Immobilization of Phosphatidylserine by Ethanol and Lysozyme on the Cell Surface for Evaluation of Apoptosis-Like Decay in Activated-Sludge Bacteria

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ABSTRACT Accurate determination of microbial viability can be crucial in microbe-dominated biosystems. However, the identification of metabolic decay in bacterial cells can be elaborate and difficult. We sought to identify apoptosis-like bacterial processes by using annexin V-fluorescein isothiocyanate (FITC) (AVF), a probe typically used to stain phosphatidylserine (PS) on exposed cell membranes. The bacterial cell wall provides a barrier that is responsible for low efficiency of direct PS staining of decayed bacterial cells. This can be overcome by pretreatment of the bacteria with 70% ethanol, which fixates the bacteria and preserves the PS status, combined with lysozyme treatment to hydrolyze the cell wall. That treatment improved the efficiency of AVF staining considerably, as shown for pure strains of an *Ochrobactrum* sp. and a *Micrococcus* sp. Using this method, decayed bacterial cells (induced by starvation) were more strongly stained, indicating externalization of PS to a greater extent than seen for cells harvested at logarithmic growth. A multispecies microbial sludge was artificially decayed by heat treatment or alternating anoxic-oxic treatment, which also induced increased AVF staining, again presumably via decay-related PS externalization. The method developed proved to be efficient for identification of bacterial decay and has potential for the evaluation of multispecies bacterial samples from sources like soil matrix, bioaerosol, and activated sludge.

IMPORTANCE Since the externalization of phosphatidylserine (PS) is considered a crucial characteristic of apoptosis, we sought to identify apoptosis-like decay in bacterial cells by PS staining using AVF. We show that this is possible, provided the bacteria are pretreated with ethanol plus lysozyme to remove a physical staining barrier and preserve the original, decay-related externalization of PS. Our work suggests that PS externalization occurs in starved bacteria and this can be quantified with AVF staining, providing a measure of bacterial decay. Since PS is the common component of the lipid bilayer in bacterial cell membranes, this approach also has potential for evaluation of cell decay of other bacterial species.

KEYWORDS bacterial viability, apoptosis-like decay, activated sludge, annexin V-FITC staining, ethanol-lysozyme treatment

Biotreatment of wastewater depends on a complex community of multiple microbial species (1, 2), of which bacteria are typically responsible for the adsorption and transformation of the majority of pollutants. The microbial viability in such a community is considered crucial in effective treatment of wastewater because the dynamics of the bacterial growth, decay, and death vastly influence the removal of pollutants (2–4). For example, it was found that cell death affected the total decay activity of the

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biomass present in activated sludge, while metabolic decay had a smaller effect (5, 6). The ratio of dead to living bacterial cells is crucial for microbial viability and has been evaluated in activated sludge for decades. However, apart from dead and living (metabolizing) cells, intact but metabolically inactive cells may be present that are either degenerated or dormant under certain environmental stress conditions (e.g., starvation or unsuitable pH), and these cannot easily be distinguished from dead cells. Microbial viability is typically experimentally determined by plate counting, enzyme activity measurements, or cell staining using various dye probes (6–8). Microbial plating is elaborate and takes days to complete. Staining methods using fluorescent dyes are faster; their use is based on differences in cell membrane permeability toward the large dye molecules, allowing for differentiation between living and dead cells. The ratio of live versus dead cells is used as an approximation for microbial viability and for evaluation of their activity. For this, propidium iodide (PI) is typically used, as the molecule cannot penetrate cells with an intact membrane and thus ideally only stains dead cells (6, 9, 10). The resultant fluorescence is due to interaction of PI with DNA. Although PI was believed to mostly stain dead cells, it is not very specific, as the intact membrane of some microorganisms cannot prevent PI from entering (9, 10). For example, up to 40% of living *Sphingomonas* sp. or *Mycobacterium* sp. cells can be stained with PI (11). Furthermore, PI staining is poorly capable of distinguishing metabolically inactive but otherwise intact bacterial cells from metabolically active cells. Alternative staining methods that use fluorogenic ester dyes determine living cells based on enzymatic activity; however, these methods suffer from low potential for differentiation of living and decayed cells (12).

Metabolic decay is a key parameter of apoptosis, a programmed cell death pathway first described for eukaryotic cells (13, 14). The externalization of phosphatidylserine (PS) on cell membranes provides a signature response of apoptosis, and this may also exist in prokaryotic cells. Since PS can be specially bound by annexins (first discovered as vascular proteins), we considered the possibility that PS can be labeled with annexin protein in an attempt to evaluate bacterial decay at a cellular level. PS is a common structural molecule of cell membranes, but it is not normally found on the external surface of the bacterial cell membrane. Noninvasive PS staining of membrane surfaces would make it a superior alternative to PI staining (15, 16).

For evaluation of eukaryotic apoptosis, annexin V-fluorescein isothiocyanate (FITC) (AVF) staining is commonly used, but few studies have used AVF to stain bacterial cells, in isolation or in complex communities. In previous studies, stressed cells of *Escherichia coli* were partially stained by AVF (17, 18), while the dye was found insufficient to be applied to bacterial communities present in sludge (15, 19). Possibly, externalization of PS on cell membrane is not common in prokaryotes, though the apoptosis-like decay was found to exist in a limited number of species (17, 20–22). Alternatively, the efficiency of AVF staining of externalized PS is too low to be reliable. Experiments were designed to investigate this in detail, in an attempt to improve the performance of AVF-aided staining of PS of bacterial cells.

We identified that the main reason for low staining efficiency of apoptosis-like decay in activated sludge bacteria was the obstruction of peptidoglycan layers of the cell wall. To overcome this staining barrier and improve the AVF staining efficiency for bacterial cells, we developed an effective approach to simultaneously preserve original PS externalization and remove the peptidoglycan layer with a pretreatment using ethanol and lysozyme. This improved the staining efficiency dramatically, and the detected externalization of PS was now found to correlate with the viability of the bacterial cells. The method presented here has potential for evaluation of decay in complex bacterial communities, such as those in activated sludge or in environmental samples.

RESULTS

Influence of the bacterial cell wall on PS staining by AVF. Initially, PS staining of otherwise untreated *Ochrobactrum* sp. cells (a Gram-negative species typically present in sludge) was performed to test whether direct staining of the bacteria by AVF was

FIG 1 Cytofluorograms of starved *Ochrobactrum* sp. cells stained by different concentration of AVF. (A to C) Cytofluorograms of cells without any treatment and with treatment at 100 µg/liter (A), 200 µg/liter (B), and 300 µg/liter (C). (D to I) Cytofluorograms of logarithmic-phase, stationary-phase, and decline-phase cells stained by AVF (100 µg/liter) after lysozyme treatment (D to G) and of ethanol-lysozyme-treated cells in logarithmic phase stained by PI (H) and by AVF (I). CT, culturing time. The PS-positive staining percentage was calculated as the sum of the percentages from the right upper quadrant and right bottom quadrant for cells stained by AVF.

possible. The bacteria were starved for 30 days (see Materials and Methods) to ensure that a fraction of decayed cells was present, and AVF fluorescence was detected by flow cytometry, analyzing at least a total of 20,000 cells per assay. Three AVF concentrations (100, 200, and 300 µg/liter final concentrations) were tested. This revealed that no staining was detectable (Fig. 1A to C). When the cells were treated with lysozyme prior to staining, AVF-dependent fluorescence was detectable (Fig. 1D to G). The percentages of AVF-stained cells differed vastly between the lysozyme-treated cells and nontreated cells, but only a small difference was observed between cells harvested during logarithmic growth and those harvested during stationary phase. These results suggested that AVF could successfully bind PS of the bacterial cell membrane, provided the cells were first treated with lysozyme. Prior to staining, dispersion of the cells was performed by ultrasonic treatment (see Materials and Methods), and to determine whether this had any effect on the staining efficiency, alternative dispersion was performed by repeated pipetting. The mean AVF staining intensities of the ultrasonic and pipetting treatments were 36.4 and 36.0, respectively (Fig. S1B in the supplemental material). The small difference of 0.4 was considered negligible, so it was concluded that ultrasonic treatment does not substantially affect the PS staining results.

To assess the barrier effect of the cell wall against AVF-dependent PS staining of cells, a Gram-negative *Ochrobactrum* sp. strain and a Gram-positive *Micrococcus* sp. strain were used and AVF staining was compared to PI staining. The autoclaved cells were treated with lysozyme prior to staining, and untreated cells were included as a control. The mean Log value of the AVF intensity was calculated from the measured green fluorescence emitted by AVF-PS, and the mean Log value of the PI intensity was determined from the red fluorescence emitted by PI-DNA. In both species, the combined treatment of autoclaving followed by lysozyme resulted in much higher AVF fluorescence intensities than single or no treatment (Fig. 2B and F).

The AVF staining intensity of autoclaved *Ochrobactrum* cells was 9.66, and that of *Micrococcus* cells was 9.89, and these values increased to 23.93 and 131.67, respectively, when autoclaving was combined with lysozyme treatment (Fig. 2I and J). In comparison to that of *Ochrobactrum* cells, the AVF staining of *Micrococcus* cells was 4.5 times higher for autoclaving-plus-lysozyme-treated cells (131.67 versus 23.93) (Fig. 2I and J). Since

FIG 2 The fluorescence intensities of differently treated logarithmic-phase cells stained with AVF and PI. Histograms of lysozyme-treated and autoclaved *Ochrobactrum sp.* cells stained with AVF (A, B) and with PI (C, D), histograms of lysozyme-treated and autoclaved *Micrococcus sp.* cells stained with AVF (E, F) and with PI (G, H), and mean staining intensities of *Ochrobactrum sp.* (I) and *Micrococcus sp.* (J).

the outer membrane of Gram-negative bacteria might also be a barrier for AVF staining, pretreatment with Na₂EDTA was also assessed, as this strong chelator destroys the outer membrane (23, 24). It was tested whether AVF staining of starved *Ochrobactrum* cells could be achieved without removal of peptidoglycan by lysozyme but with EDTA; however, no AVF staining was observed following treatment with 500 mg/liter (wt/wt) Na₂EDTA (Fig. S1A).

Effect of ethanol fixation of cells on AVF and PI staining. Since lysozyme treatment allowed PS staining with AVF, suggesting externalization of PS had taken place, while no staining was detectable in the absence of lysozyme treatment, we considered that the original state of PS needed to be preserved. This was achieved by fixation of the cells prior to staining, which would avoid externalization of PS by lysozyme. Fixation was performed with 70% ethanol, after which the cell wall was removed by lysozyme treatment, thus preserving the original status of PS. To verify whether ethanol influenced the integrity of the cell membrane, both AVF and PI were used to stain ethanol-treated cells. The ethanol-treated cells were strongly stained with PI (60.6%), revealing that the cell membrane was destroyed by ethanol and cells became fully permeable (Fig. 1H). As expected, no AVF staining of PS was visible for cells that had undergone ethanol-plus-lysozyme treatment (culture time = 1 day) or for control cells (Fig. 1I and 2A and E).

These results showed the restraint of ethanol on excess PS externalization, which was induced by lysozyme treatment, and revealed the potential of the ethanol-plus-lysozyme treatment to evaluate PS externalization of starving bacterial cells.

The staining efficiency of PI was verified with cells cultured for 1 day with treatments similar to AVF staining (Fig. 2C and G). Ethanol-treated *Ochrobactrum sp.* cells produced the highest PI fluorescence intensity, which was almost 10-fold higher than that of untreated cells (Fig. 2I), due to the ethanol-induced changes in membrane permeability. The mean Log value of the PI staining intensity of lysozyme-treated cells was close

FIG 3 Fluorescence intensities of AVF- and PI-stained cells with different starvation times (0 to 50 days). *Ochrobactrum* sp. cells stained with PI (A) and AVF (B) and *Micrococcus* sp. stained with PI (C) and AVF (D). The PS staining with AVF was completed after treatment with ethanol and lysozyme, while the staining with PI was performed directly.

to that of nontreated cells, indicating that lysozyme only hydrolyzed the cell wall and did not significantly change the membrane permeability. Autoclaved cells showed a slightly higher PI intensity than untreated *Ochrobactrum* sp. cells. In contrast, for *Micrococcus* sp., the PI staining was slightly higher for autoclaved cells than for ethanol-treated cells (Fig. 2J).

AVF and PI staining to identify starvation time of bacterial cells. To understand the staining efficiencies of AVF and PI for decayed bacterial cells, staining tests were performed on bacterial cells cultured under starvation conditions, as this is one of the most common processes related to bacterial cell decay in nature (25–27). Bacteria that had been starved for different periods of time were therefore collected. The PI staining intensities of both *Ochrobactrum* sp. and *Micrococcus* sp. cells produced weak positive correlations with starvation times ($R_1^2 = 0.480$ and $R_3^2 = 0.362$, respectively) (Fig. 3A and C). Due to inefficient PI staining for autoclaved cells, it was not surprising that PI was less able to detect decayed bacteria that had suffered from different starvation durations. Also, the recovery of membrane permeability in the PI-permeable cells might result in ineffective PI staining (10). These results were in accordance with a deficiency of PI in differentiating dead from live cells, as already reported by others (28, 29).

The AVF staining of surface-exposed PS on bacteria after various starvation times was next conducted, for which the cells were treated with ethanol-plus-lysozyme prior to staining. This revealed that AVF staining intensities increased with starvation time, producing strong correlations. A linear correlation between starvation times and fluorescence intensities was found with *Ochrobactrum* sp. cells ($R^2 = 0.886$), and a logarithmic correlation was identified for *Micrococcus* sp. cells ($R^2 = 0.950$) (Fig. 3B and D). Regarding the different correlations of the two strains, a stable phase of externalization of PS might exist during the decay process. Moreover, the decay rate of *Micrococcus* sp. cells might be higher than that of *Ochrobactrum* sp. cells. Thus, this led to a shorter time for entering the stable phase for *Micrococcus* sp. cells than for *Ochrobactrum* sp. cells. These results suggested that PS externalization on cell membranes is a measurable characteristic of bacterial cells. The AVF staining procedure applied produced a higher sensitivity for the identification of decayed microbial cells than did PI staining. These

FIG 4 (A, B) Growth curves of two strains of bacteria, *Ochrobactrum sp.* (A) and *Micrococcus sp.* (B). (C, D) Correlations between viable cell numbers and AVF staining intensities in *Ochrobactrum sp.* (C) and *Micrococcus sp.* (D). OD600, optical density at 600 nm.

results also provided evidence for externalization of PS on the cell membrane during metabolic decay, as induced by starvation, in both Gram-negative and Gram-positive bacteria. The starvation time and the viable-cell number of both species tested were also investigated to clarify the viable cell numbers during starvation (Fig. 4A and B). The number of CFU, an important indicator for the number of viable cells being present, matched well with typical bacterial growth curves in both cases. During culture in LB medium, both strains had a lag phase of 3 to 4 h, followed by a logarithmic growth phase lasting from 4 to 40 h, and then entered stationary phase lasting up to 100 h, after which the culture aged. The highest CFU of *Ochrobactrum sp.* reached 1.67×10^{10} /ml, while that of *Micrococcus sp.* reached 2.11×10^{10} /ml. After 50 days of starvation, the number of *Ochrobactrum sp.* CFU declined to 8.0×10^8 /ml and that of *Micrococcus sp.* to 6.3×10^8 /ml. A clear negative logarithmic correlation between viable cell numbers and AVF staining intensities in both strains ($R^2 = 0.792$ for *Ochrobactrum sp.* and $R^2 = 0.970$ for *Micrococcus sp.*) was found (Fig. 4C and D), suggesting that externalization of PS intensifies as starvation is prolonged during bacterial culture, together with a decrease in numbers of viable cells.

When the AVF intensities were plotted over starvation times, a distinct peak (log value of AVF intensity around 2×10^2) was found for *Micrococcus sp.* cells at day 10 (Fig. 5). Such an apoptosis-like peak was not observed with *Ochrobactrum sp.* cells. Overall, the mean fluorescence intensity was lower for *Ochrobactrum sp.* cells than for *Micrococcus sp.* cells. This might be attributed to the thickness of the peptidoglycan layer in the cell wall of Gram-positive bacteria. Although the rather vigorous ethanol-plus-lysozyme treatment might be less applicable to Gram-negative bacteria, a clear positive correlation between fluorescence intensities and starvation times of *Ochrobactrum sp.* cells was still observed.

Verification of staining approach for identification of sludge decay. To test whether the PS staining method developed could be applied to bacterial communities as well, coking wastewater sludge was used. This was artificially decayed by either heat

FIG 5 Histograms of fluorescence intensities of PS staining of *Micrococcus* sp. by AVF after treatment with ethanol and lysozyme. *Micrococcus* sp. cells with different starvation times (1 to 36 days) were stained.

treatment or alternating anoxic-oxic treatment (see Materials and Methods). Both treatments have been reported to induce bacterial decay (30–32). The artificially decayed sludge was treated with ethanol and lysozyme and then stained with AVF, and fluorescence intensity results are shown in Fig. 6. The histogram of AVF fluorescence intensities and cell count results produced obvious shifts in peaks between heat-treated activated sludge and the control (Fig. 6A).

The AVF intensities of sludge heated at 60°C, 70°C, or 115°C ranged from 66.6 to 80.6. These values were 2.2 to 2.7 times higher than the value for nonheated control cells (29.9). The fluorescence intensity of 50°C-treated sludge reached 41.7, which was 39.5% higher than that of the control. The highest fluorescence intensity was obtained following 60°C incubation, showing that this treatment most strongly induced apoptosis-like decay. Alternating anoxic-oxic treatment of the sludge for 24 to 72 h also

FIG 6 Histograms of staining intensities of decayed-sludge samples induced by heat treatment (A) and alternating anoxic-oxic treatment (B) and the mean staining intensities of differently treated sludge samples (C). AOA, anoxic-oxic alternating.

produced a peak shift compared to the peak for the untreated control (Fig. 6B), indicating that this treatment also resulted in decay of bacterial cells. The AVF intensities of the treated sludge ranged from 34.3 to 46.7, which was 20.4% to 63.9% higher than those of the control.

These results indicated that sludge decay induced either by heating or by alternating anoxic-oxic treatment was identified by the AVF staining method developed. Ethanol-plus-lysozyme pretreatment combined with AVF staining was effectively verified on decayed sludge, illustrating the potential of this approach for evaluating the decay of bacteria in complex communities like those in environmental samples.

DISCUSSION

Evidence that PS staining is obstructed by an intact bacterial cell wall. Annexin V-FITC (AVF) is widely used to detect PS on the external side of apoptotic eukaryotic cell membranes (33). However, direct AVF staining of *Ochrobactrum* sp. cells derived from a culture with declining viability (starvation for 30 days) was ineffective at all three AVF concentrations tested. This is thought to be the result of PS not being directly available for staining on the surface of decaying bacterial cells. The results in Fig. 1 suggested that some barrier shielded PS and prevented AVF staining. The cell wall of Gram-positive bacteria, as well as the outer membrane of Gram-negative bacteria, could be responsible for this effect. Lysozyme promotes the hydrolysis of peptidoglycan, the main component of multiple cross-linked linear glycans in a bacterial cell wall (34, 35), so it was tested whether pretreatment with this enzyme could improve AVF staining. Indeed, lysozyme treatment of cells resulted in effective staining but produced similar fluorescence intensities for cells harvested at different growth phases (Fig. 1D to G). These results suggest that lysozyme pretreatment might have maximally exposed the available PS of the cells, due to induced cell lysis. To our best knowledge, there is no published evidence that lysozyme can induce externalization of PS in bacterial cells. Thus, the staining of PS to evaluate the degree of bacterial decay can only take place when excess exposure of PS (as induced by lysozyme) is prevented.

Better results were obtained when the cells were treated with ethanol plus lysozyme prior to AVF staining (Fig. 2E and 5). This resulted in significant differences in AVF fluorescence intensities between cells harvested during logarithmic growth and cells that had entered the declining starvation phase. The inclusion of a fixation step with ethanol prior to AVF staining is important to identify the degree of decayed cells that are present.

To verify whether the cell wall prevented the AVF staining, autoclaved cells were stained with and without lysozyme treatment. Following autoclaving, all cells will be dead, but lysozyme may still be able to expose PS that would otherwise not be available to the dye. Indeed, the staining intensities of cells treated by autoclaving and lysozyme were 2.47 and 13.3 times higher than those of cells only being autoclaved, as was demonstrated for *Ochrobactrum* sp. and *Micrococcus* sp. cells (Fig. 2I and J). The cytometric multigraph overlays of the two bacterial species with different treatments also showed distinctions of positive PS staining in lysozyme-treated bacterial cells and autoclaving-plus-lysozyme-treated cells (Fig. S2 and S3), indicating that the cell wall indeed prevented the staining of PS by AVF. Whereas Gram-positive bacteria have a relatively thick cell wall, Gram-negative bacteria further possess an outer membrane, which may also function as a staining barrier. This effect was investigated with the application of EDTA, which at high concentration destroys the outer membrane (23, 24). However, this treatment alone did not improve AVF staining of starved *Ochrobactrum* sp. cells, although in combination with lysozyme, AVF staining was successful. Since combined EDTA and lysozyme treatment was needed, we conclude that the peptidoglycan layer is the most likely major barrier that prevents AVF from binding to Gram-negative bacteria. This is in line with previously published results showing that the cell wall could hinder PS expression on the bacterial surface (36). The cell wall can likewise be a staining barrier during fluorescent *in situ* hybridization (FISH), where hydrolyzation of peptidoglycan is also needed to improve staining efficiency (37). Thus,

in this study, the peptidoglycan layer of bacterial cell walls was demonstrated to be the main obstruction to staining by AVF to evaluate bacterial apoptosis-like decay.

Influence of lysozyme, autoclaving, and ethanol on bacterial cell membrane permeability. The influence of lysozyme treatment on bacterial membrane permeability was investigated using PI as the staining dye, and the treatment was compared for bacteria treated by autoclaving and by ethanol fixation. Ethanol-treated cells were stained by PI, demonstrating that the fixation had destroyed cellular integrity and increased membrane permeability (Fig. 2C and G). A similar result was found by Branco et al. (38). The slightly higher intensity of PI staining of autoclaved cells compared to that of controls, observed with both bacterial species (Fig. 2D, H), indicated that even though bacteria were autoclaved, membrane integrity was still retained to some extent. Thus, even though the bacterial cells were killed by autoclaving, their membranes were not completely destroyed. Obvious differences in the PI staining intensity of ethanol-treated cells were found between the two bacterial species, most likely due to the peptidoglycan layers of the Gram-positive *Micrococcus* sp. cells being thicker than those of the Gram-negative *Ochrobactrum* sp. cells. Since ethanol can induce shrinkage of the thicker peptidoglycan layers, PI might be more easily trapped in the thicker but shrunken layers of the cell wall, resulting in a lower PI intensity for the ethanol-treated *Micrococcus* sp. cells than for the *Ochrobactrum* sp. cells. The similar absence of PI staining of lysozyme-treated cells of both species revealed that the cell membrane permeability was not influenced by lysozyme treatment. To summarize, lysozyme treatment was demonstrated to keep the cell membrane of bacteria intact, as verified by PI staining, while both ethanol treatment and autoclaving were fully destructive of viability. Ethanol-treated cells were highly permeable due to cells being completely damaged, while the permeability of autoclaved bacterial cells was slightly increased, so that PI staining was less efficient for the identification of dead cells killed by autoclaving treatment.

Hypothetical mechanism of PS staining in bacterial cells. According to AVF staining on the two tested species of bacteria at different starvation times, the intensities obtained correlated positively with the starvation times (0 to 50 days). A logarithmic correlation was found in *Micrococcus* sp. cells (starvation time, 0 to 36 days). From the first discovery of hallmarks of apoptosis in *Xanthomonas campestris*, evidence of apoptosis-like events has been observed in an increasing number of other bacteria, such as cyanobacterium *Trichodesmium* spp. (39), *Escherichia coli* (17), *Streptococcus pneumoniae*, and *Haemophilus influenzae* (21). These findings highlight the possibility that widely divergent organisms possess remarkably conserved and previously unrecognized programmed cell death pathways, found in both Gram-positive and Gram-negative bacteria. The PS externalization related to this process can be used to evaluate bacterial decay of various species via AVF staining, providing a very promising technique for environmental studies.

According to the results obtained, a hypothetical mechanism of PS staining of bacterial cells is proposed (Fig. 7). In both Gram-negative and Gram-positive bacteria, a cell wall covers the cell membrane, which again is composed of a lipid bilayer (37, 40). The bacterial cell walls of Gram-positive and Gram-negative bacteria differ, but in both cases, peptidoglycan layers are a common primary component. Eukaryotic (mammalian) cells lack a cell wall that might prevent AVF from binding to PS. However, bacterial cells can have a cell wall of about 2 nm in thickness (41). The size of annexin V molecules was crystallographically determined as above 4 nm ($a = 155.87 \text{ \AA}$, $c = 37.34 \text{ \AA}$) (42), implying that obstruction of AVF by the peptidoglycan layer of a prokaryotic cell wall is quite likely. For this reason, prior hydrolysis of the peptidoglycan in the cell wall is necessary for AVF-dependent PS staining of bacterial cells. In eukaryotic cells, two intracellular enzymes maintain the PS balance on the cell membrane. Scramblase transfers PS from the inner leaflet to the outer leaflet, while phospholipid transferase transfers the PS from the outer leaflet to the inner leaflet (43). In prokaryotic cells, the externalization of PS might be catalyzed by intracellular enzymes with similar

FIG 7 Assumptive mechanism of staining of exposed PS on bacterial cell membrane by AVF with ethanol and lysozyme treatment.

functions. After the cell wall is hydrolyzed with lysozyme, the bacteria would turn on the response for PS externalization. According to our results, the staining by AVF on lysozyme-treated cells was effectively eliminated by ethanol fixation. There are two possible explanations for this. First, ethanol might have prevented the combination of PS and AVF. Second, ethanol (which is lethal to bacterial at the concentration used) might quickly extinguish all metabolic activity, including any (enzymatic) reaction of PS externalization. Subsequently, starved cells were used to verify the effect of ethanol fixation on PS staining, and it was shown that ethanol-plus-lysozyme-treated starved *Micrococcus* cells were effectively stained (Fig. 5). Since PS staining of ethanol-fixed starved cells still produced positive staining, it can be concluded that ethanol does not affect the combination of PS and AVF. The most likely explanation, therefore, is that ethanol inactivated all enzymatic activity, no longer allowing additional PS to be transferred during lysozyme hydrolysis. Hence, ethanol helped to preserve the original amount of PS present on the exterior of the cell membrane that was induced by cell decay and prevented any excess PS externalization induced by lysozyme hydrolysis. Consequently, the PS externalization represents the original state of apoptosis-like decay of bacterial cells. This result suggests that ethanol fixation followed by lysozyme hydrolysis treatment is a promising approach for detection of the original status of PS on external membrane surfaces of bacterial cells and can be used as an indicator for bacterial decay.

In this study, the cell wall was found to be an obstruction to PS staining by AVF, while the ethanol-plus-lysozyme treatment was efficient in removing the staining barrier and preserving the original status of externalization of PS. This work reveals that the ethanol-plus-lysozyme treatment combined with PS staining is an effective approach for quantitative evaluation of apoptosis-like decay in bacterial cells based on PS externalization. The method was verified using pure cultures of bacteria and a multi-organism sludge, showing good efficiency for identification of bacterial decay. The method has potential for evaluation of multispecies bacterial samples, such as soil microbiomes, bioaerosols, and activated sludge.

MATERIALS AND METHODS

Identification of test bacteria from activated sludge. Two strains of phenol-degrading bacteria were isolated from activated sludge, sampled from a coking wastewater treatment plant in Shaoguan, Guangdong Province, China. To identify their genera, their 16S rRNA genes were amplified by primers 27F/1492R and sequenced using an Illumina HiSeq 2500. The sequences were analyzed by nucleotide BLAST (Basic Local Alignment Search Tool) at NCBI (National Center for Biotechnology Information), and

high similarities to an *Ochrobactrum* sp. and a *Micrococcus* sp., respectively, were identified. Both strains had a high ability to degrade phenol (concentration range from 250 to 750 mg/liter), with removal efficiencies of over 95% in 60 h (Fig. S4 and S5). The strains were stored in glycerol (60%, vol/vol) at -20°C in a cryogenic refrigerator for 3 months.

Preparation of bacterial cultures. Both strains were cultured in 100 ml LB medium (10 g/liter peptone, 5 g/liter yeast extract, and 10 g/liter NaCl) for 24 h in a thermostatic shaker at 30°C and 180 rpm. The cultures were then incubated for 0.5 to 50 days to accumulate bacterial cells with different degrees of starvation. For each time point, 1 ml of culture (containing $[0.5 \text{ to } 1.0] \times 10^{10}$ CFU) was extracted and centrifuged at 8,000 rpm for 3 min (TGL-18 M; Luxiangyi, Shanghai, China), after which the cell pellet was diluted in 30 ml 0.01 M phosphate-buffered saline (PBS) (pH 7.2). This diluted cell suspension was dispersed by ultrasonic treatment (JY92-II DN; Xinzhi, Bo Ning, China) at 50 W and 25°C for 120 s (5-s working time with 5-s time interval). Mechanical dispersion was performed as an alternative to ultrasonication by repeated pipetting with a 5-ml Eppendorf pipette for 5 min until cell aggregates were no longer observed.

Fixation of bacterial cells. Cells were fixed with ethanol to preserve their PS status, in order to prevent interference by intracellular reactions that respond to sudden stress in subsequent procedures. For this, 1 ml bacterial suspension was centrifuged as described above, but the cell pellet was suspended into 20 ml 70% (vol/vol) ethanol and thoroughly mixed. Both fixed and nonfixed cells were prepared for staining. The suspensions were then used for lysozyme treatment or cell staining.

Lysozyme treatment and cell staining detected by flow cytometry (FCM). When indicated, the cells were treated with lysozyme prior to staining. For this, following the initial centrifugation of 1 ml cell culture, the pellet was resuspended in 30 ml PBS and lysozyme (100 mg/liter final concentration) was added. The mixture was incubated at 37°C in a water bath for 1 h, after which the cell suspension was filtered using nylon filters (200- μm mesh) to remove cell aggregates. Staining was then performed as described above. Alternatively, the bacteria were pretreated with PBS containing 500 mg/liter EDTA for 10 min prior to staining. In one set of experiments, the cells were autoclaved at 120°C for 15 min prior to staining. In these experiments, lysozyme treatment, which was performed following autoclaving, was included as indicated.

Staining was performed by using an AVF-plus-PI apoptosis determination kit (Sangon Biotech, Shanghai, China). First, 0.1 ml of the diluted cell suspension was mixed with 0.9 ml HEPES buffer containing Ca^{2+} . Then, either 10 μl PI or 5 μl AVF was added and, after mixing, the suspension was stored in the dark for 20 min. The cells were then analyzed by flow cytometry (Epics XL; Beckman Coulter, USA). Because of the small size of the bacteria, a side scatter (SSC) threshold was used. The maximum events of a testing sample were set as 20,000, which represented approximately 20,000 cells being analyzed for every sample. FCM was performed with detector settings as listed in Table S1 in the supplemental material.

Different treatments for inducing decay of activated sludge. Activated sludge was collected from a 2.5-liter laboratory-scale coking wastewater aerobic treatment reactor with good performance. A total of 2.0 ml sludge was mixed with 8.0 ml PBS, and this was treated for 20 min at 50°C , 60°C , 70°C , or 115°C for 20 min to induce microbial decay. In addition, 20 ml of the original sludge was diluted with 80 ml PBS and used for alternating anoxic-oxic treatment, which was performed with the combination of a timing controller and an air pump. The treatment consisted of alternating periods of 6 h aeration and 6 h nonaeration for total times of 24 h, 48 h, and 72 h.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, PDF file, 0.4 MB.

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