast microscopy and scanning confocal laser microscopy [SCLM]), used with or without image analysis, were found to be suitable for enumerating viable cells when membrane integrity was used as a physical indicator. The results of this physical method for determining cell viability were compared with results obtained with a commercial fluorescent viability probe.

MATERIALS AND METHODS

Organisms and flow cells. *P. fluorescens* CC-840406-E and *S. enteritidis* ATCC 4931 were cultured in 1 g of glucose per liter of basal salts medium [3 mM $Na_2HPO_4 \cdot 7H_2O$, 2 mM KH_2PO_4, 0.8 mM $(NH_4)SO_4$, 0.4 mM $MgSO_4 \cdot 7H_2O$, 0.02 mM $Ca(NO_3)_2 \cdot 4H_2O$, 0.8 μM $FeSO_4 \cdot 7H_2O$] and in 0.3% (wt/vol) Trypticase soy broth (TSB) (BBL, Becton Dickinson, Cockeysville, Md.), respectively. Plasmolysis assays were initiated by inoculating flow cells with log-phase cells obtained from 50-ml batch cultures, as described previously (18, 22). The flow cells were then mounted on the stage of either a Zeiss Photomicroscope III microscope or a Nikon FXA–Bio-Rad MRC-600 SCLM while a continuous nutrient flow for high-magnification phase-contrast, low-magnification dark-field, or SCLM analysis was maintained.

Modified flow cells were required to achieve Köhler illumination for phase-contrast microscopy of the same areas studied by laser microscopy (i.e., during *S. enteritidis* salt stress studies). These flow cells (1.3 by 50 by 80 mm) were constructed by using glass coverslips (no. 1 thickness; Corning Glassware), a silicone gasket, polycarbonate plastic blocks (Lexan; General Electric), and type S/P medical-grade silicone tubing (inside diameter, 1.5 mm; outside diameter, 3.18 mm; Dow Corning Co., Baxter, Ill.). The flow cells were mounted on a flow cell base which was custom milled to align with the stage mounts of the Nikon FXA microscope.

Defining NaCl concentrations for plasmolysis of *P. fluorescens* biofilms. Studies were conducted to determine NaCl concentrations which induced cellular plasmolysis but did not damage the cells or inhibit cell growth after the salt stress was relieved. A suspension of log-phase *P. fluorescens* cells was used to inoculate flow cells irrigated with glucose-M salts medium (laminar flow velocity, 0.13 cm s^{-1}). Approximately 20 to 40 attached cells per microscopic field (at high magnification) were present following the inoculation procedure. Cells were cultivated under flowing conditions for 1 h in the absence of salt stress before we switched to a reservoir containing growth medium (glucose-M salts medium) amended with 0.3, 1.0, 1.5, or 2.0 M NaCl. The cells were then irrigated with flowing NaCl-amended growth medium for 1 h before we switched back to the growth medium. Application of 1-h-on–1-h-off salt stress was repeated three times during the 6-h experiment. Changes in the cell protoplast size were monitored by high-magnification phase-contrast microscopy, measured as described below, and reported as the mean cell area of 10 individual cells.

Pulse treatment of viable and nonviable *P. fluorescens* with 1.5 M NaCl. Log-phase *P. fluorescens* cells were used to inoculate flow cells as described above. Cells were grown in the flow cells for 1 h in the presence of glucose-containing growth medium before we switched to growth medium amended with 1.5 M NaCl. Photomicrographs were obtained immediately before addition of salt and 5 min after salt stress. Images were also digitized for on-line computer measurement of cell responses (i.e., changes in cross-sectional area) to NaCl (see below). Initially, all bacteria in the flow cells were viable, as confirmed by photomicroscopy and image analysis, as well as by visual confirmation of cell elongation and division. A 2-ml pulse of 75% ethanol (exposure time, 2 min) was then used to kill all of the cells, after which introduction of salt was repeated to document whether the cells retained the ability to plasmolyze.

Low-magnification dark-field microscopy was also used in conjunction with image analysis to quantify the response of a large number of cells exposed to 1.5 M NaCl. Bacteria were cultivated in flow cells as described above. Ten random fields, each including approximately 100 individual cells, were then measured before and after application of NaCl. A single representative field of analysis was also used to acquire two images of the same microscopic field (one before salt application and one after salt application) for difference imagery. These images were then digitally subtracted to quantify the number of plasmolysis-responsive cells. Cells whose light-scattering properties did not change following salt stress, either in terms of difference imagery for individual cells or in terms of mean cell area for populations of cells, were considered nonviable. A comparative dark-field analysis of killed cells was performed as described above.

Pulse treatment of viable and nonviable *S. enteritidis* biofilms with 1.5 M NaCl. Nonviable *S. enteritidis* cultures were prepared by killing biofilm cells, which had been cultured in 0.3% TSB under flowing conditions, with either heat or formalin. Heat-killed *S. enteritidis* biofilms were obtained by immersing the flow cell chamber and connected tubing in a heated (65°C) water bath (Lab-Lite Magnastir, Melrose Park, Ill.) for 10 min. Formalin-killed biofilms were prepared by injecting 4% formalin (36 to 38% [wt/vol] formaldehyde; BDH) into the flow cell, allowing 10 min for cell inactivation, and washing the biofilm with sterile growth medium.

Biofilms containing a mixture of viable and heat-killed cells were prepared by reinoculating flow cells containing heat-killed cells with log-phase *S. enteritidis* cells. Similarly, biofilms containing a mixture of viable and formalin-killed cells were prepared by reinoculating flow cells containing formalin-killed cells with log-phase *S. enteritidis* cells while the nutrient flow was maintained. NaCl plasmolysis assays were then performed by using viable, heat-killed, formalin-killed, and mixed viable-nonviable cell preparations. Prior to initiation of the plasmolysis assays, the condition of viable and nonviable *S. enteritidis* cells was determined by using high-magnification phase-contrast microscopy and cell elongation-division. The plasmolysis assay was performed by injecting 2 ml of TSB amended with 1.5 M NaCl into the flow cell.

The physical response (plasmolytic contraction of the cell membrane and cytoplasm from the cell wall) of the cells was then documented by high-magnification phase-contrast photomicroscopy and/or SCLM. During SCLM plasmolysis assays, NaCl was added to the fluorescein stock solution to obtain a final concentration of 1.5 M. This solution was then injected into the flow cell for use as a negative stain (5), which permitted detection of changes in the cell membrane and location of the protoplast. Lastly, a BacLight Live/Dead viability probe (Molecular Probes, Inc., Eugene, Oreg.) was used in conjunction with NaCl to evaluate the suitability of plasmolysis as a physical indicator of cell viability. The BacLight assay reagent (0.3 ml) was injected into a flow cell containing either viable cells, nonviable cells or viable-nonviable cell mixtures, and the preparation was incubated for 15 min in the dark. A 1.5 M NaCl solution was then injected into the same chamber. Cells were then observed by both photomicroscopy and SCLM and analyzed as described above.

Dark-field microscopy, phase-contrast microscopy, and SCLM. A Zeiss model PMIII microscope was equipped with either a ×10 dark-field (0.22 numerical aperture) or a ×100 phase-contrast (1.3 numerical aperture) objective lens (6, 22). Dark-field and phase-contrast photomicrographs were taken by using Ektachrome color or Tmax black-and-white film (Kodak, Rochester, N.Y.). During phase-contrast microscopy, a green interference filter was used to reduce chromatic aberration. SCLM optical thin sections were obtained by using a model MRC-600 Lasersharp system (Bio-Rad Microscience, Ontario, Canada) equipped with an argon laser source and mounted on a Nikon model FXA microscope equipped with a ×60 objective (1.4 numerical aperture) (18, 20). A blue high-sensitivity filter was used for imaging fluorescence in the green spectrum (i.e., fluorescein). Dual-channel photodetectors and filter sets were used for simultaneous imaging of green and red fluorescence emitted from cells labeled with the BacLight viability probe. Single or dual-channel images were acquired in either the horizontal (*xy*) or vertical (*xz*) plane of analysis, as described previously (18). The Nikon model FXA microscope was equipped with a computer-controlled *xy* motorized stage, which permitted relocation of multiple fields before and after salt treatment.

Fluorescent probes. Fluorescein (molecular weight, 289; Sigma Chemical Co., St. Louis, Mo.), which was used for negative staining, was prepared as a 0.1% (wt/vol) solution (pH 7.0) and was aseptically injected with a syringe and a 27-gauge needle into the flow cell prior to SCLM analysis of the biofilms (5). To determine the efficacy of plasmolysis as a physical indicator of viability, killed and living bacteria were also assayed by using SCLM and the BacLight Live/Dead viability probe. Equal volumes of BacLight reagents A and B were added to sterile 10% TSB to prepare the viability probe (final concentration, 3 μg/ml). Approximately 0.3 ml of this solution was aseptically injected into the flow cell chamber. Following a 15-min reaction period, the attached cells were imaged by using dual-channel SCLM, and their fluorescent response was quantified. In accordance with the instructions provided with the BacLight kit, cells which stained green were considered viable and red cells were considered nonviable.

Digital imaging and analyses. A computer image analysis of the growth and plasmolytic response of *P. fluorescens* cells was performed by using a Dage model 66 ISIT camera directly mounted on the Zeiss model PMIII microscope equipped with a high-magnification phase-contrast objective. The results were analyzed by using an IBAS 2000 computer or a PC-based image analysis system (22). On-line dark-field microscopy image analysis, in conjunction with difference imagery, was also performed as previously reported (19).

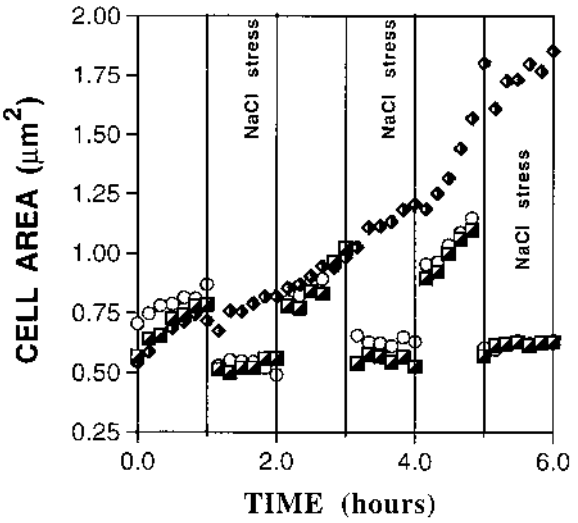

FIG. 1. Response of attached *P. fluorescens* cells to a range of intermittently applied salt concentrations (0.3, 1.0, and 1.5 M NaCl). Cells were cultured in flow cells in the absence of NaCl, and then salt was applied at 1.0, 3.0, and 5.0 h. Salt stress was relieved at 2.0, 4.0, and 6.0 h. Changes in cell protoplast area, as well as the ability of the cells to increase in area over time, were quantified by performing a high-magnification, phase-contrast image analysis. Symbols: ◆, 0.3 M NaCl; ◪, 1.0 M NaCl; ○, 1.5 M NaCl.

High-magnification phase-contrast photomicrographs were scanned with a 35-mm slide scanner (Nikon Coolscan; Nikon Inc., Melville, N.Y.) used in conjunction with Adobe Photoshop (Adobe Systems, Inc., Mountain View, Calif.). Processed images were then imported into Ultimage (GTFS, Inc., Santa Rosa, Calif.) for digital analysis of cell number and cell area before and after salt treatment. Longitudinally attached cells were selected from each field for cell area analysis. All other cells were digitally deleted.

Processing and analysis (biomass area determinations) of dual-channel SCLM images were performed by using a Northgate host computer and software provided by Bio-Rad. Bleed-through of the green fluorescence into the red channel was subtracted from all dual-channel images to improve image quality. Digital optical thin sections were directly printed by using Adobe Photoshop and a Primera photo-quality printer (model 76000; Fargo Electronics, Eden Prairie, Minn.).

RESULTS AND DISCUSSION

Effect of NaCl concentration on plasmolysis and growth of sessile *P. fluorescens*. *P. fluorescens* biofilms grown in flow cells were intermittently exposed to hypertonic NaCl solutions to determine the concentration(s) of NaCl which induced detectable cell plasmolysis (determined directly by phase-contrast microscopy) without causing cell death or irreversibly interfering with the cells' ability to grow after salt stress was removed. Accordingly, attached bacteria were cultivated on surfaces in flow cells for 1 h by using 1 g of glucose per liter of M salts medium (to confirm cell viability by increases in cell area) before the medium was changed to glucose-M salts medium amended with 0.3, 1.0, 1.5, or 2.0 M NaCl. When *P. fluorescens* biofilms were exposed to 0.3 M NaCl, the cell area continued to increase at a constant rate (Fig. 1), but the cells did not visibly plasmolyze. At higher salt concentrations (1.0 to 1.5 M NaCl) all cells plasmolyzed, as confirmed by decreases in cell area measurements (Fig. 1). The apparent decreases in the cell area of plasmolyzed bacteria during phase-contrast microscopy observations were the result of condensation or shrinkage of the cell protoplast, which decreased the area of phase-dense cell material. When plasmolyzed, cells ceased to increase in cell area; however, cells were able to resume growth upon removal of salt stress (Fig. 1). When 2.0 M NaCl was used, cells did not resume growth after the medium was switched back to unamended growth medium (data not shown).

The rate of cell growth between periods of salt stress appeared to vary inversely with the concentration of NaCl (at 0.3 M NaCl the growth rate was $0.32 \pm 0.13\ h^{-1}$, at 1.0 M NaCl the growth rate was $0.29 \pm 0.07\ h^{-1}$, and at 1.5 M NaCl the growth rate was $0.24 \pm 0.06\ h^{-1}$) (Fig. 1), suggesting that the cells were not able to switch between saline and nonsaline conditions without incurring a metabolic penalty. Cells did not grow between periods of salt stress when 2.0 M NaCl was used (data not shown); therefore, it was assumed that the recovery period (1 h) was not sufficient for cells to regulate their internal water and/or transmembrane ionic gradients. However, 2 M NaCl was not toxic to the *P. fluorescens* cells (i.e., the cells eventually grew and divided following NaCl removal).

Extremely hypertonic solutions are known to inhibit or kill certain bacteria (which is of significance to food processors) (38); however, physiological mechanisms which developed over evolutionary time permit the survival as well as the growth of many bacteria under a broad range of less extreme osmotic conditions commonly encountered in nature (halobacteria employ unique mechanisms for salt tolerance and are not considered here). Early studies by Scheie (37) provided evidence that significant numbers of *Escherichia coli* cells subjected to hypertonic solutions of sucrose or NaCl (concentrations up to 1.0 M) grew to form colonies when they were transferred to agar plates having the same osmotic strength.

Studies to examine the effect of water activity on microorganisms are frequently conducted by using suspended bacteria grown in batch culture (12, 16), even though most organisms from terrestrial or aquatic environments primarily live attached to surfaces as biofilms. In soils which undergo periodic dehydration, salts become concentrated in hydroscopic water associated with particles which many bacteria have colonized. In general, the in situ response to water stress and survival of attached bacteria under water stress conditions are poorly understood, but remain a fundamental part of the natural life cycle of indigenous soil and sediment bacteria.

Sperber (38) described two possible scenarios by which bacteria could regulate their internal water activity so as to retain cell turgor and metabolic activity following plasmolysis. The first scenario requires that a cell retains enzymatic function even though a solute with reduced water activity has entered the cell. The second scenario involves the cell internally accumulating a different solute (distinct from the external compound causing hyperosmotic conditions) which balances the intracellular water activity required for enzyme function. Numerous reports support the hypothesis that many bacteria accomplish osmotic regulation through accumulation and loss of amino acids (11, 12, 16, 36).

The nature of the growth medium, including the ion concentration, is significant and may well have an impact on the extent of cell plasmolysis. For example, Scheie (37) demonstrated the tendency for some media to provide protection against bacterial plasmolysis, whereas other medium constituents promoted plasmolysis. In addition, bacteria isolated from marine or saline environments and bacteria isolated from nonsaline systems may respond differently. Gram-positive bacteria have also been shown to plasmolyze when they are subjected to hypertonic media (27, 36), but their thick peptidoglycan layer can make the response more difficult to quantify than the response of gram-negative bacteria. It is thus essential to conduct control experiments, such as those described above, for different media or different bacteria, in order to assure that the salt concentrations are sufficient to induce a detectable but nontoxic plasmolytic response.

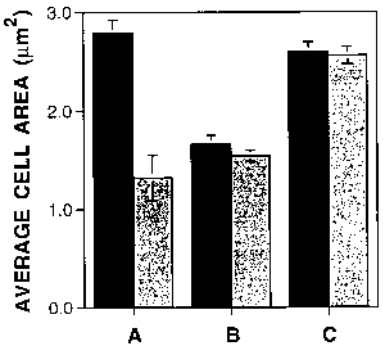

FIG. 2. Response of viable (columns A), formaldehyde-killed (columns B), and heat-killed (columns C) *S. enteritidis* cells to a 1.5 M NaCl solution. Flow cells were inoculated and subsequently irrigated with sterile growth medium, which allowed the development of biofilms. The biofilms were then treated with 4% (vol/vol) formaldehyde (columns B) for 5 min or heated in a heated water bath (65 ± 1°C) for 10 min (columns C) before the salt was added. High-magnification phase-contrast microscopy and digital image analysis were used to quantify changes in cell area ($n > 12$ fields, each containing ~25 cells) due to plasmolysis. Nonviable cells (columns B and C) were not responsive to the salt solution, whereas viable cells (columns A) readily plasmolyzed, as shown by the reduction in cell area. The error bars represent the 95% confidence intervals. Solid columns, no NaCl; shaded columns, NaCl.

Plasmolysis as a physical indicator of bacterial viability. The need for direct methods for determining the metabolic condition of water-stressed organisms, the survival of introduced bacteria, and the efficacy of antimicrobial treatments has been stressed previously (35, 42). A nondestructive, nontoxic method provides an advantage over fluorochemical techniques by allowing temporal studies to be conducted without adversely affecting the microbial populations. Extrinsically applied osmotic stress has proven to be useful for elucidating structure-function details of the bacterial cell and cell membrane (9, 11, 12, 17, 36). From such studies and ultrastructural studies, it has become clear that a primary function of the cell membrane is selective regulation of both intra- and extracellular ions and solutes necessary for sustained metabolic activity and ultimately cell reproduction. The presence of a functional cell membrane may consequently serve as an index of viability which is independent of cell growth. Below we discuss the utility of various microscopic techniques for detecting plasmolytic responses of sessile bacteria.

Phase-contrast microscopy. Phase-contrast microscopy was used to quantify the responses of both viable and nonviable cell populations and individuals to salt stress. When *S. enteritidis* cells were grown in flow cells and exposed to 1.5 M NaCl, an approximately 50% reduction in cell protoplast area was observed (the mean cell area before NaCl stress was 2.8 ± 0.8 μm^2, and the mean cell area after NaCl stress was 1.35 ± 0.7 μm^2) (Fig. 2). In contrast, heat-killed and formalin-killed populations of *S. enteritidis* were unresponsive to NaCl treatment. The results obtained with a mixed population of viable and heat-killed *S. enteritidis* cells clearly illustrate the tendency for only viable cells to plasmolyze in the presence of NaCl (Fig. 3). In this case, all six viable cells exhibited growth during a 10-min period before salt application, during which each cell underwent a significant, detectable change (increase) in cell area. Nine killed cells did not grow during the same interval, nor did they change in cell area following NaCl application, presumably because the cell membrane had been breached during the killing procedure.

In general, some increase in osmotic pressure must occur for cells to plasmolyze (37). For example, once cells have equilibrated with high salt conditions, slight changes in solution osmotic strength may not necessarily result in plasmolysis. Alternatively, if cells are grown at low salt concentrations, a small change (i.e., from 0.2 to 0.4 M NaCl) may be sufficient to induce plasmolysis. The magnitude of osmotic change may also influence the extent and speed of plasmolysis (7, 37). For example, Caldwell and Lawrence (7) showed that the rate of *P. fluorescens* plasmolysis was dependent on magnesium sulfate concentration, with 0.3 M $MgSO_4$ causing a gradual reduction in the areas of affected cells and 1.5 M $MgSO_4$ causing much more rapid changes in cell area.

Furthermore, if cells were repeatedly plasmolyzed and deplasmolyzed, they could be killed even though the salt concentration was sublethal when a recovery period was provided. Rapid cycling between a hypertonic solution (medium containing 1.5 M NaCl) and growth medium was found to exceed the ability of *P. fluorescens* cells to regulate their internal water; this treatment apparently damaged the cell membrane and resulted in cell death. Figure 4A and B show a typical field of bacteria following the first plasmolysis event, in which all cells plasmolyzed following treatment with 1.5 M NaCl. Following four cycles of plasmolysis and deplasmolysis within a 15-min period, cells became unresponsive to fluxes in salt concentration and appeared to be phase light, presumably after they lost their cytoplasmic contents to the bulk phase. These cells did

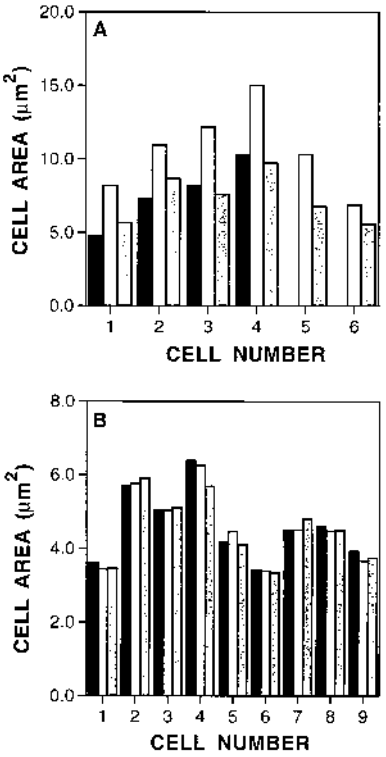

FIG. 3. Response of a mixed culture of viable (A) and nonviable (B) *S. enteritidis* cells to a 1.5 M NaCl solution. Flow cells were inoculated and subsequently irrigated with sterile growth medium, which allowed the development of biofilms. The flow cells were then placed in a heated water bath (65 ± 1°C, 10 min), which killed all of the cells. Viable *S. enteritidis* cultures were then used to reinoculated the flow cells, which resulted in a mixed population of viable and nonviable cells. Phase-contrast microscopy and digital image analysis were then used to detect changes in the cell areas (following 1.5 M NaCl stress) of individual cells present in the field of analysis. Data for six viable cells (A) and nine nonviable cells (B) are shown. The bars represent the areas of selected cells at time zero (solid bars), after a 10-min incubation period (lightly shaded bars), and after salt was added to the cultures (darkly shaded bars).

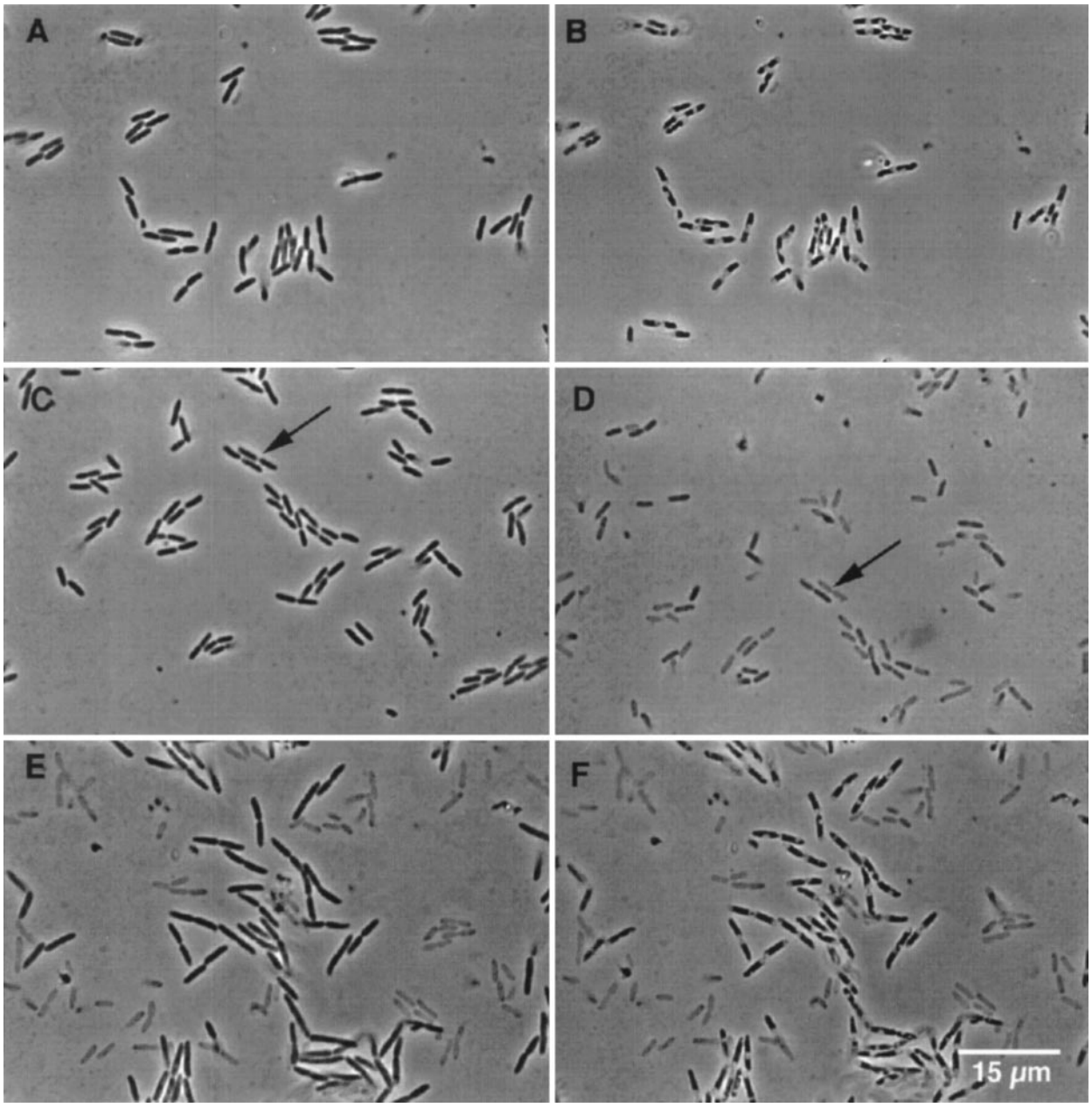

FIG. 4. High-magnification phase-contrast images of *P. fluorescens* cells before and after repeated osmotic stress (rapid switching between growth medium and medium amended with 1.5 M NaCl). (A and B) Actively growing cells before (A) and after (B) application of NaCl. Note that all cells underwent plasmolysis after pulse application of 1.5 M NaCl. (C) New field of viable bacteria before repeated application of salt stress. (D) Same bacteria as in panel C, now appearing phase light and no longer capable of plasmolytic response or cellular division even after extended periods. (E) Following a 16-h period of survivor regrowth, both viable (phase-dark) and nonviable (phase-light) cells were observed in the same field as the field in panel D. (F) Plasmolytic response (and subsequent growth) confirmed that the phase-dark cells were viable, whereas the phase-light cells were nonviable.

not resume growth even after an extended recovery period and were thus confirmed to have died (Fig. 4C and D). After ~16 h of cultivation in the absence of salt, surfaces adjacent to attached dead cells eventually became recolonized by surviving bacteria and their progeny (Fig. 4E). These phase-dark, viable cells could be plasmolyzed by 1.5 M NaCl, whereas adjacent dead cells could not be plasmolyzed (Fig. 4F), clearly demonstrating the sensitivity of this method for detecting living cells on the basis of a functional cell membrane. It is worth noting that rapid, repeated changes in osmotic strength are not common to many natural environments; therefore, most bacteria have not adapted to tolerate these conditions.

Dark-field microscopy. Computer-enhanced dark-field microscopy was used as a direct method to detect the plasmolytic response of populations of attached *P. fluorescens* cells. Cells were osmotically challenged by changing the substrate reservoir from a reservoir containing 10% TSB to a reservoir containing 10% TSB amended with 1.5 M NaCl. The mean dark-field areas (obtained from 15 random fields) for unstressed and salt-stressed *P. fluorescens* cells are shown in Fig. 5. Before

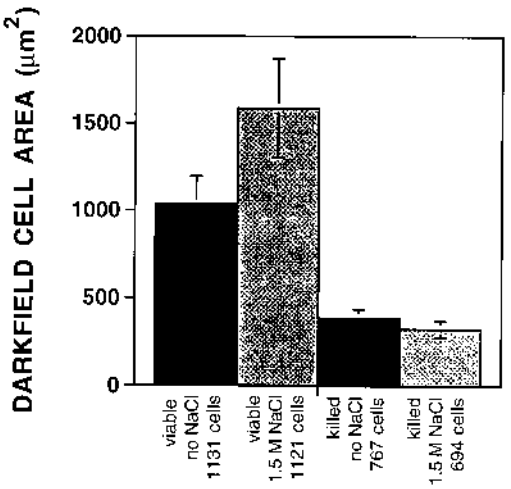

FIG. 5. Response of attached viable and nonviable *P. fluorescens* cells to a solution of 1.5 M NaCl. Cells were cultured in flow cells in growth medium for 1 h, after which a 1.5 M solution of NaCl was applied. Changes in the cell protoplast area, as well as the ability of the cells to increase in area over time, were quantified by performing a low-magnification, dark-field image analysis. Cells were killed with 75% ethanol.

plasmolysis, viable cells had a mean field area of 1,035 ± 162 μm^2 (n = 1,131 cells), whereas after cells were plasmolyzed, this value increased by ~50% to 1,588 ± 284 μm^2 (n = 1,121 cells) because of a large increase in light scattering due to the compacted (plasmolyzed) cytoplasmic materials. By performing difference imagery (image subtraction [19]) with an individual microscopic field before and after plasmolysis, it was determined that all of the *P. fluorescens* cells present within the field exhibited a detectable, plasmolysis-induced increase in light scattering. In early studies in which packed cell volumes of *Photobacterium fischeri* cells were measured directly, the authors concluded that when this organism was challenged with hypertonic solutions, it shrank as much as 27% (14).

P. fluorescens cells were killed by directly injecting a 75% ethanol solution into the flow cell for a 2 min period. Ethanol rapidly penetrates cells, dehydrating the cytoplasm, denaturing proteins, and rendering the membrane permeable. This treatment, like the heat and formalin treatments, also resulted in the loss of the cells' ability to respond to changes in osmotic pressure. Figure 5 shows the effect of ethanol on the mean dark-field area for unstressed and salt-stressed *P. fluorescens* cells. The mean dark-field area for killed bacteria (which because of ethanol treatment exhibited a decrease in overall light scattering compared with living cells) in the absence of 1.5 M NaCl was 384 ± 51 μm^2 (n = 767 cells), whereas in the presence of NaCl the area was 323 ± 46 μm^2 (n = 694 cells). Difference imagery failed to detect differences in dark-field area between individual treated and untreated bacteria within the same microscopic fields of analysis.

Dark-field microscopy has previously been integrated with digital imaging to quantitatively analyze the growth, development, and behavior of attached bacteria in flowing microenvironments (22, 24, 25). Light-scattering applications have also been used to study osmotically induced changes in growing suspensions of bacteria. For example, Koch (17) performed turbidometric measurements on *E. coli* cells by using narrow-beam spectrophotometry to demonstrate that the apparent optical density of bacteria increased with increasing external osmotic pressure. Earlier, Mager et al. (26) demonstrated that suspensions of viable gram-negative bacteria in solutions with high osmotic pressures had greater optical densities than suspensions of the same living bacteria in distilled water. Killed cells failed to show any change in optical density when the osmotic pressure of the suspending liquid was changed. While the mechanisms leading to changes in culture optical density used by Mager et al. are analogous to the mechanisms in operation during the present study, the batch culture approach lacked the sensitivity to identify changes in individual cells and could not determine the condition of cells which are attached to surfaces.

SCLM. SCLM, used in conjunction with negative staining, was also found to be suitable for detecting changes in *S. enteritidis* cell morphology due to plasmolysis. Typical fluorescence patterns of negatively stained bacteria are shown in Fig. 6A. This image shows that the extracellular (bulk phase) concentrations of fluorescein were higher than the intracellular fluorescein concentrations, which caused the cells to appear darker relative to the brighter background. Following plasmolysis, the cells appeared to concentrate fluorescein between their cell walls and cell membranes, resulting in a bright fluorescent zone where the cytoplasmic membrane had contracted away from the cell wall (Fig. 6B). In general, the patterns of cell plasmolysis appeared to be consistent with the patterns expected for actively dividing cells, as opposing cell poles contained condensed cytoplasmic materials even though many of the cells did not show evidence of septation. The three degrees

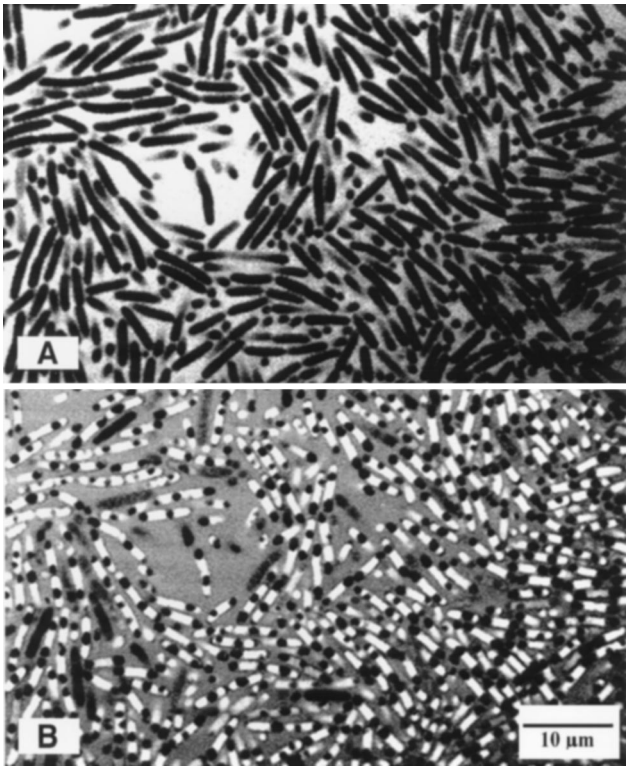

FIG. 6. Horizontal (*xy*) optical thin sections of a negatively stained *S. enteritidis* biofilm in growth medium (A) and in salt solution (B). Flow cells were inoculated and irrigated with sterile growth medium for 24 h at room temperature. A pulse of 1.5 M NaCl was then applied to ascertain the viability of morphologically similar biofilm cells. Most cells were positively stained following the addition of a salt and fluorescein solution; however, a small number of cells (1%) remained negatively stained. Direct observation by phase-contrast microscopy confirmed that all cells plasmolyzed in the salt solution. An artifact, stemming from the confocal nature of the SCLM system, resulted in this apparent lack of response of some cells to NaCl in the presence of fluorescein.

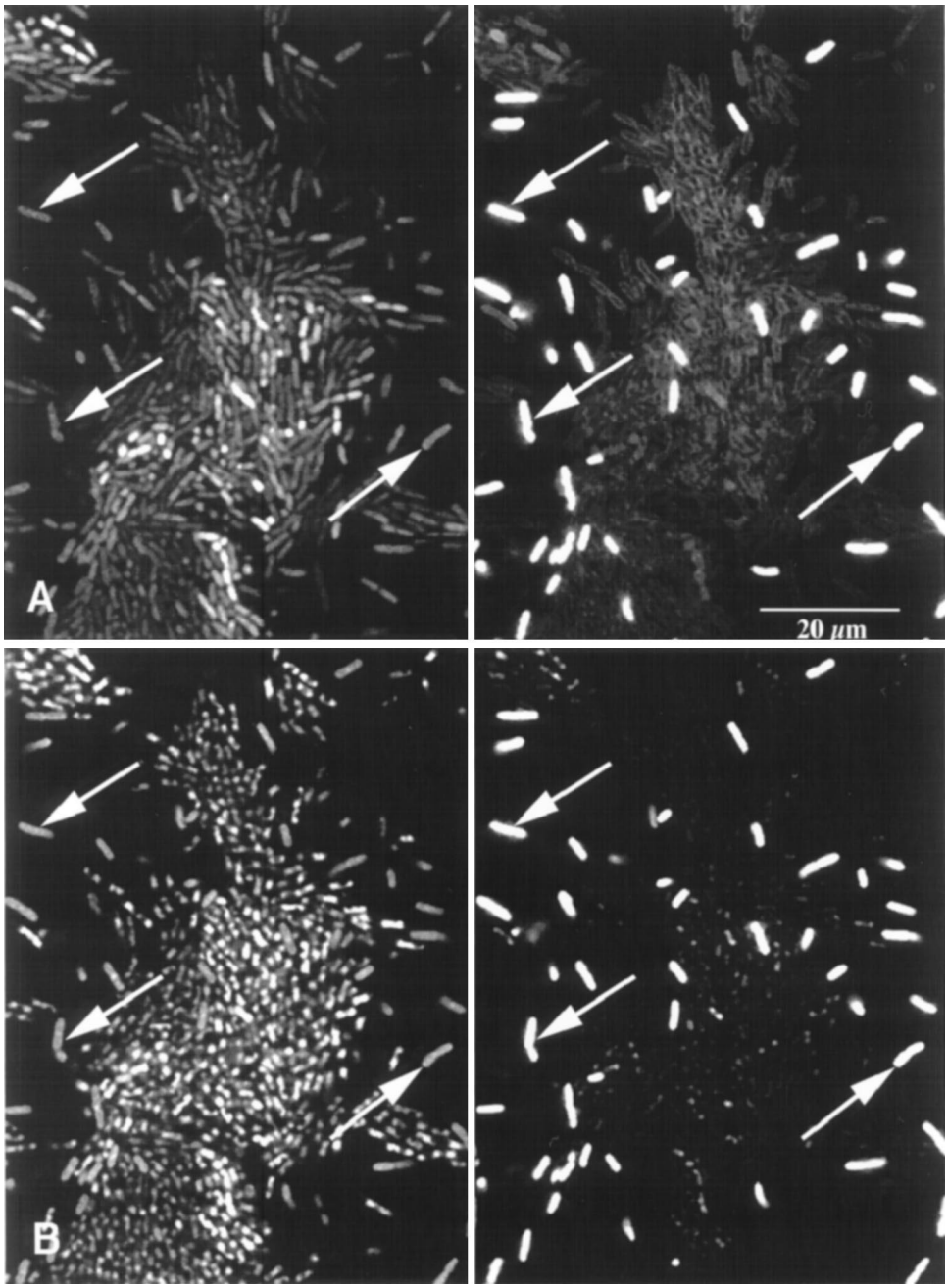

FIG. 7. Dual-channel (red-green) horizontal (*xy*) thin sections showing the response of a mixed culture of formaldehyde-killed and viable *S. enteritidis* cells stained with the BacLight viability probe in growth medium (A) and in salt solution (B). The arrows indicate the positions of the same (representative) nonviable bacteria for each image pair. In panel A, note that while cells located at the arrows emitted green fluorescence (left channel), they also emitted much stronger red fluorescence (right channel), indicating that they were not viable. When a 1.5 M NaCl salt solution was added to the system (B), the cells indicated by the arrows were incapable of plasmolysis, which confirmed that they were not viable.

of plasmolysis described by Scheie (37) did not include the observed patterns of plasmolysis documented during the present study; however, Scheie focused on the response of planktonically cultivated *E. coli* cells instead of the response of *S. enteritidis* biofilm bacteria used during the present study.

A small percentage of the cells in Fig. 6B did not show evidence of plasmolysis when SCLM and negative staining were used; however, the nature of the confocal image (actually a horizontal optical thin section ~0.2 μm thick) does not preclude the possibility that plasmolysis occurred in other locations of the cells not shown in the thin section. Phase-contrast analysis of the same microscopic field subsequently confirmed that all of the bacteria shown in Fig. 6B actually plasmolyzed (data not shown). Fluorescein staining thus provides a method for effective differentiation between the fluorescence patterns of living cells (which plasmolyze) and dead cells (which negatively stain but do not plasmolyze). While this approach was quite effective for identifying cells positioned horizontally along an attachment surface, it was less appropriate for examining the response of cells randomly oriented in

three dimensions within a biofilm. Consequently, the plasmolysis method for defining cell viability may be best suited for examining cells located at the base of the biofilm (the biofilm-substratum interface) when SCLM is used. Notably, this cell population is generally considered the population that is most resistant to biocides and antimicrobial treatments (21) and is thus of interest.

A commercial fluorescent viability probe (the BacLight Live/Dead viability probe) was also used to examine living and killed cells to determine how chemical indicators of membrane permeability correlated with a physical indicator of membrane function (plasmolysis). SCLM photomicrographs (Fig. 7A) show a mixture of living and heat-killed *S. enteritidis* cells; living and dead cells both emitted green fluorescence and hence appeared in the left channel of the dual-channel image. However, only dead cells emitted high-intensity, red fluorescence and could therefore be clearly identified in the right channel of Fig. 7A. Dead cells, as indicated by the Live/Dead probe, did not plasmolyze when they were exposed to 1.5 M NaCl (Fig. 7B, arrows in the left channel). Thus, a combination of two methods to indicate bacteria viability confirmed the status of the cells both physically and chemically. With this approach, quantitative measurements of cell viability could be made either manually or by using digital image analysis.

Conclusions. Defining cell death is philosophically and experimentally difficult (32), as cells may not actually be dead but may simply be unresponsive to the set of conditions provided. However, no consensus regarding a definitive indicator of viability has been reached, and consequently a number of approaches are still used to define the number of viable cells in various cell populations. It is thus apparent that an index of viability which is independent of the growth of bacteria and is also nontoxic would be both advantageous and is necessary.

Using bacterial plasmolysis as an indication of cell membrane integrity provides a rapid, inexpensive method for determining cellular viability. High-magnification phase-contrast microscopy may be used without epifluorescence hardware, and counts may be obtained by manual counting. Combining plasmolysis viability detection methods with computer image-processing hardware provides a more analytical way to determine the number of living and dead cells present on surfaces or obtained from solution. When dark-field computer-enhanced microscopy is used, it is also possible to analyze larger populations of bacteria. Furthermore, when plasmolysis is used in conjunction with SCLM and fluorescent probes, such as fluorescein, determinations may be made at the base of thick microbiological films. Overall, these approaches should prove to be valuable for the evaluation of the efficacy of biocides or antimicrobial agents against biofilms formed by bacteria known to undergo plasmolysis.

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REFERENCES

1. **Atlas, R. M.** 1984. Use of microbial diversity measurements to assess environmental stress, p. 540–545. *In* M. J. Klug and C. A. Reddy (ed.), Current perspectives in microbial ecology. ASM Press, Washington, D.C.
2. **Betts, R. P., P. Bankes, and J. G. Banks.** 1989. Rapid enumeration of viable micro-organisms by staining and direct microscopy. Lett. Appl. Microbiol. **9:**199–202.
3. **Bottomley, P. J., and S. P. Maggard.** 1990. Determination of viability within serotypes of a soil population of *Rhizobium leguminosarum* bv. *trifolii*. Appl. Environ. Microbiol. **56:**533–540.
4. **Brock, T. D.** 1987. The study of microorganisms *in situ*: progress and problems, p. 1–20. *In* M. Fletcher, T. R. G. Gray, and J. G. Jones (ed.), Ecology of microbial communities. Cambridge University Press, Cambridge.
5. **Caldwell, D. E., D. R. Korber, and J. R. Lawrence.** 1992. Imaging of bacterial cells by fluorescence exclusion using scanning confocal laser microscopy. J. Microbiol. Methods **15:**249–261.
6. **Caldwell, D. E., and J. R. Lawrence.** 1986. Growth kinetics of *Pseudomonas fluorescens* microcolonies within the hydrodynamic boundary layers of surface microenvironments. Microb. Ecol. **12:**299–312.
7. **Caldwell, D. E., and J. R. Lawrence.** 1989. Microbial growth and behavior within surface microenvironments, p. 140–145. *In* T. Hattori, Y. Ishida, Y. Maruyama, R. Y. Morita, and A. Uchida (ed.), Recent advances in microbial ecology. Japan Scientific Societies Press, Tokyo.
8. **Daley, R. J.** 1979. Direct epifluorescence enumeration of native aquatic bacteria: uses, limitations, and comparative accuracy, p. 29–45. *In* J. W. Costerton and R. R. Colwell (ed.), Native aquatic bacteria: enumeration, activity, and ecology. American Society for Testing and Materials publication no. 695.
9. **Dhavises, G., and G. D. Anagnostopoulos.** 1979. Influence of amino acids and water activity on the growth of *Escherichia coli* B/r/1. Microbios Lett. **7:**105–115.
10. **Fischer, A.** 1900. *In* A. C. Jones (ed.), The structure and function of bacteria, p. 4–9. Oxford Press, Oxford.
11. **Geesey, G. G., and R. Y. Morita.** 1981. Relationship of cell envelope stability to substrate capture in a marine psychrophilic bacterium. Appl. Environ. Microbiol. **42:**533–540.
12. **Hua, S.-S. T., V. Y. Tsa, G. M. Lichens, and A. T. Noma.** 1982. Accumulation of amino acids in *Rhizobium* sp. strain WR1001 in response to sodium chloride salinity. Appl. Environ. Microbiol. **44:**135–140.
13. **Jensen, V.** 1968. The plate count technique, p. 158–170. *In* T. R. G. Gray and D. Parkinson (ed.), The ecology of soil bacteria. University of Toronto Press, Toronto.
14. **Johnson, F. H., and E. N. Harvey.** 1937. The osmotic and surface properties of marine luminous bacteria. J. Cell. Comp. Physiol. **9:**363–380.
15. **Kaprelyants, A. S., and D. B. Kell.** 1992. Rapid assessment of bacterial viability and vitality by rhodamine 123 and flow cytometry. J. Appl. Bacteriol. **72:**410–422.
16. **Killham, K., and M. K. Firestone.** 1984. Salt stress control of intracellular solutes in streptomycetes indigenous to saline soils. Appl. Environ. Microbiol. **47:**301–306.
17. **Koch, A. L.** 1984. Shrinkage of growing *Escherichia coli* cells by osmotic challenge. J. Bacteriol. **159:**919–924.
18. **Korber, D. R., G. A. James, and J. W. Costerton.** 1994. Evaluation of fleroxacin activity against established *Pseudomonas fluorescens* biofilms. Appl. Environ. Microbiol. **60:**1663–1669.
19. **Korber, D. R., J. R. Lawrence, K. E. Cooksey, B. Cooksey, and D. E. Caldwell.** 1989. Computer image analysis of diatom chemotaxis. Binary: Comput. Microbiol. **1:**155–168.
20. **Korber, D. R., J. R. Lawrence, M. J. Hendry, and D. E. Caldwell.** 1993. Analysis of spatial variability within mot^+ and mot^- *Pseudomonas fluorescens* biofilms using representative elements. Biofouling **7:**339–358.
21. **Korber, D. R., J. R. Lawrence, H. M. Lappin-Scott, and J. W. Costerton.** 1995. Growth of microorganisms on surfaces, p. 15–45. *In* H. M. Lappin-Scott and J. W. Costerton (ed.), Microbial biofilms. Cambridge University Press, Cambridge.
22. **Korber, D. R., J. R. Lawrence, B. Sutton, and D. E. Caldwell.** 1989. Effect of laminar flow velocity on the kinetics of surface recolonization by mot^+ and mot^- *Pseudomonas fluorescens*. Microb. Ecol. **18:**1–19.
23. **Kurath, G., and R. Y. Morita.** 1983. Starvation-survival physiological studies of a marine *Pseudomonas* sp. Appl. Environ. Microbiol. **45:**1206–1211.
24. **Lawrence, J. R., D. R. Korber, and D. E. Caldwell.** 1989. Computer-enhanced darkfield microscopy for the quantitative analysis of bacterial growth and behavior on surfaces. J. Microbiol. Methods **10:**123–138.
25. **Lawrence, J. R., D. R. Korber, and D. E. Caldwell.** 1992. Behavioral analysis of *Vibrio parahaemolyticus* variants in high- and low-viscosity microenvironments using computer-enhanced microscopy. J. Bacteriol. **174:**5732–5739.
26. **Mager, J., M. Kuckzynski, G. Schatzberg, and Y. Avi-Dor.** 1956. Turbidity changes in bacterial suspensions in relation to osmotic pressure. J. Gen. Microbiol. **14:**69–76.
27. **Marquis, R. E.** 1968. Salt-induced contraction of bacterial cell walls. J. Bacteriol. **95:**775–781.
28. **Mitchell, P., and J. Moyle.** 1956. Osmotic function and structure in bacteria. Symp. Soc. Gen. Microbiol. **6:**150–180.
29. **Morita, R. Y.** 1985. Starvation and miniaturisation of heterotrophs with special emphasis on maintenance of starved viable state, p. 111–130. *In* M. Fletcher and G. D. Floodgate (ed.), Bacteria in their natural environments. Academic Press, Inc., London.
30. **Nix, P. G., and M. M. Daykin.** 1992. Resazurin reduction tests as an estimate of coliform and heterotrophic bacterial numbers in environmental samples. Bull. Environ. Contam. Toxicol. **49:**354–360.
31. **Novitsky, T. J.** 1984. Monitoring and validation of high purity water systems

with the *Limulus* amebocyte lysate test for pyrogens. Pharm. Eng. **4:**21–33.
32. **Postgate, J.** 1989. A microbial way of death. New Sci. **20:**43–47.
33. **Postgate, J. R., J. E. Crumpton, and J. R. Hunter.** 1961. The measurement of bacterial viabilities by slide culture. J. Gen. Microbiol. **24:**15–24.
34. **Rodriguez, G. G., D. Phipps, K. Ishiguro, and H. F. Ridgway.** 1992. Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. Appl. Environ. Microbiol. **58:**1801–1808.
35. **Roszak, D. B., and R. R. Collwell.** 1987. Survival strategies of bacteria in the natural environment. Microbiol. Rev. **51:**365–379.
36. **Schall, B. F., G. V. Marathe, and B. K. Ghosh.** 1981. Stereological analysis of plasmolysis in logarithmic-phase *Bacillus licheniformis*. J. Bacteriol. **146:** 391–397.
37. **Scheie, P. O.** 1969. Plasmolysis of *Escherichia coli* B/r with sucrose. J. Bacteriol. **98:**335–340.
38. **Sperber, W. H.** 1983. Influence of water activity on foodborne bacteria—a review. J. Food Prot. **46:**142–150.
39. **Stubberfield, L. C. F., and P. J. A. Shaw.** 1990. A comparison of tetrazolium reduction and FDA hydrolysis with other measures of microbial activity. J. Microbiol. Methods **12:**151–162.
40. **Tabor, P. S., and R. A. Neihof.** 1982. Improved method for determination of respiring individual microorganisms in natural waters. Appl. Environ. Microbiol. **43:**1249–1255.
41. **Ward, D. M., R. Weller, and M. M. Bateson.** 1990. 16S rRNA sequences reveal uncultured inhabitants of a well-studied thermal community. Nature (London) **345:**63–65.
42. **Yu, F. P., and G. A. McFeters.** 1994. Rapid in situ assessment of physiological activities in bacterial biofilms using fluorescent probes. J. Microbiol. Methods **20:**1–10.