Role of Autofluorescence in Flow Cytometric Analysis of Escherichia coli Treated with Bactericidal Antibiotics

Sabine Renggli,a,b Wolfgang Keck,a Urs Jenal,b Daniel Ritza
Actelion Pharmaceuticals, Ltd, Allschwil, Switzerlandb; Biozentrum, University of Basel, Basel, Switzerlandb

Bactericidal antibiotics kill by different mechanisms as a result of a specific interaction with their cellular targets. Over the past few years, alternative explanations forcidality have been proposed based on a postulated common pathway, depending on the intracellular production of reactive oxygen species. Detection of hydroxyl radicals relies on staining with specific fluorescent dyes that can penetrate the cell and are detected using flow cytometry. Flow cytometry has become an important tool in microbiology to study characteristics of individual cells within large heterogeneous cellular populations. We show here that Escherichia coli treated with different bactericidal antibiotics exhibits increased autofluorescence when analyzed by flow cytometry. We present evidence suggesting that this change in autofluorescence is caused by altered cell morphology upon antibiotic treatment. Consistent with this view, mutant cells that fail to elongate upon norfloxacin treatment show no increased auto-fluorescence response. Finally, we present data demonstrating that changes in autofluorescence can impact the results with fluorescent probes when using flow cytometry and confound the findings obtained with specific dyes. In summary, recent findings that correlate the exposure to cidal antibiotics with the production of reactive oxygen species need to be reconsidered in light of such changes in autofluorescence. Conclusive evidence for an increase of hydroxyl radicals after treatment with such drugs is still missing.

With flow cytometry, it is possible to measure several parameters simultaneously and details about physical and biological properties of single particles in a population are obtained (1, 2). It is an established technique for studies in cell and developmental biology. In the field of microbiology, the use of flow cytometry has increased significantly over the past decades (3). Early experiments by Steen et al. showed how effects of antibiotics on bacterial cells can be assessed with flow cytometry (4–7). More recently, Tracy et al. demonstrated the potential and the increased importance of flow cytometry in microbiology (1). In line with this, a recent proposal for a common mechanism of cell death induced by bactericidal antibiotics is based on data obtained with flow cytometry (8–10).

Flow cytometry offers the great advantage to analyze bacterial populations at the single-cell level. It combines direct and rapid assays to determine numbers, cell size distribution, and additional biochemical and physiological characteristics of individual cells, revealing the heterogeneity present in a population (2). Moreover, in combination with appropriate fluorescent probes, flow cytometry provides a powerful and sensitive tool to describe physical and biochemical characteristics of individual cells within a bacterial population, rather than average values that are not necessarily representative for the entire population.

Still, with bacteria the potential of this technology has not been fully exploited (1, 3). The main problems are small cell size, the low per-cell content of macromolecules, and the selection of the most suitable fluorescent dyes that had been originally developed for mammalian cells (1–3). For example, prokaryotes do not share the permeability or the uptake kinetics of eukaryotic cells, and bacterial efflux mechanisms efficiently pump out many fluorochromes (1, 2). As a consequence, bacterial membranes are less permeable for fluorescent dyes (2). These potential difficulties indicate the need for careful protocol development and choice of appropriate controls to extract reliable and biologically relevant information from flow cytometry experiments (1).

In the present study, it was attempted to validate the use of fluorescent probes for hydroxyl radical detection in order to assist the screening for new antibacterial agents with bactericidal activity. It became apparent that Escherichia coli exhibits autofluorescence under standard conditions in flow cytometric analysis. These background signals need to be taken into account when bacteria are analyzed with fluorescent probes. The extent of fluorescence observed was directly linked to cell length and was found to mask any potential effects induced by changes in reactive oxygen species.

MATERIALS AND METHODS

Media and growth conditions. E. coli strains were grown aerobically at 37°C using Luria-Bertani medium (LB; Becton Dickinson, Allschwil, Switzerland). Chloramphenicol (Fluka, Sigma-Aldrich Chemie GmbH, Buchs, Switzerland) was used for selective growth of mutant constructs, and ampicillin, kanamycin (Sigma-Aldrich), and norfloxacin (Sigma-Aldrich) were added to liquid cultures as test substances. The concentrations used are specified for each experiment. For the growth rate determination in media with dipyridyl or thiourea, dipyridyl was added to a final concentration of 500 μM or thiourea in solid form to a final concentration of 150 mM, respectively.

Chemicals. Chemicals used in the present study were purchased from Sigma-Aldrich Chemie GmbH (Buchs, Switzerland), except for hydroxyphenyl fluorescein (HPF; Molecular Probes, Invitrogen AG, Basel, Switzerland) (excitation/emission maxima 490/515 nm) and phosphate-buffered saline (PBS, pH 7.4; Gibco, Invitrogen AG, Basel, Switzerland).
Bacterial strains and genetic construction of mutants. All *E. coli* experiments were performed with the *E. coli* K-12 strain MG1655 (ATCC 700926/47076) or with mutants derived from it.

A ΔrecA knockout strain was constructed by PCR amplification of resistance cassettes, including flanking regions of homology (50 bp) to the target gene and integration in the chromosome of the recipient strain by transformation using the λ Red system (11). Transformants with the desired resistance phenotype were confirmed by colony PCR. The knockout was transferred with P1 phage transduction into MG1655. Transductants with the desired phenotype were again confirmed by colony PCR. Protocols for construction of strains were adapted from Sambrook et al. (12).

Determination of MIC. MICs were determined following guidelines of the Clinical and Laboratory Standards Institute. Briefly, stock solutions of the antibiotics were 2-fold serially diluted in 20% dimethyl sulfoxide, and bacteria were added in Mueller-Hinton broth (Becton Dickinson) from a fresh plate at a final concentration of 10^6 per ml. Growth was evaluated after 20 h of incubation at 37°C without agitation.

Flow cytometry. Bacterial cells grown overnight at 37°C were diluted 1:100 into 10 ml of fresh medium (LB) to an optical density (OD) of 0.04 in a 100-ml flask and grown at 37°C to an OD_{600} of ~0.3 before antibiotics were added. An untreated sample was used as positive control.

Cells from 1 ml of bacterial culture were collected at different treatment time points by centrifugation, and samples were directly stained or resuspended into 1 ml of ice-cold PBS and incubated for 15 min at 37°C in 1 ml of buffer solution (0.85% sodium chloride in water filtered through a 0.2-μm pore-size syringe filter) or HPF-staining solution (5 μM HPF in buffer solution). For fixation, the cells were resuspended into 1 ml of ice-cold fixing solution (4% formalin in 1X PBS) and incubated for 30 min at room temperature. Bacteria were washed once and then resuspended in buffer solution.

Live and fixed cell samples were washed once with buffer solution after staining. Finally, cells were diluted 1:10 into buffer for fluorescence-activated cell sorting (FACS) analysis. Data with fixed cells were collected using a flow cytometer (FACSAria; Becton Dickinson) with a 488-nm blue laser and a 500- to 560-nm emission filter (fluorescein isothiocyanate [FITC]) at a flow rate of 2. The following PMT voltage settings were used for FACS analysis: 450 (forward scatter [FSC]), 350 (side scatter [SSC]), and 825 (FL1/FITC). The data for live cells (see Fig. 5 and see Fig. S2 in the supplemental material) were collected on a different flow cytometer with the same laser and emission filter at low flow rate (CyAn ADP; Beckman Coulter, Nyon, Switzerland). The PMT voltage settings were 400 (FSC), 450 (SSC), and 825 (FL1/FITC). At least 100,000 cells were collected for each sample. To avoid background signal from particles smaller than bacteria, the detection threshold was put to 1,000 (or 0.2%) in SSC analyses.

RESULTS

Treatment with ampicillin and norfloxacin causes increased autofluorescence when measured by flow cytometry. *E. coli* treated for 3 h with norfloxacin showed an increased fluorescence compared to untreated cells measured by flow cytometry in the FITC photomultiplier tube (PMT) (Fig. 1A). With ampicillin, a bimodal distribution was observed after 3 h of treatment. While one subpopulation showed a higher fluorescence, the other subpopulation displayed no difference in fluorescence compared to the untreated control (Fig. 1A).

To further investigate the conditions resulting in elevated autofluorescence, cultures treated with norfloxacin and ampicillin were examined at different time points (Fig. 1B). With norfloxacin, autofluorescence increased over time. After ampicillin treatment, the cells exhibited a unimodal distribution with higher fluorescence compared to untreated cells after 1 h. At 3 h posttreatment, a bimodal distribution was observed as described above.

Finally, the concentration dependency of the autofluorescence was analyzed by exposing *E. coli* to different concentrations of norfloxacin and ampicillin for 3 h (Fig. 1C and D). Drugs were added at three different concentrations corresponding to 0.5×, 2×, and 4× the MIC for ampicillin and 0.3×, 3×, and 9× the MIC for norfloxacin. With norfloxacin, the fluorescence levels increased with higher concentrations but plateaued at concentrations corresponding to 3× the MIC. With ampicillin, the bimodal distribution was only seen at low concentrations. Higher concentrations resulted in a unimodal distribution showing no increase in fluorescence compared to untreated cells. Thus, the results show that the levels in autofluorescence observed are both dependent on the treatment time and drug concentration.

Autofluorescence correlates with an increase in cell length. Norfloxacin interacts with DNA gyrase and topoisomerase IV and stabilizes complexes of these enzymes with DNA (13, 14). Treatment of bacteria with norfloxacin results in DNA replication fork arrest and the induction of the SOS response, the main DNA damage response in *E. coli* (14–16). This in turn leads to the activation of certain genes that inhibit cell division, resulting in cell filamentation (17–19). Ampicillin binds to penicillin-binding proteins (PBPs) and inhibits, especially at low concentrations, cell division by binding to PBP3 (FtS1) required for septum formation, causing filamentation (20, 21). Consequently, treatment with either antibiotic can lead to increased cell length, depending on the concentration used. Furthermore, since the mechanism of action of either antibiotic is dependent on growth, one would expect cells to be more elongated after 3 h of treatment than after 1 h. Based on these assumptions, it was hypothesized that cell length might influence autofluorescence.

Cells were therefore analyzed under a light microscope after treatment with antibiotics for 3 h. Treatment of bacteria with norfloxacin resulted in cell elongation, and upon treatment with ampicillin, even longer filaments were observed in addition to lysed cells (Fig. 2A to C).

It had previously been shown that flow cytometry can be used to detect changes in morphology (22–24). These changes are measured by forward and side scatter (FSC and SSC). The magnitude of FSC is suggested to be roughly proportional to the size of the cell and SSC was assumed to be related to the internal granularity of the cell (1, 5, 20, 23–25). Changes in cell morphology and autofluorescence resulting from ampicillin and norfloxacin treatment correlated with changes in the FSC and SSC of drug-treated cultures (Fig. 2D to F). For norfloxacin treated cells, a shift in FSC and SSC was detected (Fig. 2E). For ampicillin, two populations were observed, consistent with the bimodal distribution seen for the fluorescence intensity (Fig. 2F). The lower population (Fig. 2F, population 1) displayed no difference in FSC and SSC compared to untreated cells, while in the upper population (Fig. 2F, population 2) a clear shift in FSC and SSC was observed. This shift was even more pronounced than the shift seen for norfloxacin-treated cells, confirming previous observations (20, 22, 23).

To further investigate the two distinct populations seen after ampicillin treatment, gates (Fig. 2F, populations 1 and 2) were set in the FSC-SSC dot plot, and the fluorescence intensity distribution for each population was analyzed. As seen in Fig. 3, the population with the lower signal intensity in the FSC and SSC channels (population 1) showed a fluorescence intensity distribution similar to that of the subpopulation with the lower fluorescence signal in the bimodal distribution. Conversely, the population
with higher FSC and SSC signals (population 2) displayed the same fluorescence intensity distribution as the subpopulation that showed increased fluorescence. This strongly indicates that the increased autofluorescence observed correlates with a shift in FSC and SSC and with altered drug-induced cell morphology.

Finally, to examine whether the increase in fluorescence correlates with cell length, fluorescence (FITC) was plotted against cell size (FSC) for the experimental conditions used and a linear correlation could be seen between the two parameters (see Fig. S1 in the supplemental material). This suggests that fluorescence per cell unit remains constant and that cell length is the determining factor for the autofluorescence observed.

A mutant that fails to elongate upon norfloxacin treatment does not show increased autofluorescence. To further examine the correlation between cell morphology and autofluorescence, a ΔrecA strain was used. RecA initiates the SOS response in E. coli. The process is activated by the formation of RecA filaments on single-stranded DNA that accumulate upon DNA damage or stalled replication. RecA filaments catalyze the autocleavage of the LexA repressor, leading to the derepression of several genes of the SOS regulon. Some of these proteins contribute to the inhibition of cell division resulting in cell filamentation (17–19). To confirm that cell length is causing increased autofluorescence upon drug treatment, a ΔrecA strain, which fails to induce the SOS response, was examined after treatment with norfloxacin and compared to a wild-type strain grown under the same conditions. As a control, the mutant strain was also treated with ampicillin at a concentration where no induction of the SOS response was observed.

First, MICs were determined for the ΔrecA knockout strain. The ampicillin MIC remained at 8 μg/ml, as in the wild type, whereas the norfloxacin MIC shifted from 0.08 to 0.04 μg/ml. Next, antibiotic concentrations for treatment were chosen such that the wild type and the knockout strain were treated with the same concentrations relative to the MIC. In addition, it was confirmed that the growth rate for both strains was similar over the duration of the experiment (data not shown). The autofluorescence of the norfloxacin-treated ΔrecA deletion strain was reduced compared to the wild-type strain, whereas no differences for the ampicillin-treated cells and untreated cells were observed (Fig. 4A). As expected, norfloxacin-treated cells from the knockout strain were shorter compared to the drug-treated wild-type cells (Fig. 4C and Fig. 2B). The wild-type strain showed a clear shift in FSC and SSC, whereas for the ΔrecA strain only a slight shift was observed (Fig. 2E and Fig. 4F). Thus, these data further support the hypothesis that increased autofluorescence is a consequence of drug-induced cell elongation.
Autofluorescence can interfere with specific fluorescent probes in flow cytometric analysis of *E. coli*. The fluorescent dye HPF was used in combination with flow cytometry to detect drug-induced hydroxyl radicals (8, 26–36). HPF is a fluorescein derivative that is used to selectively detect highly reactive oxygen species such as hydroxyl radicals. The intrinsic fluorescence of the probe in the absence of hydroxyl radicals is negligible (37).

Based on the data obtained with HPF, a model had been proposed for a common mechanism of cell death induced by bactericidal antibiotics via the production of oxygen radicals (8). An increase in fluorescence had been observed after treatment with bactericidal antibiotics and taken as experimental evidence for the presence of intracellular hydroxyl radicals. Here, we wanted to investigate whether the autofluorescence observed in our experiments had any consequences on the fluorescence increase seen with HPF staining by Kohanski et al. (8). Wild-type cells were treated with ampicillin, kanamycin, and norfloxacin as described previously (8) and unstained samples were compared to stained samples. Staining with HPF does not increase the fluorescence intensity of untreated control cells and ampicillin-treated cells but does result in a marginal positive fluorescence intensity shift for norfloxacin- and kanamycin-treated cells (Fig. 5). This suggests that hydroxyl radicals might be present upon kanamycin and norfloxacin treatment. However, the main increase in fluorescence upon norfloxacin treatment results from autofluorescence rather than from the reaction of HPF with intracellular hydroxyl radicals. Thus, this example demonstrates that experiments using HPF or other fluorescent dyes in combination with flow cytometry require correction for cell length-dependent autofluorescence and that conclusions from studies lacking such controls need to be revised.

**DISCUSSION**

The results in this study show that *E. coli* treated with ampicillin and norfloxacin displays higher autofluorescence compared to untreated cells when measured by flow cytometry. Furthermore,
the autofluorescence depends on the incubation time and antibiotic concentration. Based on observations from microscopy and specific parameters measured with flow cytometry (FSC and SSC), the cause of increased autofluorescence is cell elongation. For ampicillin-treated cells a strong autofluorescence was only observed when elongated cells were still present. With ampicillin concentrations greater than the MIC, cells lysed completely after 3 h of treatment, and only a unimodal population with low fluorescence was detected. When a bimodal distribution was seen, the stronger fluorescent signal was due to the elongated cells, while lysing cells were responsible for the weaker fluorescent signal. This is supported by previous publications using FSC and SSC as a readout to measure cell morphology (20, 22).

Similarly, *E. coli* autofluorescence correlated with cell elongation after norfloxacin treatment. Since SOS induction in *E. coli* upon treatment with fluoroquinolones is dose dependent, a less-pronounced increase in cell length was observed at lower drug concentrations. Moreover, a recA mutant strain that fails to induce the SOS response-associated cell filamentation showed only a marginal increase in autofluorescence when treated with norfloxacin, corroborating the finding that increased cell length is the cause for increased autofluorescence.

![Autofluorescence of *E. coli* recA deletion strain. (A) The distribution of fluorescence intensity is plotted in a histogram. The autofluorescence of wild-type (solid lines) and ΔrecA (dotted lines) strains exposed for 3 h to no drug (black), norfloxacin (red; 0.25 μg/ml for wild-type and 0.125 μg/ml for the ΔrecA strain corresponding to 3× the MIC), and ampicillin (5 μg/ml corresponding to 0.5× the MIC) is compared. (B to D) Microscopy pictures of the *E. coli* recA deletion strain after 3 h of treatment with 0.125 μg of norfloxacin/ml (C) or 5 μg of ampicillin/ml (D) and untreated control (B). The white scale bar corresponds to 10 μm. (E to G) Dot plots with FSC and SSC of the *E. coli* recA deletion strain after 3 h of treatment with 0.125 μg of norfloxacin/ml (F) or 5 μg of ampicillin/ml (G) and untreated controls (E). Microscopy pictures were taken with a light microscope in phase 3 (Axioskop 2 Mot Plus; Carl Zeiss AG). A magnification of ×1,000 was used.](http://jb.asm.org/)

FIG 4
Using HPF as an example, we demonstrate that autofluorescence can mask potential specific effects detected with fluorescent dyes when used in combination with flow cytometry. In particular, the autofluorescence histograms shown here are reminiscent of data presented by Kohanski et al. (8), which led the authors to propose a common mechanism of cellular death induced by bactericidal antibiotics. In contrast to that study, all of the samples here were also analyzed without staining. These experiments clearly demonstrated that HPF does not increase or only marginally increases fluorescence in cells treated with bactericidal drugs compared to the untreated control. Hence, it has to be concluded that hydroxyl radicals are not present in sufficient quantities after treatment with bactericidal antibiotics to induce a significant fluorescence response. The data presented here may also help to explain the apparently contradictory finding that low levels of ampicillin produce more hydroxyl radicals than high levels (8) since filaments leading to a higher autofluorescence signal could only be detected at lower ampicillin concentrations. Furthermore, the change in autofluorescence might also be responsible for the observations made by Kohanski et al. after the addition of thiourea or dipyridyl since, in our study, the doubling times of observations made by Kohanski et al. after the addition of thiourea or change in autofluorescence might also be responsible for the observed differences in autofluorescence, the data presented in these studies can be interpreted differently, and unstained controls are needed to avoid misinterpretations.

As our analyses suggest that fluorescence per cell unit remains constant for the experimental conditions used and thus fluorescence is increasing proportionally to the cell length, there may not be any molecular basis for increased autofluorescence other than elongation of the cells. Still, Durodie et al. suggested that cellular death pathway in E. coli or the natural peptide toxin, CcdB (27). As both compounds activated the SOS response, which was demonstrated by the induction of the LexA regulated gfp reporter construct, bacteria were elongated upon treatment (27). Similarly, Liu et al. used HPF to report that hydroxyl radicals were involved in cell killing by the bacterial topoisomerase I cleavage complex (28). These authors used an inducible topA mutant that led to rapid bacterial cell death due to the accumulation of topoisomerase I cleavage complex. The accumulation of the cleavage complex also resulted in the induction of SOS response and cell elongation. Furthermore, Davies et al. demonstrated that hydroxyurea induced hydroxyl radical-mediated cell death in E. coli (26). Hydroxyurea inhibits class I ribonucleotide reductase, leading to a depletion of deoxynucleoside triphosphate pools and finally to replication fork arrest. Again, this results in the induction of the SOS response. Finally, in a more recent publication, Kohanski et al. linked the HPF-detected formation of radicals, resulting from the treatment with low levels of bactericidal antibiotics, to an increase in mutation rates (29). A correlation between the change in mutation rates and peak HPF signals, as described in that publication, may need to be revised.

Several publications also used other fluorescent dyes to measure various cell characteristics through a similar approach with flow cytometry (30, 34, 36, 42–44). For example, Dwyer et al. sought to show that antibiotic-induced bacterial cell death exhibits physiological and biochemical hallmarks of apoptosis (34). For this, they used different fluorescent dyes specific for various characteristic markers of apoptosis. However, all compounds that exhibited an increase in fluorescence also induced cell elongation (34). Furthermore, the study demonstrated that SOS response, specifically recA, is required for the apoptotic characteristics to be observed upon norfloxacin treatment. This is in line with our findings showing that autofluorescence of the recA deletion strain after norfloxacin treatment was reduced compared to the norfloxacin-treated wild-type strain. Importantly, a sulA knockout strain cannot be used as a control for the effects of cell filamentation because of sulA-independent filamentation upon norfloxacin treatment (34, 45, 46). Altogether, considering cell-length-associated differences in autofluorescence, the data presented in these studies can be interpreted differently, and unstained controls are needed to avoid misinterpretations.

As our analyses suggest that fluorescence per cell unit remains constant for the experimental conditions used and thus fluorescence is increasing proportionally to the cell length, there may not be any molecular basis for increased autofluorescence other than elongation of the cells. Still, Durodie et al. suggested that cellular protein content increases when cells are treated with antibiotics (47). Moreover, for aquatic and environmental microbes, auto-fluorescence was assumed to be due to phototrophic pigments and some organic compounds in addition to certain physicochemical properties of the fluochrome (2). Further investigations will be needed to understand the molecular reasons for autofluorescence in more detail.

ACKNOWLEDGMENTS

We thank Andreja Knezevic and Daniela Sabato for technical support, Jonathan Delers for help with the microscope, and Barbara Wieland for thoughtful suggestions during manuscript development. We also express

FIG 5 Comparison of fluorescence upon staining with HPF. The fluorescence of live E. coli with (dashed lines) or without (solid lines) HPF stain after exposure to no drug (black) or antibiotics (0.25 μg of norfloxacin/ml, red; 5 μg of ampicillin/ml, blue; 5 μg of kanamycin/ml, green) for 3 h was measured. No differences were detected when the fluorescence of unstained live cells was compared to unstained fixed cells in a control experiment (see Fig. S2 in the supplemental material).
our deepest gratitude and heartfelt thanks to the late Alex Böhm for his contributions and, in particular, for providing strains.

REFERENCES