Response of single bacterial cells to stress gives rise to complex history dependence at the population level

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Edited by Bruce R. Levin, Emory University, Atlanta, GA, and approved February 2, 2016 (received for review June 12, 2015)

Most bacteria live in ever-changing environments where periods of stress are common. One fundamental question is whether individual bacterial cells have an increased tolerance to stress if they recently have been exposed to lower levels of the same stressor. To address this question, we worked with the bacterium *Caulobacter crescentus* and asked whether exposure to a moderate concentration of sodium chloride would affect survival during later exposure to a higher concentration. We found that the effects measured at the population level depended in a surprising and complex way on the time interval between the two exposure events: The effect of the first exposure on survival of the second exposure was positive for some time intervals but negative for others. We hypothesized that the complex pattern of history dependence at the population level was a consequence of the responses of individual cells to sodium chloride that we observed: (*i*) exposure to moderate concentrations of sodium chloride caused delays in cell division and led to cell-cycle synchronization, and (*ii*) whether a bacterium would survive subsequent exposure to higher concentrations was dependent on the cell-cycle state. Using computational modeling, we demonstrated that indeed the combination of these two effects could explain the complex patterns of history dependence observed at the population level. Our insight into how the behavior of single cells scales up to processes at the population level provides a perspective on how organisms operate in dynamic environments with fluctuating stress exposure.

bacterial memory | single cell | cell cycle | priming | synchronization

Bacteria are constantly challenged by their environment (1). Are bacterial cells able to respond better to environmental changes if they have experienced similar conditions in the recent past? It has been demonstrated that bacterial populations respond faster to a change of nutrient source when the forthcoming nutrient source has been presented in the recent past (2, 3). Similarly, bacterial populations that were exposed to sublethal stress levels showed increased survival of a higher stress level of the same type (4–6). Theoretical and experimental studies indicate that basing cellular decisions on environmental cues perceived in the past can be advantageous in dynamic environments (3, 7, 8), suggesting that such history-dependent behavior can be the result of adaptive evolution in dynamic environments.

In this study we addressed the question of memory on a single-cell level. We asked whether weak stress events provide individual cells with increased tolerance against future stress. Memory effects usually have been studied on the basis of population measurements (4, 9–12). Using population measurements, it is difficult to determine whether history dependence is a consequence of the behavioral changes in individuals or of a shift in the composition of the population as a result of past events. By using single-cell analysis, we investigated how the behavior of individuals scaled up to history-dependent behavior observed on the population level.

We used *Caulobacter crescentus* as a model system (Fig. 1*A*). *C. crescentus* is an asymmetrically dividing bacterium abundant in aquatic environments (13). A surface-attached stalked cell divides into a stalked daughter cell and a swarmer daughter cell. The stalked cell remains attached to the surface, and the swarmer cell enters a motile phase during which it disperses. Following the motile phase, the swarmer cell differentiates into a sessile stalked cell by shedding its flagellum, forming a stalk, and initiating replication (14). Because surface-attached stalked cells cannot move away from stressors in the natural environment, one might expect this bacterium to have evolved ways of responding to recurrent exposure to stress in a history-dependent manner. This reasoning suggests that *C. crescentus* is a good model system for analyzing the history-dependence of bacterial stress responses.

C. crescentus was grown in microfluidic devices to observe single cells in dynamic environments (Fig. 1*B*). This approach has emerged recently as a powerful experimental tool for studying behaviors of individual cells over extended periods of time (15–17). The asymmetric division of *C. crescentus* into stalked and swarmer cells allowed us to monitor attached stalked cells over a long time period during which swarmer progenies were continuously washed out (15). With this setup the number of cells in the microfluidic device remained approximately constant, and environmental conditions could be changed in a controlled way. We used time-lapse microscopy to image stalked cells over the course of these experiments and analyzed the images to reconstruct patterns of division and survival of individual cells after exposure to stress (Fig. S1). For all experiments reported here we used the same criterion for survival: Cells were considered to have survived a stress event if they divided at least once within 2 h after exposure (Fig. S2).

We used sodium chloride as stressor. Bacterial cells are known to respond to an increase in the external salt concentration by accumulating metabolites, either by synthesis or uptake from the

Significance

The ability to memorize information from the past is well known in complex organisms. Less is known about whether unicellular organisms such as bacteria also store information about past events and use these "memories" to inform their current behavior. We used an experimental setup that allowed us to follow single bacterial cells through repeated exposure to salt stress and asked whether past exposure allowed cells to cope better with the stress. Although the responses of individual cells were largely independent of past events, we saw the emergence of memory-like behavior at the level of the population. These results reveal differences in how past events can modulate the behavior of individuals and groups of bacteria.

Author contributions: R.M. and M.A. designed research; R.M. performed research; R.M. analyzed data; and R.M. and M.A. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Freely available online through the PNAS open access option.

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1511509113/-/DCSupplemental.

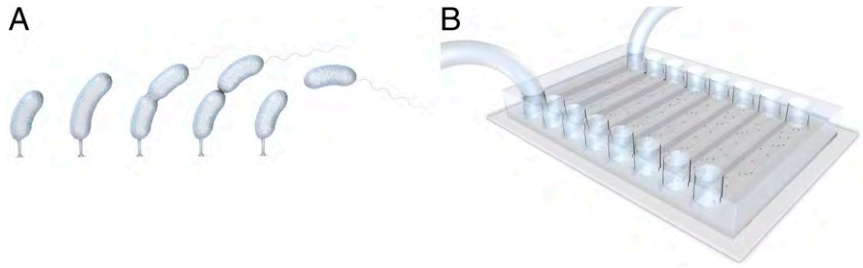

Fig. 1. We performed single-cell experiments with the bacterium *C. crescentus* in microfluidic devices to investigate whether tolerance to a stressor is influenced by past exposure. (*A*) *C. crescentus* divides asymmetrically into a surface-attached stalked cell and a motile swarmer cell. The figure shows five stages of the cell-division cycle of a stalked cell, which lasts about 60 min. (*B*) We followed individual stalked cells over several successive divisions and analyzed whether their survival during exposure to sodium chloride depended on exposure to lower concentrations of sodium chloride in the past. Experiments were conducted using microfluidic chips with eight parallel channels. Environmental conditions were controlled by flowing medium through the channels (tubing is shown for one channel). Stalked cells were attached to the glass surface. The number of *C. crescentus* cells in the chip was approximately constant because the flow of medium removed the motile swarmer cells after cell division. Image courtesy of Stephanie Stutz (stephaniestutz.ch/).

medium, to counteract the loss of water (18, 19). Sodium chloride has been used in other studies to characterize specific stress responses of *C. crescentus* (20–22). Our experimental system allowed us to expose *C. crescentus* to short periods of elevated levels of sodium chloride and examine whether the responses of single cells to this stressor were modulated by events in the recent past. Overall, these experiments revealed that the responses of single cells to stress can give rise to surprising and nontrivial patterns observed at the population level. In such cases, single-cell observations are essential for understanding the cellular basis of how bacteria react in dynamic environments with recurrent exposure to stressors.

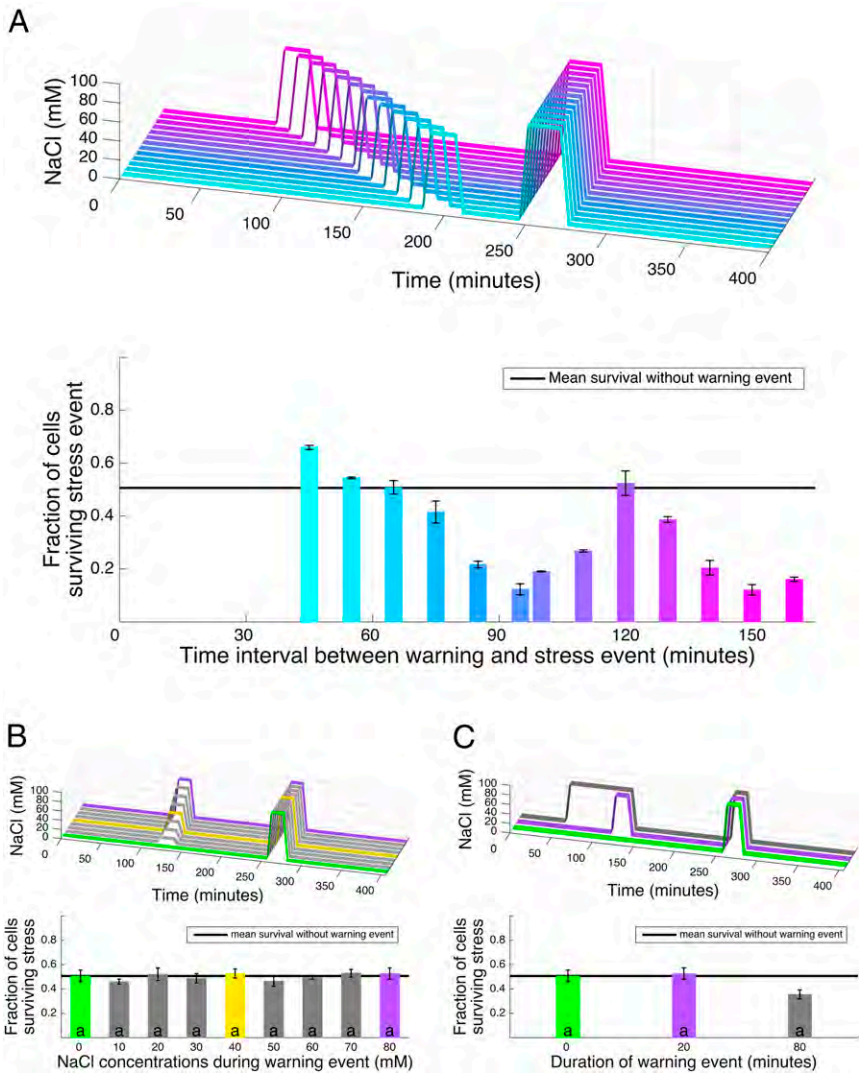

Fig. 2. Responses of individual cells to salt stress gave rise to complex patterns of history dependence at the population level. Cells were exposed to two events of sodium chloride exposure, the warning event and stress event. We varied the time between the two events (*A*), the concentration of the warning event (*B*), and the duration of the warning event (*C*) in a series of experiments. (*A–C*, *Upper*) Graphical representations of the time course of sodium chloride in different experimental treatments. (*A–C*, *Lower*) The fraction of surviving cells for each of these treatments. Error bars denote SEMs. (*A*) Cells were exposed to a warning event of 80 mM sodium chloride and a stress event of 100 mM sodium chloride. Both events lasted 20 min. In the lower panel the fraction of surviving cells is shown for different time intervals between the warning and the stress events. Data reported in the lower panel were obtained from experiments (2×200 cells for all recovery time intervals except for 120 min, where there are 5×100 cells). Survival of the stress event was dependent on the time interval between the two events (ANOVA $P < 0.001$). Also see Dataset S1. (*B*) Survival of a stress event 120 min after a warning event did not depend significantly on the sodium chloride concentration of the warning event (ANOVA; $P = 0.89$; five or more independent experiments per condition with 100 cells analyzed per experiment). Cells were exposed for 20 min to 0 (green trajectory), 10, 20, 30, 40 (yellow trajectory), 50, 60, 70, or 80 (purple trajectory) mM sodium chloride; after a 120-min time interval, they were exposed to a stress event of 100 mM sodium chloride. The identical significance-group letters (shown in each bar) indicate the absence of significant differences (Tukey post hoc ANOVA test; $P < 0.05$). Also see Dataset S2. (*C*) Survival of a stress event 120 min after a warning event did not depend significantly on the duration of the warning event. Cells were exposed to warning events of 0, 20, or 80 min; survival of a 100-mM stress event 120 min later did not depend significantly on the duration of the warning event (ANOVA; $P = 0.07$; four or more independent experiments per condition with 100 cells analyzed per experiment). The identical significance-group letters shown in the bars indicate the absence of significant differences between treatments (Tukey post hoc ANOVA test; $P < 0.05$). Also see Dataset S3.

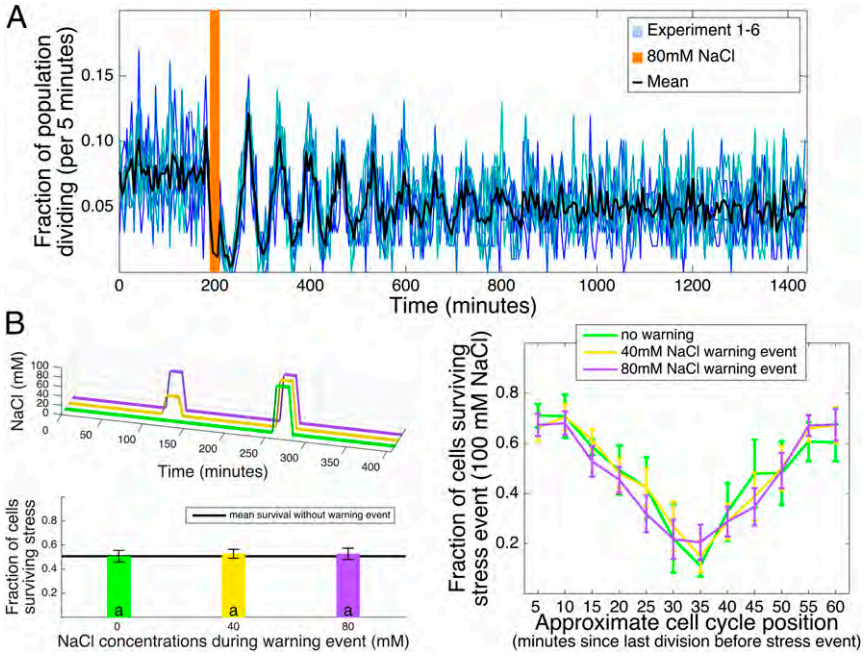

Fig. 3. Analysis of the division activity of single cells revealed two effects: synchronization of cell divisions (*A*) and cell-cycle position–dependent survival (*B*). See Fig. S1 for a graphical representation of the divisional activity of single cells. (*A*) A single 80-mM sodium chloride exposure event (corresponding to the warning event in Fig. 2*A*) synchronized cell divisions. Six shades of blue represent the results of six independent experiments. For each experiment, the fraction of a population of 100 cells dividing within a 5-min interval is shown; the black trace represents the mean number of divisions over the six experiments. After the 80-mM sodium chloride event (vertical orange stripe), cells divided synchronously. See Figs. S5 and S6 for a more detailed analysis of the synchronization effect. Also see Dataset S4. (*B*) Single-cell analysis revealed that a cell's survival after exposure to 100 mM sodium chloride (the stress event) is dependent on the cell's position in the cell cycle. Survival is shown for three experimental treatments in which cells were exposed to different sodium chloride concentrations during the warning event: 0 mM (green), 40 mM (yellow), or 80 mM (purple) sodium chloride for 20 min. The panels on the left show a subset of the treatments from Fig. 2*B*. The panel on the right shows the fraction of the cells at specific cell-cycle positions at the onset of stress (at a resolution of 5 min) that survived the 100-mM stress event. The cell-cycle position at the onset of stress is approximated for each cell by the time that had passed since the last division. Cells in the middle of the cell cycle showed a higher sensitivity to sodium chloride than cells close to the beginning of the end of the cell cycle (ANOVA; $P < 0.01$). Also see Dataset S5.

Results and Discussion

We first asked whether individual cells of *C. crescentus* would survive exposure to high levels of sodium chloride better if they had been exposed previously to lower concentrations of the same stressor. We exposed cells growing in a microfluidic chip to two subsequent events with a range of time intervals between the two events (Fig. 2*A*, *Upper*). We worked with a range of sodium chloride concentrations that have been demonstrated to impact *C. crescentus* growth (20–23). For the first event we used a concentration of 80 mM (unless stated otherwise), and for the second event we used 100 mM sodium chloride. (Fig. S3 shows the results of exposure to single events.) We refer to the first event as the "warning event" and the second event as "stress event."

We expected that exposure to the warning event would increase survival of the stress event. The basis for this expectation was that the warning is expected to activate the salt stress response (20, 23, 24), and this response increases survival of a second exposure to sodium chloride. Furthermore, we expected that the magnitude of this protective effect would decrease as the time interval between the warning event and the stress event increased. The cellular molecules that mediate the salt stress response—sigma factors, transcripts, and proteins—are expected to be diluted during growth and division, so that the protective effect they confer is expected to diminish with increasing time.

However, and surprisingly, these expectations were not borne out by the outcome of our experiments. The experiments showed that when we increased the time interval between the warning and stress events from 45 min to 160 min, the effect on cell survival did not decrease monotonically but instead showed a seemingly periodic fluctuation (Fig. 2*A*, *Lower*). This cyclic pattern was quite robust against changes in the experimental parameters. When we changed the sodium chloride concentration of the warning events or its duration, we obtained similar values for survival of the stress events (Fig. 2 *B* and *C*). Together, these results establish that, with increasing time between warning and stress, the fraction of the individuals that survive the stress event cycles in a robust and unexpected way.

This finding raised the question about the cellular basis of this unexpected survival pattern. When we investigated the division activity of single cells in more detail, we discovered two effects that together could explain these survival patterns: (*i*) exposure to nonlethal levels of sodium chloride led to synchronization of the cell division cycle, and (*ii*) the probability that an individual cell would survive the stress event depended on that cell's position in the cell cycle. We now describe these two effects and then discuss how together they can give rise to the survival pattern that we observed.

Analysis of the division history of single cells revealed that exposing cells to the warning event (80 mM sodium chloride) led to a transient synchronization of cell cycles in a population. Although cell-division events in a population were distributed approximately uniformly in time before the warning event, cell divisions were partially synchronized after the event (Fig. 3*A*). The degree of synchronization decreased with time, and synchronization was lost after about 16 h. Detailed analysis of the division and survival of single cells revealed that the synchronization was primarily a consequence of changes in the timing of cell division in response to the warning event. (See Fig. S4 for a more detailed analysis.) Upon exposure, cells halted cell division,

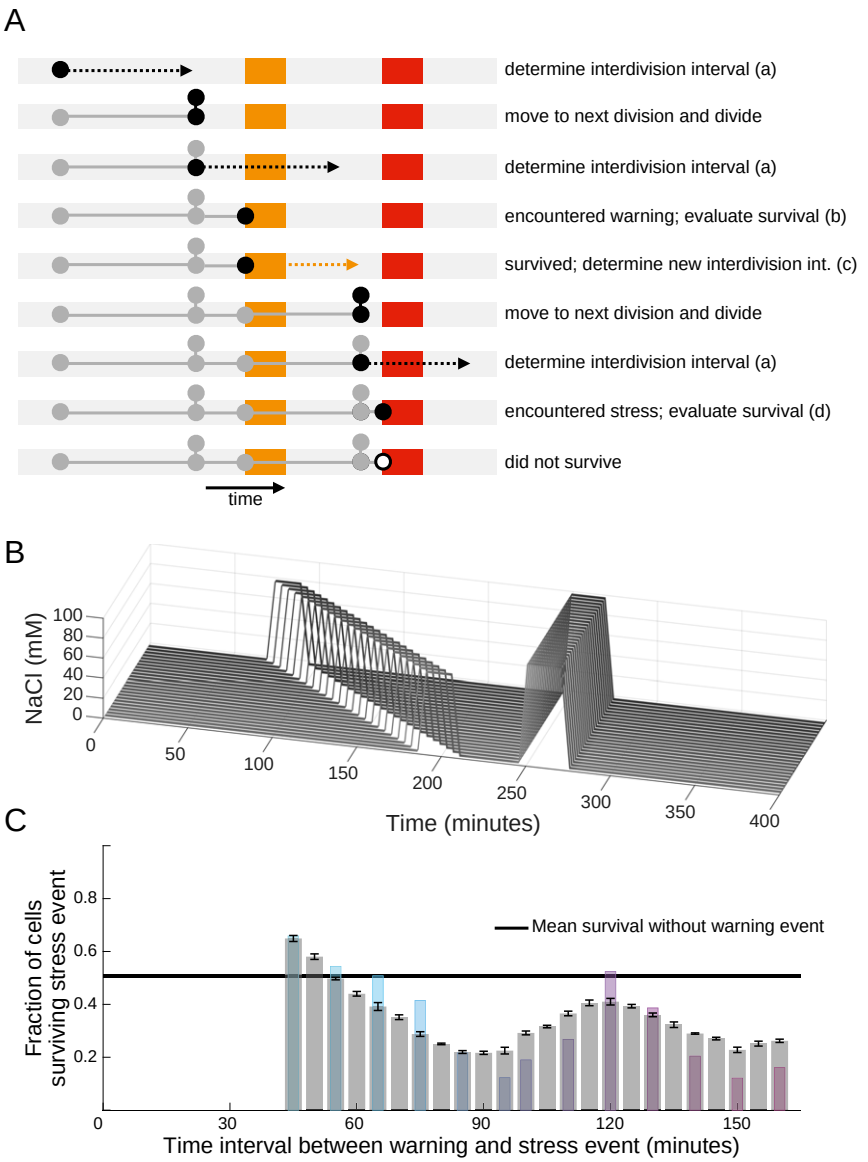

Fig. 4. Simulations of cell divisions can reproduce the complex patterns of history dependence observed in experiments (Fig. 2*A*). (*A*) Schematic representation of nine consecutive steps (each represented by one horizontal line) in the individual-based computer simulation. At each step, the black circle shows the current temporal position of the cell; gray circles indicate past positions. Single cells were initialized with a randomly determined cell-cycle position. For each cell, a next division time point was drawn from an experimentally derived distribution [indicated as (a) in the text column at the right]. If the cell reached this time point without encountering a warning or stress event, it divided (marked by a second filled circle above the horizontal line). When cells encountered a sodium chloride event, their probability of survival was determined as a function of their cell-cycle position [indicated as (b) in the text at the right]. For cells that survived, the time to the next division was again drawn from experimentally derived distributions [marked as (c) in the text at the right] (also see Fig. S8). This procedure was repeated until the cell died or the end of the simulation was reached [marked as (d) in the text at the right]. (*B*) In these simulations, cells were exposed to a warning event (exposure to 80 mM sodium chloride) and a stress event (exposure to 100 mM sodium chloride). We ran simulations for time intervals of 45–160 min (at a 5-min resolution) between the warning and stress events, schematically represented as gray trajectories. For each time interval we performed five simulations with 1,000 cells each simulation. (*C*) The fraction of surviving cells is represented by gray bars; experimental data from Fig. 2*A* are superimposed as colored bars for comparison. Error bars denote SEMs for the data from the simulations (ANOVA; $P < 0.001$). The simulations explain ~80% of the variability of the experimentally observed survival (linear regression of the mean survivals derived by simulations versus experimentally observed mean survivals for each time interval between the warning and stress events; R^2, 0.78). Also see Dataset S1.

and most cells divided again about 60 min after exposure, irrespective of their position in the cell cycle at the onset of the warning event. (See Fig. S5 for more details on the delay effect.) As a consequence, cell-division cycles in these populations were synchronized. Synchronization of the cell-cycle position was robust against changes in experimental parameters and also was observed if the duration of the warning period or the concentration of sodium chloride during the warning period was altered (Fig. S6). One possible molecular mechanism for the delay in cell division that leads to synchronization has been established by previous work: In *C. crescentus*, exposure to diverse stressors leads to the degradation of the essential replication initiation factor DnaA (25–27) by the Lon protease. It is possible that DnaA degradation in response to exposure to sodium chloride induced a halt in the cell-division cycle that led to synchronization.

The second effect we discovered was that survival of the stress event depended on the cell's position in the cell cycle; cells that

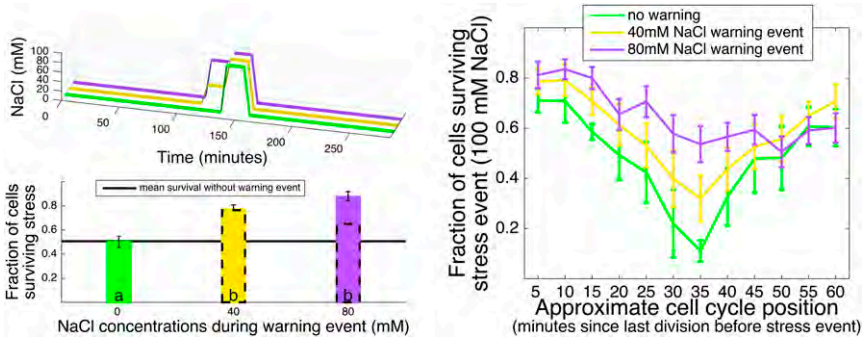

Fig. 5. (*Upper Left*) Cells exposed to 100 mM sodium chloride immediately after a warning event (40 mM or 80 mM, yellow and purple trajectories, respectively) showed a higher probability of survival than unwarned cells (green trajectory). (*Lower Left*) When calculating survival of the stress event, we corrected for the mortality imposed by the warning event. To do so, we divided the fraction of cells that survived both the warning and subsequent stress event by the fraction of cells that survived only the warning event of 40 or 80 mM, respectively. The dashed line represents the uncorrected survival (ANOVA; $P < 0.001$ for both corrected and uncorrected survival means; four or more independent experiments per condition with 100 cells analyzed per experiment). Significance groups (a/b/c for 0/40/80 mM, respectively) for uncorrected survival are displayed within the bars. Error bars denote SEMs. (*Right*) The higher survival of cells that were previously exposed to a warning event becomes apparent when analyzing survival of cells as a function of the time since the last division. Also see Dataset S5.

had just divided or were about to divide had a survival probability of about 70%, whereas cells in the middle of the cell cycle had a survival probability of about 20%. Fig. 3*B* shows the survival dependency on the cell-cycle position of cells that had been exposed to a 0-mM, 40-mM, or 80-mM sodium chloride warning event 2 h before a 100-mM sodium chloride stress event. Importantly, although the cell-cycle position had a strong effect on survival, prior exposure to the warning event had no substantial or statistically significant direct effect; the curves that show survival for cells from the three different warning regimes lay on top of each other. This latter observation means that 120 min after exposure to the warning event we found no evidence that individual cells would have a memory of this event. Survival of the stress event was dependent only on the cell's position in the cell cycle and was not, in any way that we could detect, dependent on whether the cell had been exposed to sodium chloride 120 min earlier. That a cell's survival depends on its position in the cell cycle again points to a potential role of DNA replication in determining whether cells survive exposure to sodium chloride. Experiments with a strain in which DnaA is overexpressed or in which Lon is knocked out [and DnaA is stabilized as a consequence (25)] could provide insights into the possible role of DNA replication in cell-cycle synchronization and the dependence of survival on the cell-cycle position that we observed here.

We then hypothesized that together the two effects described above can lead to the nontrivial patterns of history-dependent stress responses that we reported in Fig. 2*A*. The basis of this hypothesis is as follows: Because the warning event synchronized cell divisions, the exact timing of the stress event determined whether the synchronized population encountered the stress at a moment when cells were sensitive to sodium chloride, i.e., in the middle of the cell cycle (Fig. 3*B*), or at a moment when they were robust to sodium chloride, i.e., at the beginning and end of the cell cycle. As a consequence, one would expect that increasing the time interval between warning and stress event from 45 min to 160 min, as shown in Fig. 2*A*, could lead to the cyclic pattern of survival that we observed.

We used computational modeling to test whether these two effects—synchronization and cell-cycle dependent survival—can indeed explain the cyclic survival patterns observed in Fig. 2*A*. The model was based on parameters that we retrieved from our single-cell experiments: the distribution of interdivision intervals of cells in the absence of sodium chloride; the probability, depending on their position in the cell cycle at the beginning of the warning or stress event, that cells would survive event; and, for the cells that did survive, the distribution of interdivision intervals following exposure to the warning or stress events (these intervals are longer than interdivision intervals in the absence of stress, as shown in Fig. S5). Importantly, the model did not include any aspect of a cellular memory; the fates of individual cells depended only on their position in the cell cycle, which was the only cellular trait that we modeled, and were not influenced by their histories. A schematic of the model is shown in Fig. 4*A*.

To test whether we could recover the complex pattern that we had found in our experiments, we used this computational model to predict how survival of the stress event would depend on the time interval between warning and stress (Fig. 2*A*). Indeed, the computational model predicted that if the time interval between warning and stress event was initially set at 45 min and then increased, average survival would show a cyclic pattern that was qualitatively similar to our experimental results (Fig. 4*B*). The outcome of the computational model thus supports the notion that in our experimental system the history-dependent survival patterns observed at the population level emerged from the combination of two effects, namely the cell-cycle synchronization that is a consequence of exposure to the warning event and cell-cycle–dependent survival of the stress event. Once these effects are known and taken into account, survival probabilities can be determined with substantial precision based on the temporal distance between the two events. The qualitative fit between our model (which did not include cellular memory) and the experimental survival patterns (Fig. 4*B*) supports the notion that such complex patterns of history dependence can arise without individual cells forming a cellular memory of past event, other than the effect these events have on the timing of the cell cycle.

This conclusion raised a question about the match between our study and previous work on the response to salt stress in *C. crescentus*. In response to osmotic stress this bacterium expresses the alternative sigma factor σ^T, which activates transcription of genes involved in general stress response (21). Thus one would expect that after the warning event of 80 mM sodium chloride that we used in our experiments cells would have elevated levels of this sigma factor or of the transcripts or proteins of genes regulated by it, and that these elevated levels would make these cells more tolerant to subsequent exposure to higher levels of sodium chloride. As a consequence, one would expect to find a certain degree of cellular memory, that is, that cells would show elevated salt tolerance at least for a certain period after induction of the salt stress response. Why did we not see this effect in our experiments?

An additional experiment resolved this apparent contradiction: When we subjected cells to a stress event that immediately followed a warning event, with no intervening period, we did indeed observe that the warning event increased survival of the stress event (compare Figs. 3*B* and 5). For these experiments, and in contrast to the experiments presented above, we could not determine whether a given cell died during the warning or the stress event. However, we could correct for the average mortality during the warning event, and this analysis revealed that cells that were exposed to 80 mM or 40 mM of sodium chloride had a higher probability of surviving a subsequent exposure to 100 mM sodium chloride than cells without previous exposure. This result indicates that this bacterium is able to form a cellular memory of past exposure to salt, but this cellular memory is short lived, so that it does not manifest in the analysis of situations in which the previous exposures occurred further in the past, as in the experiments reported in Fig. 2*A*. It is interesting to consider whether the increased survival of warned cells might be based partially on changes in the timing of cell division; if so, modulation of the cell-division cycle might be an important aspect of the protective effect of the salt-stress response and possibly of other bacterial stress responses as well.

Overall, our results reveal a striking difference between the behavior of single cells and the dynamics at the population level. Whether an individual cell would survive a stress event could be predicted to a large degree simply by knowing the position of the individual cell in the cell cycle. Knowledge of the history of the cell was not needed to make this prediction. Therefore individual cells did not show evidence for cellular memory in the conventional sense. However, past events had an indirect effect on survival that manifested at the level of the population: Past events influenced the distribution of cell-cycle positions of the cells in the population and thereby influenced the average stress tolerance in the population. The patterns of history dependence measured at the population level therefore did not simply reflect cellular memory of individual cells but emerged from single-cell behavior in nontrivial ways.

Investigating how the behavior of single cells scales up to history dependence at the population level is an important goal. Many microorganisms live in dynamic environments where the quality and quantity of nutrients and biological, physical, and chemical stressors change continuously. Therefore understanding how microorganisms operate in such dynamic environments is a fundamental question. In addition, such understanding also has potentially relevant applications, for example in the context of pathogens that are exposed to fluctuating concentrations of antibiotics during treatment or microorganisms in technical systems that are exposed to dynamic operating conditions. Single-cell measurements help to achieve a deeper understanding of history-dependent processes in microbial populations.

Materials and Methods

Time-Lapse Microscopy. Stalked cells of *C. crescentus* strain UJ590 (15) were grown in microfluidic devices and were exposed to different concentrations of sodium chloride (*SI Materials and Methods*). Images were acquired every 5 min, and cell division events were recorded (Fig. S7).

Computational Modeling. Individually based simulations were programmed in Matlab R2015b and were based on experimentally determined interdivision intervals and survival probabilities (Fig. S8).

Statistics. All statistical analyses were performed in Matlab R2015b.

Data. All data are made available as *Supporting Information*. Each figure legend includes a reference to the corresponding dataset. Figs. 2–5 are based on Datasets S1–S5, and Figs. S1–S8 are based on Datasets S6–S13. All datasets follow the same structure described in Dataset S14.

ACKNOWLEDGMENTS. The idea of exposing *C. crescentus* to a mild sodium chloride event followed directly by a sodium chloride stress event was proposed by Markus Arnoldini, who also conducted initial experimental tests with a setup similar to the one described in this work. Daan Kiviet adapted the mother machine design to fit eight channels on one chip and produced the silicon wafers. We thank Yves Barral, Dagmar Iber, the editor, and two anonymous reviewers for helpful comments and Stefanie Conrad, Désirée Schmitz, Chérine Baumgartner, Aliénor von Roten, Benjamin Lüthi, Gabrielle Siegrist, and Thomas Röösli for help with the image analysis. This work was supported by a grant from the Swiss National Science Foundation (to M.A.), by ETH Zurich and by Eawag.

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