A Single-Amino-Acid Substitution in Obg Activates a New Programmed Cell Death Pathway in *Escherichia coli*

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ABSTRACT **Programmed cell death (PCD) is an important hallmark of multicellular organisms. Cells self-destruct through a regulated series of events for the benefit of the organism as a whole. The existence of PCD in bacteria has long been controversial due to the widely held belief that only multicellular organisms would profit from this kind of altruistic behavior at the cellular level. However, over the past decade, compelling experimental evidence has established the existence of such pathways in bacteria. Here, we report that expression of a mutant isoform of the essential GTPase ObgE causes rapid loss of viability in *Escherichia coli*. The physiological changes that occur upon expression of this mutant protein—including loss of membrane potential, chromosome condensation and fragmentation, exposure of phosphatidylserine on the cell surface, and membrane blebbing—point to a PCD mechanism. Importantly, key regulators and executioners of known bacterial PCD pathways were shown not to influence this cell death program. Collectively, our results suggest that the cell death pathway described in this work constitutes a new mode of bacterial PCD.**

IMPORTANCE **Programmed cell death (PCD) is a well-known phenomenon in higher eukaryotes. In these organisms, PCD is essential for embryonic development—for example, the disappearance of the interdigital web—and also functions in tissue homeostasis and elimination of pathogen-invaded cells. The existence of PCD mechanisms in unicellular organisms like bacteria, on the other hand, has only recently begun to be recognized. We here demonstrate the existence of a bacterial PCD pathway that induces characteristics that are strikingly reminiscent of eukaryotic apoptosis, such as fragmentation of DNA, exposure of phosphatidylserine on the cell surface, and membrane blebbing. Our results can provide more insight into the mechanism and evolution of PCD pathways in higher eukaryotes. More importantly, especially in the light of the looming antibiotic crisis, they may point to a bacterial Achilles' heel and can inspire innovative ways of combating bacterial infections, directed at the targeted activation of PCD pathways.**

Received 6 November 2015 **Accepted** 19 November 2015 **Published** 22 December 2015

Citation Dewachter L, Verstraeten N, Monteyne D, Kint CI, Versées W, Pérez-Morga D, Michiels J, Fauvart M. 2015. A single-amino-acid substitution in Obg activates a new programmed cell death pathway in *Escherichia coli*. mBio 6(6):e01935-15. doi:10.1128/mBio.01935-15.

Editor Vanessa Sperandio, UT Southwestern Medical Center Dallas

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The term programmed cell death (PCD) is used for all genetically encoded pathways resulting in the cell's demise. Although many different types of PCD exist, it is best known under the form of apoptosis. Apoptosis is a process present in multicellular eukaryotic organisms which is involved in embryogenesis, maintenance of tissue homeostasis, and elimination of potentially dangerous cells (1, 2). This type of cell death is associated with many physiological changes, including chromatin condensation, DNA fragmentation, exposure of phosphatidylserine on the cell surface, and plasma membrane blebbing (1–3). Other PCD mechanisms present in metazoans include autophagy and programmed necrosis (3).

Although PCD pathways provide a clear benefit to the survival and development of multicellular organisms, the existence of such pathways is not restricted to higher eukaryotes. PCD is present in unicellular eukaryotes (4, 5) as well as in bacteria (6–11).

While the existence of PCD in bacteria might seem counterintuitive, it has long been known that these pathways function in developmental programs like fruiting body formation of *Myxococcus xanthus* and sporulation of *Bacillus subtilis* (12). More recently, a particular PCD pathway of *Staphylococcus aureus* was also shown to play a role in biofilm development (9). Moreover, it was argued that bacterial PCD might also function as an altruistic mechanism promoting the survival of the bacterial population under stressful conditions such as nutrient limitation and phage infection (12, 13). If true, bacterial PCD—as is the case for eukaryotic PCD mechanisms—functions in both the development of multicellular structures and the prolonged preservation of the "organism," the bacterial population.

We here report on a new bacterial PCD pathway in *Escherichia coli*. This pathway is triggered by the expression of a mutant iso-

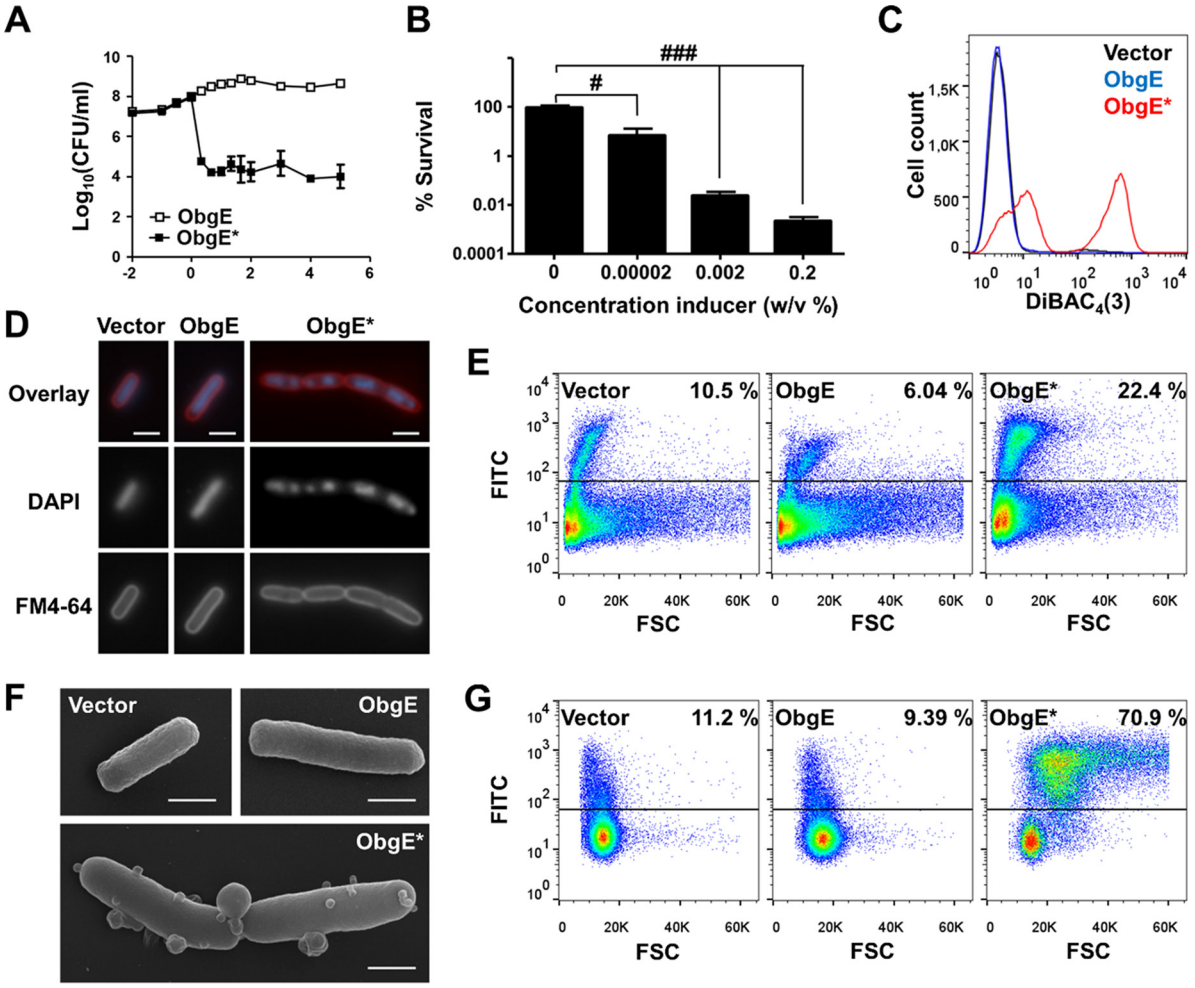

FIG 1 Expression of ObgE* triggers a PCD pathway. (A) The number of CFU per milliliter of an exponential-phase culture was monitored. At time zero, expression of ObgE or ObgE* from pBAD/His A was induced by adding 0.2% arabinose. Error bars represent standard errors of the means. (B) ObgE*-mediated toxicity at different induction levels. Error bars represent standard errors of the means. Pairwise comparisons were made between the control condition (no inducer) and induced cultures. #, $P \leq 0.05$; ###, $P \leq 0.0001$. (C) $DiBAC_4(3)$ staining as a measure of membrane depolarization. Fluorescence intensity was measured by flow cytometry. (D) Microscopic analysis of induced cells stained with the blue DNA dye DAPI and the red membrane-specific dye FM4-64. Bars, 2 μm. (E) DNA fragmentation was determined using the TUNEL assay. Percentages shown represent the fraction of the population with DNA damage. FSC, forward scatter. (F) SEM images showing the formation of blebs upon ObgE* expression. Bars, 1 μm. (G) Staining of externalized phosphatidylserine by FITC-conjugated annexin V. Percentages shown represent the fraction of the population that displays phosphatidylserine on the cell surface. Cells with compromised membrane integrity were omitted from this figure.

form of the essential GTPase ObgE (Obg of *E. coli*). This mutant isoform carries a single-amino-acid substitution, K268I, located in the GTPase domain. Biosynthesis of $ObgE_{K268I}$ (here designated ObgE*) rapidly elicits cell death and is associated with physiological changes that are markers of PCD. These physiological changes include loss of membrane potential, chromosome condensation, DNA fragmentation, exposure of phosphatidylserine on the cell surface, and membrane blebbing. We demonstrate that ObgE* triggers a PCD pathway that differs fundamentally from other, previously described bacterial PCD mechanisms.

ObgE*-mediated cell death. We previously explored the role of specific amino acid residues of ObgE in sensitivity toward hydroxyurea by screening a mutant *obgE* overexpression library constructed by error-prone PCR (14). This study additionally yielded a mutant allele encoding $ObgE_{K268I}$ (ObgE*) that, upon controlled expression, rapidly leads to cell death and is the focus of the current study.

Cell death caused by ObgE* was investigated by determining the number of CFU per milliliter at different time points before and after induction of protein expression with 0.2% arabinose (Fig. 1A). ObgE* causes a decrease in the number of CFU per milliliter of more than 4 orders of magnitude and this in less than 40 min after induction of expression. ObgE*-mediated toxicity was also investigated at different induction levels 2 h after the addition of inducer (Fig. 1B). Significant killing can already be seen at very low induction levels (0.00002% arabinose and higher), highlighting the strong toxicity of this protein. Protein expression levels were verified by Western blot analysis. To confirm that ObgE* causes cell death rather than a switch to a nongrowing state, cells expressing ObgE* were subjected to LIVE/DEAD staining. The result of this staining confirms that ObgE* is bactericidal and leads to eventual loss of membrane integrity (see Fig. S1 in the supplemental material). These experiments were carried out in an *E. coli* strain that retains the chromosomal *obgE*

gene. We therefore conclude that *obgE** is a dominant negative allele which, when expressed, causes rapid cell death in *E. coli*.

ObgE* triggers a PCD mechanism. To demonstrate that the cell death mechanism triggered by ObgE* is programmed, a wide array of PCD markers were investigated. The investigated markers are loss of membrane potential, chromosome condensation, DNA fragmentation, phosphatidylserine exposure, and membrane blebbing.

It was previously shown that bacterial PCD is associated with a loss of membrane potential (7, 8). Similarly, depolarization of the mitochondrial inner membrane is also a key characteristic of the intrinsic pathway of eukaryotic apoptosis (2). By using the potential-sensitive $DiBAC_4(3)$ [bis-(1,3-dibutylbarbituric acid) trimethine oxonol] dye, we found that loss of membrane potential also occurs during ObgE*-mediated cell death (Fig. 1C).

A second marker of PCD, chromosome condensation, was visualized using the blue DNA stain 4′,6-diamidino-2-phenylindole (DAPI). DAPI staining of cells expressing ObgE* shows multiple condensed foci of DNA per cell, whereas the DNA of cells containing the empty vector or cells expressing ObgE occupies the entire cell volume (Fig. 1D). In these experiments, membranes were visualized by concomitant staining with the lipophilic dye FM4-64. In addition to DNA condensation, it is clear that ObgE* also causes a defect in cell division, leading to the formation of cell chains up to 10 cells long.

The third marker of PCD that was detected is fragmentation of DNA, which is also a key characteristic of apoptosis. DNA fragmentation was evaluated using the TUNEL (terminal deoxynucleotidyltransferase dUTP nick end labeling) assay. This assay makes use of a terminal deoxynucleotidyltransferase that couples free 3′ OH groups of DNA to a dUTP residue that is covalently coupled to fluorescein isothiocyanate (FITC), linking fluorescence to DNA damage. Compared to cells carrying the empty vector or cells expressing wild-type ObgE, ObgE* leads to an increase in FITC fluorescence, indicating an increase in DNA damage (Fig. 1E).

Fourth, externalization of phosphatidylserine, a phospholipid that is normally localized in the inner leaflet of the cell membrane, was demonstrated using FITC-conjugated annexin V (Fig. 1G). Annexin V is a protein that specifically binds to phosphatidylserine and is excluded from cells with intact cell membranes. To discriminate between cells exposing phosphatidylserine on the cell surface and cells that have lost membrane integrity, the membrane-impermeant DNA dye propidium iodide (PI) was used. PI-positive cells with compromised membrane integrity are included in Fig. S2 in the supplemental material. An increase in FITC-annexin V binding can be seen upon expression of ObgE*, clearly showing the externalization of phosphatidylserine during the progression of cell death.

A final physiological characteristic that is associated with both apoptosis and programmed necrosis is the formation of protrusions of the membrane, called membrane blebs (2). Because of the drastic structural changes of the eukaryotic plasma membrane during PCD, the membrane structure of *E. coli* during ObgE*-mediated cell death was visually inspected by means of scanning electron microscopy (SEM). Images show extensive membrane blebbing when ObgE* is expressed (Fig. 1F).

Combined, the loss of viability upon expression of ObgE* and the concomitant phenotypic changes that we observed (loss of membrane potential, DNA condensation and fragmentation, exposure of phosphatidylserine at the cell surface, and severe membrane deformations resembling apoptotic blebs) provide convincing evidence that the pathway triggered by ObgE* is indeed a PCD pathway.

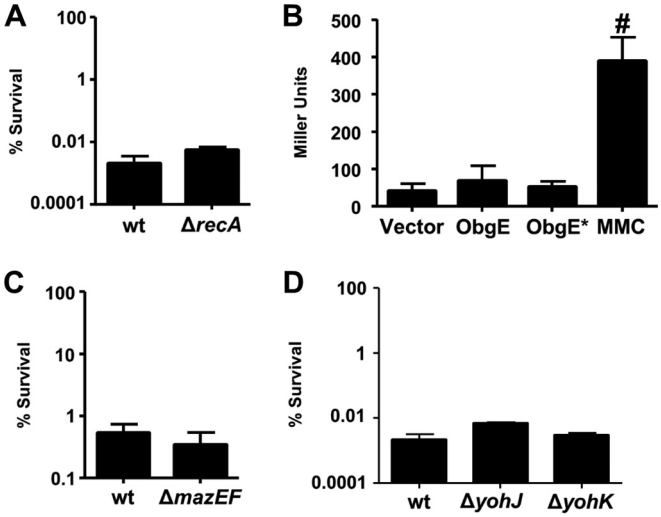

FIG 2 ObgE*-mediated PCD differs fundamentally from previously described bacterial PCD pathways. (A) ObgE*-mediated toxicity in *E. coli* WM2949 pJAT8-*araE* and its Δ*recA* derivative. (B) A β-galactosidase assay with *E. coli* MG1655 λ*sfiA*::*lacZ* shows no activation of the SOS response when ObgE* is expressed. As a positive control, MMC was used at a concentration of 5 μg/ml. #, $P \leq 0.05$ versus empty-vector control. (C) ObgE*-mediated toxicity in *E. coli* MC4100 and its Δ*mazEF* derivative. (D) ObgE*-mediated toxicity in *E. coli* WM2949 pJAT8-*araE* and its Δ*yohJ* and Δ*yohK* derivatives. wt, wild type. Error bars represent standard errors of the means.

ObgE*-mediated PCD is distinct from previously described bacterial cell death mechanisms. To unravel the mechanism underlying ObgE*-mediated PCD, we investigated the involvement of key regulators of previously described bacterial PCD mechanisms. Because ObgE*-triggered cell death occurs in *E. coli* and exhibits the main markers of eukaryotic apoptosis, the most likely candidate pathway is so-called apoptosis-like death (ALD) (6–8). Although the molecular pathway involved in ALD has not been fully uncovered yet, it was previously shown that this pathway is activated by treatment with DNA-damaging agents like nalidixic acid, norfloxacin, or mitomycin C (MMC) (6–8), requiring the multifunctional enzyme RecA and its role in the SOS response (6–8). It was also reported that ALD can be activated by treatment with bactericidal antibiotics like gentamicin and ampicillin. In addition to a possible specific effect of these antibiotics on the activation of RecA, it is thought that they lead to the formation of DNA-damaging hydroxyl radicals that can activate ALD similarly to other DNA-damaging agents (7). In the presence of DNA damage, the SOS response is activated by RecA and leads to the expression of over 40 genes involved in DNA damage repair (8). In the case of overwhelming DNA damage, however, high levels of activation of the SOS response presumably lead to ALD rather than repair, thereby preventing a futile attempt to repair DNA that is beyond salvage (8). However, deletion of the *recA* gene did not restore viability in the presence of ObgE* (Fig. 2A) and had no effect on any of the PCD markers investigated (see Fig. S3 in the supplemental material). Moreover, a β-galactosidase assay with *E. coli* MG1655 λ*sfiA*::*lacZ* (15), in which *lacZ* expression is controlled by the promoter of the SOS gene *sfiA* (also known as *sulA*),

showed that expression of ObgE* does not result in activation of the SOS response (Fig. 2B), although this strain is also sensitive to ObgE* expression (data not shown). The absence of an SOS response in the presence of ObgE* might seem surprising since the TUNEL assay revealed that expression of ObgE* leads to DNA damage. However, this can be explained as follows. PCD as described in eukaryotes occurs in a hierarchically ordered manner, with some events preceding others (2). Likewise, DNA fragmentation presumably is one of the later steps of ObgE*-mediated PCD since the fraction of cells showing DNA damage is lower than the fraction of cells that have lost viability, have lost their membrane potential, or expose phosphatidylserine on the cell surface at the same time point. It is therefore likely that cells showing DNA fragmentation are lethally injured and are no longer capable of activating the SOS response to repair this damage. Together, these results suggest that RecA plays no direct role in ObgE*-mediated cell death. This is consistent with previous reports suggesting that RecA and ObgE act complementarily rather than interdependently (16, 17).

Another PCD pathway described in *E. coli* is mediated by the type II toxin-antitoxin (TA) module MazEF (6). To exclude a possible role for the MazF toxin in the execution of ObgE*-mediated cell death, we tested toxicity in *E. coli* MC4100 Δ*mazEF* and the corresponding wild-type strain, *E. coli* MC4100 (6). Expression of ObgE* in *E. coli* MC4100 Δ*mazEF* did not result in altered survival compared to *E. coli* MC4100 (Fig. 2C). Therefore, the *mazEF* module does not play a role in the execution of ObgE*-mediated cell death. Moreover, the *E. coli* strain used throughout this study (*E. coli* WM2949) is defective in the production of the extracellular death factor (EDF)—a quorum-sensing peptide that is essential for the toxic action of MazF (6)—further indicating that this pathway is not involved in ObgE*-mediated cell death.

A final bacterial PCD pathway of interest is mediated by (anti-)holin-like activities and has so far been described only in *Staphylococcus aureus* and *Pseudomonas aeruginosa* (9, 18). The *cidAB* and *lrgAB* loci are the regulators of this PCD mechanism in *S. aureus*. We therefore investigated a potential role for the homologous locus in *E. coli*, *yohJK*. Single-gene knockouts of both *yohJ* and *yohK* were tested for their sensitivity toward ObgE*-mediated cell death (Fig. 2D). Deletion of *yohJ* or *yohK* did not alter the viability in the presence of ObgE*, excluding any role for these genes in the execution of cell death and also arguing against a protective function toward ObgE* toxicity. The *P. aeruginosa alpB* gene that presumably functions as a holin contributing to PCD does not have a homologue in *E. coli*.

Other bacterial PCD pathways have either previously been shown not to be active in *E. coli* (10), are induced by specific triggers that do not occur during ObgE* expression (11), or are associated with differentiation and formation of specialized structures (12) and can thus be excluded.

Conclusions. Serendipitous discovery of a mutant *obgE* allele, $obgE_{K268I}$, led to the discovery of a new cell death pathway in *E. coli*. Cell death caused by ObgE* is associated with many markers of PCD. Importantly, ObgE*-mediated cell death does not proceed through any of the previously described bacterial PCD mechanisms, indicating that it is a fundamentally different bacterial PCD pathway. ObgE* can therefore be used as a unique research tool to activate and study this newly discovered cell death mechanism in *E. coli*.

Obg proteins are P-loop GTPases that are found in all domains of life. They are involved in a myriad of important metabolic processes and are essential for bacterial viability (19, 20). Although the role of Obg in bacterial physiology is clearly invaluable, its precise functions have not been elucidated yet. A role for Obg has been described in ribosome assembly, the stringent response, DNA replication, chromosome segregation, and antibiotic tolerance (19–21). It was previously suggested that Obg might function as a cell cycle checkpoint to coordinate some of these processes with cell division (17, 19).

The mechanism of ObgE*-mediated PCD is still unclear. However, considering the proposed role for ObgE as a cell cycle checkpoint, it is tempting to speculate that the K268I mutation might cause a defect in cell cycle regulation, resulting in erroneous programmed cell death. Eukaryotic cell cycle regulators are able to halt cell division as well as induce PCD when deemed necessary (2). Likewise, it was shown previously that ObgE is important for the onset of cell division since both overexpression and depletion of ObgE result in filamentation (17, 22). Moreover, we have identified a mutant isoform of ObgE that is capable of inducing PCD. This leads us to hypothesize that wild-type ObgE might be capable of inducing PCD under specific physiological conditions, possibly through posttranslational modification or protein-protein interactions, and that aberrant regulation of this capability in ObgE* leads to constitutive PCD. The prospect of ObgE controlling both the onset of cell division and the activation of PCD—like eukaryotic cell cycle regulators do—is an intriguing one that will be investigated further.

Future research will be directed into unravelling the genetic and molecular basis of ObgE*-mediated PCD, the conservation of this cell death pathway in other organisms, and discovering natural and physiologically relevant conditions that trigger the PCD pathway that is artificially activated by ObgE*. This will in turn reveal the biological relevance of this cell death pathway, the mechanism behind ObgE*-mediated PCD, and the potential of this pathway as a target for the future development of a new class of antibacterials.

Strains, plasmids, and growth conditions. In this work, *E. coli* WM2949 (23) with plasmid pJAT8-*araE* (24) was used except when stated otherwise. In this strain, the chromosomal *araFGH* and *araE* genes were deleted and *araE* expression is controlled by a constitutive promoter on the pJAT8 plasmid, thereby allowing homogeneous expression from the P_{BAD} promoter (23, 24). Single-gene knockouts of *yohJ* and *yohK* were constructed in *E. coli* WM2949 pJAT8-*araE* by transducing the corresponding knockouts from the Keio collection (25) as described previously (15). Deletion of the correct gene was confirmed by PCR with primers SPI10966 (GCGCTGATTTCTTAA TGTGA) and SPI10967 (ATCAATGTCAGCGGTAATGA) for the deletion of *yohJ* and primers SPI10968 (CGTAGGTCAGAAAGGAT CAG) and SPI10969 (CGCGTGAAACATACATCAAA) for the deletion of *yohK*.

To investigate ObgE*-mediated effects, strains were transformed with pBAD/His A (Invitrogen), pBAD/His A-*obgE* (pCMPG13901), or pBAD/His A-*obgE** (pCMPG13828). The influence of the N-terminal His tag present in these constructs was investigated by additionally expressing ObgE and ObgE* in their native forms from the pBAD33 vector. The presence or absence of the His tag did not affect the results.

For all phenotypic and toxicity tests, overnight cultures were diluted 100 times in lysogeny broth (LB) containing the appropriate antibiotics, incubated for 2 h at 37°C with continuous shaking

at 200 rpm, and induced with 0.2% arabinose for 2 h under the same conditions.

Toxicity tests. To characterize ObgE*-mediated toxicity (Fig. 1A), the number of CFU per milliliter was determined at several time points before and after induction by plating out serial dilutions on LB containing the appropriate antibiotics and 1.5% agar. For all other toxicity tests, the number of CFU per milliliter was determined analogously after 2 h of induction of either ObgE or ObgE*. The percent survival was calculated by dividing the number of cells surviving ObgE* expression by the number of cells present after ObgE expression under the same conditions.

Membrane potential. Loss of membrane potential was determined using the membrane potential-sensitive dye $DiBAC_4(3)$ (Life Technologies), as described previously (21). Fluorescence was measured using a BD Influx cell sorter equipped with 488-nm (green) and 561-nm (red) lasers and standard filter sets.

DNA fragmentation. DNA fragmentation was quantified by the TUNEL assay (Apo-Direct kit; BD Biosciences) as described previously (7). Fluorescence was measured by means of flow cytometry.

DNA condensation. For DAPI (Sigma-Aldrich) and FM4-64 (Life Technologies) staining, cultures were washed and dissolved in 10 mM $MgSO_4$. The dyes were added simultaneously at a concentration of 10 μg/ml, and cultures were incubated for 10 min in the dark at room temperature. Cells were visualized with the Zeiss Axio Imager Z1. Standard DAPI and Texas Red filters were used.

Phosphatidylserine exposure. Exposure of phosphatidylserine at the cell surface was measured by the FITC-annexin V/dead cell apoptosis kit (Molecular Probes) as described previously (7). Fluorescence was measured by means of flow cytometry.

Scanning electron microscopy. *E. coli* WM2949 Δ*recA* samples were prepared as described above. Cultures were fixed overnight at 4°C in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) and postfixed in 2% OsO_4 in the same buffer. After serial dehydration, samples were dried at critical point and coated with platinum by standard procedures. Observations were made in a Tecnai FEG ESEM Quanta 200 microscope (FEI), and images were processed by SIS iTEM (Olympus) software.

***β*-Galactosidase assay.** To measure activation of the SOS response, cultures were induced with 0.2% arabinose for 3 h prior to sample collection. The β-galactosidase assay and the calculation of Miller units as a measure of expression level were carried out as described previously (26).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01935-15/-/DCSupplemental.

Figure S1, PDF file, 0.1 MB.
Figure S2, PDF file, 0.3 MB.
Figure S3, PDF file, 0.3 MB.

ACKNOWLEDGMENTS

We thank John Cronan, Jr. (University of Illinois), Jay Keasling (University of California), Abram Aertsen (KU Leuven), and Hanna Engelberg-Kulka (Hebrew University of Jerusalem) for strains and plasmids. We are also thankful to Abram Aertsen (KU Leuven) and Rob Lavigne (KU Leuven) for fruitful discussions.

L.D. received a fellowship from the Fund for Scientific Research, Flanders (FWO). C.I.K. is a recipient of a fellowship from the Institute for the Promotion of Innovation through Science and Technology in Flanders (IWT Vlaanderen). This work was supported by grants from the FWO (G.0413.10, G.0471.12N, and G0B2515N), KUL-BOF CREA/13/019, and the Interuniversity Attraction Poles Program initiated by the Belgian Science Policy Office.

We apologize to those authors whose work could be referred to only by citation of review articles.

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