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Protein Aggregates: an Aging Factor Involved in Cell Death[▽]

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In a previous study, we demonstrated the presence of protein aggregates in an exponentially grown *Escherichia coli* culture. In light of these observations, protein aggregates could be considered damage to cells that is able to pass from one generation to the next. Based on the assumption that the amount of aggregate protein could represent an aging factor, we monitored this amount in a bacterial culture during senescence. In doing so, we observed (i) a significant increase in the amount of aggregate protein over time, (ii) a proportional relationship between the amount of aggregate protein and the level of dead cells, (iii) a larger amount in dead cells than in culturable cells, (iv) a heterogeneous distribution of different amounts within a homogenous population of culturable cells entering stasis, and (v) that the initial amount of aggregate protein within a culturable population conditioned the death rate of the culture. Together, the results presented in this study suggest that protein aggregates indeed represent one aging factor leading to bacterial cell death.

The accumulation of damage is currently assumed to be responsible for the ubiquitous progressive decline in the functional capacity of aging cells or organisms. The aging process was believed to be specific to eukaryotes. However, Ackermann et al. (2) were the first to demonstrate signs of replicative aging in bacteria, using *Caulobacter crescentus.* Interestingly, cytokinesis is intrinsically asymmetrical in this bacterium. This finding was in good agreement with the assumption suggesting that the distinction between organisms that age and those that do not depends upon asymmetrical fission. In this respect, aging could evolve only together with rejuvenating reproduction. However, two recent studies reported aging in organisms that appear to divide symmetrically, i.e., *Schizosaccharomyces pombe* and *Escherichia coli* (5, 16). Taking into account these earlier findings, an asymmetrical distribution of damage could be considered a strategy to cope with damage as an alternative to repair (1, 12, 17). As a possible general cell response, this strategy may thus represent a more fundamental aspect of all cellular organisms (eukaryotes or prokaryotes that divide symmetrically or not). All of the aforementioned studies were based on observations of signs of replicative aging, but these were never made toward the end of aging or death. In addition, no information is currently available on what kinds of aging factors, such as damaged or toxic molecules, are critical enough to lead to cell death during senescence. Among potential aging factors, protein aggregates appear to be an interesting candidate. Indeed, Nystrom's group has shown that oxidized protein in aggregate form is partitioned in progenitor cells during yeast cytokinesis (3, 9, 10), and in mammalian cells this phenomenon occurs via the aggresomes (11). Finally, Lindner et al. (13) have recently shown a link between protein aggregates and signs of replicative aging in *E. coli* in a nonstressed population. Interestingly, under the same experimental conditions, we have previously shown the existence of aggregates enriched with abnormal proteins (14). Together, these observations led us to investigate the possibility that protein aggregates represent one aging factor that affects not only sibling-specific fitness but also bacterial cell death.

In this study, using *E. coli* as a model organism, we observed that the relative amount of aggregate protein within cells appears to represent at least one aging factor, conditioning bacterial culture fate during bacterial senescence.

MATERIALS AND METHODS

Bacterial strains and media. Strains (*E. coli* MG1655 and mutants) were grown aerobically in liquid Luria-Bertani (LB) medium in a rotary shaker at 37°C and 200 rpm. Overnight cultures were diluted 100-fold in LB and allowed to grow to an optical density at 600 nm (OD_{600}) of 0.5. Deletion mutants were constructed by P1 transduction in the same parental strain (MG1655) and selection of kanamycin resistance for the Δ*dnaK*::*kan* (JW0013), Δ*clpX*::*kan* (JW0428), and Δ*clpB*::*kan* (JW2573) mutants (4). Kanamycin resistance genes were removed as described previously (7) except for the Δ*dnaK*::*kan* strain. The *hpx* mutant was a kind gift from F. Barras. The *skx* strain (*katE*::Tn*10 katG*::Tn*10 sodA49 sodB*::MudPR3, *zij*::minikan-omega) and plasmid pSodA pDT1-19 (pBR322 carrying the *lacI* gene and the *sodA* gene under P*tac* control) were provided by D. Touati.

Protein aggregate preparation. Briefly, and as described previously (14), aggregate proteins were isolated from cells grown in LB medium at the indicated time and then washed twice with phosphate buffer (pH 7, 0.05 M, 4°C) by centrifugation at 5,500 × *g* for 20 min at 4°C. Cells were resuspended in phosphate buffer and lysed by four cycles of French press treatment, and all samples were treated with 200 μg/ml DNase and 50 μg/ml RNase. French press crude extracts were then centrifuged for 30 min at 18,000 × *g* (4°C) to obtain pellets. These were then resuspended in buffer A (50 mM Tris, 150 mM NaCl, pH 8) with 1% Triton X-100 and incubated at 4°C for 3 h. This procedure was repeated with 0.5% Triton X-100. Pellets were then washed with buffer A and centrifuged for 30 min at 18,000 × *g* to obtain insoluble protein aggregates. Finally, the insoluble protein aggregates were solubilized in rehydratation buffer {7 M urea, 2 M thiourea, 4% [wt/vol]) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate [CHAPS], 100 mM dithiothreitol).

Radioselectan equilibrium density gradients. Cells were grown in LB medium and collected after either 10 h or 48 h. Cells were then washed in cold phosphate buffer (pH 7.4, 0.05 M) and concentrated in cold 26.45% radioselectan (sodium and meglumine amidotrizoate; final volume, 10 ml; final cell concentration, 5×10^{10} cells/ml). Gradients were prepared as described previously (6) using 10 ml of 37% radioselectan in each polycarbonate centrifuge tube (25 by 89 mm) layered with 1 ml of a bacterial suspension (5×10^{10} cells) without a loss of resolution. Gradients were spun at 55,000 rpm in a Ti70 rotor using a Beckman tabletop ultracentrifuge at 4°C for 2 h. After collection, the cells in the sample were pelleted and rinsed with cold phosphate buffer (pH 7.4, 0.05 M). For each

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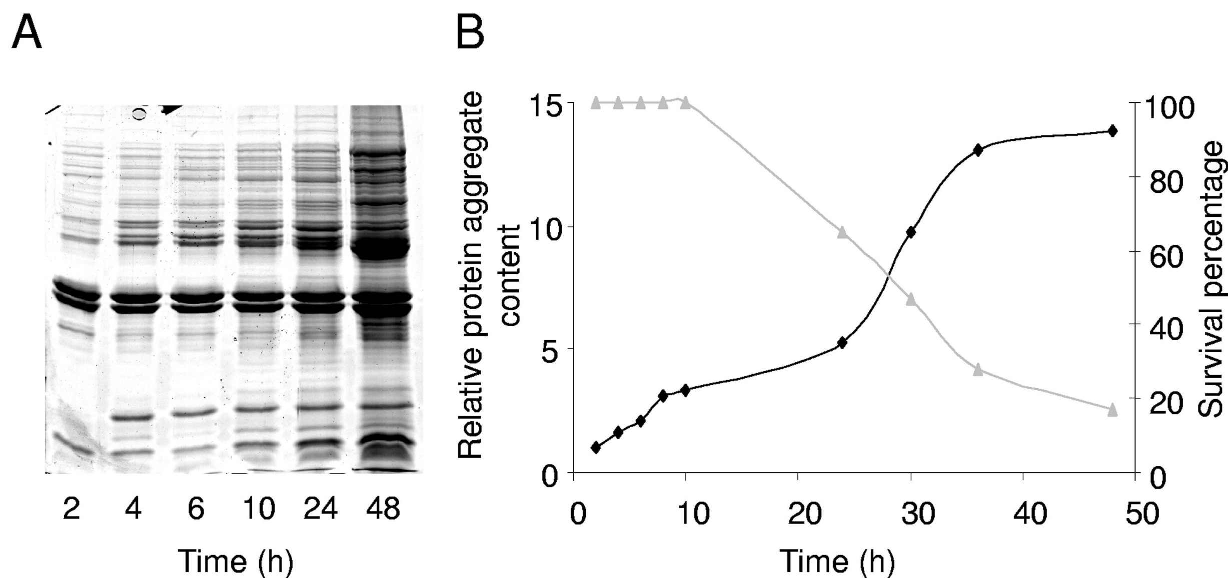

FIG. 1. Aggregate protein accumulates over time. (A) Coomassie blue-stained SDS-polyacrylamide gel showing the relative amount of aggregate protein (per mg of soluble protein) at three time points during exponential (2, 4, and 6 h) or stationary (10, 24, and 48 h) phase. At each point, samples were prepared from equal amounts of cells as determined by OD. (B) Representative results for (i) relative aggregate content for each time during exponential (2, 4, and 6 h) or stationary (10, 24, 30, 36, and 48 h) phase, quantified using Quantity One software (Bio-Rad) (dark line), and (ii) percent survival at the same time (gray line). Analyses were repeated three times to confirm reproducibility.

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cell preparation and separation step, total cell, intact cell, and culturable cell concentrations were determined by microscopic counting, by use of a Live/Dead Baclight bacterial viability kit (Molecular Probes), and by plating on LB agar, respectively, after serial dilution in cold phosphate buffer (0.05 M, pH 7.4°C).

Starvation assays and viability measurements. Cells were grown in LB medium to an OD_{600} of 0.5. Cells were then harvested and washed twice with phosphate buffer (pH 7, 0.05 M, 4°C) by centrifugation at 5,500 × *g* for 20 min at 4°C. Cells were then resuspended in the same initial volume of culture with phosphate buffer (0.05 M pH 7.4) at 37°C. The survival rate was monitored after 24 h of phosphate buffer starvation. For this, culturable bacteria were assayed by plating samples from suspensions onto LB agar plates after serial dilution in cold phosphate buffer (0.05 M, pH 7.4°C). Colonies were counted after 24 h of incubation at 37°C.

RESULTS

The amount of aggregate protein increases with time of culture. In a previous study (14), we demonstrated and characterized the existence of aggregates enriched in abnormal proteins in exponentially grown *E. coli* (wild-type strain MG1655). Here we proposed to evaluate the relative amounts of aggregate protein during different stages of *E. coli* growth. For this purpose, we analyzed the amount of aggregate protein per mg of soluble protein at various time points during exponential phase (2, 4, and 6 h) and stationary phase (10, 24, 30, 36, and 48 h). As depicted in Fig. 1A, sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis analysis revealed an important increase in the relative amount of aggregate protein during the time of culture. As shown in Fig. 1B, this increase exhibited an exponential pattern, with more than a 10-fold increase between the exponential (2 h) and later stationary (48 h) phases of culture. These results demonstrate that the relative amount of aggregate protein drastically increased with time of culture. Interestingly, this increase occurred concomitantly with cell death, suggesting a first link between these two parameters (Fig. 1B).

Levels of reactive oxygen species and abnormal proteins affect the amount of aggregate protein. As shown previously, in exponentially grown *E. coli* (14), higher levels of aggregate protein are found in mutants with mutations in genes involved in defenses against oxidative stress or heat shock than in wild-type strains. Having shown that the relative amount of aggregate protein increases during growth in the wild-type strain, we wanted to know if these mutants also display an increased relative amount of aggregate protein with time. For this purpose we used several mutants deficient in reactive oxygen species defenses (*skx*, *hpx*, and *hpx* pSodA mutants) or heat shock responses (Δ*clpB*, Δ*clpX*, and Δ*dnaK* mutants) and evaluated the relative amount of aggregate protein in the exponential phase (2 h) and at two points in the stationary phase (24 and 48 h). As depicted in Fig. 2A and B, SDS-polyacrylamide gel electrophoresis analysis revealed that for all tested mutants, the relative amount of aggregate protein increased with time. At each tested time point, two of the three tested mutants deficient in the heat shock response (Δ*clpX* and Δ*dnaK* mutants) showed greater relative amounts of aggregate protein than the wild type, not only in the exponential phase (as previously described) (14) but also in the stationary phase (Fig. 2A and B). This was not the case, however, for the Δ*clpB* mutant, which instead showed the same pattern as the wild type (Fig. 2A and B). At each tested time point, the relative amount of aggregate protein also increased for mutants with mutations in genes involved in defenses against oxidative stress, such as the *skx* (deficient in cytosolic superoxide dismutase and catalases) and the *hpx* (deficient in catalase and alkyl hydroperoxidase) mutants, compared to the wild type (Fig. 2A and B). Interestingly, overproduction of Mn-superoxide dismutase in the wild type (pSodA) provoked a decrease in the relative amount of aggregate protein compared to that in wild-type cells, not only

FIG. 2. Levels of aberrant proteins modulate the amount of aggregate protein during stasis. (A) Coomassie blue-stained SDS-polyacrylamide gel showing the relative amounts of aggregate protein (per mg of soluble protein) from different genetic backgrounds prepared during exponential phase (2 h) or twice during stationary phase (24 and 48 h). At each time point, samples were prepared from equal amounts of cells as determined by OD. Six times and three times more material were loaded in samples from 2 and 24 h, respectively, compared to samples from 48 h. (B) Relative aggregate content for each genetic background over time. At each time point (2, 24, and 48 h), relative aggregate content was quantified using Quantity One software (Bio-Rad). Analyses were repeated three times to confirm reproducibility. wt, wild type.

in the exponential phase (as previously described) (14) but also in the stationary phase (only at 24 h). This phenomenon was most pronounced when Mn-superoxide dismutase was overexpressed in the *hpx* mutant (*hpx* pSodA), not only in the exponential phase (as previously described) (14) but also in the stationary phase (at both 24 and 48 h) (Fig. 2A and B). Taken together, these results indicate that at each tested time of culture, the relative amount of aggregate protein is modulated by levels of reactive oxygen species and/or abnormal protein.

Relationship between amount of aggregate protein and cell survival during stasis. The presence of protein aggregates has been proposed to reflect a cell disorder which may ultimately lead to bacterial death (15). In order to test this assumption, we measured the CFU and the relative amount of aggregate protein at two different time points during the stationary phase (24 and 48 h) for the wild type and all mutants described above. As depicted in Fig. 3, for all tested strains (wild type and

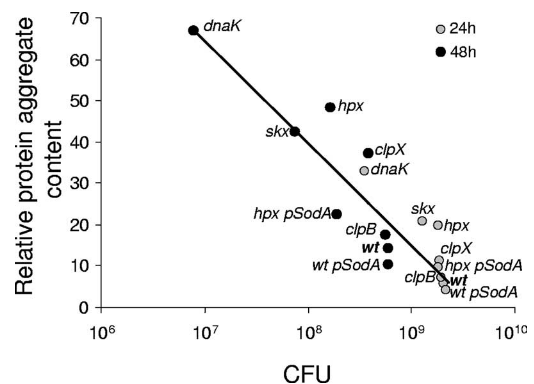

FIG. 3. Relative amount of aggregate protein conditions bacterial culture fate during stasis. The relative amount of aggregate protein (per mg of soluble protein) as a function of culturable cell number (expressed as CFU) is shown for eight genetic backgrounds analyzed at two time points during stasis (24 h [gray circles] and 48 h [black circles]). wt, wild type.

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mutants) at 24 and 48 h, the larger the relative amount of aggregate protein, the higher the concentration of dead cells. This result suggests a link between the relative amount of aggregate protein and the percentage of dead cells present in the culture during stasis.

Population asymmetry: dead cells contain an increased amount of aggregate protein compared to culturable cells. We next wondered if the increase in the amount of aggregate protein within the culture was mainly the result of a larger relative amount present in the dead cell subpopulation. For this purpose, we used a nondestructive separation technique allowing us to separate culturable and dead bacteria during stasis from the same population on the basis of a slight difference in cell density (8). Using this separation technique, we isolated two cell subpopulations from a 48-hour stationary-phase culture. As previously described (8), we observed that more than 90% of the cells in the low-density fraction were able to form colonies, compared to only a small percentage of the cells in the high-density fraction (Fig. 4A). Evaluation of the relative amount of aggregate protein in each subpopulation revealed more than a fivefold increase in the high-density fraction (enriched in dead cells) compared to the low-density fraction (enriched in culturable cells) (Fig. 4B and C). This result demonstrates that the increase in the amount of aggregate protein observed within a population during stasis relates mainly to an increase in the relative amount inside the dead cell subpopulation. Finally, this result also shows that the amount of aggregate protein among cells of a population is highly heterogeneous and related to the culturability.

An increase in the amount of aggregate protein occurs before cell death. The increases in relative amount of aggregate protein observed in dead cells could occur either after or before the loss of culturability. To investigate this further, we used the same nondestructive separation technique as that previously described, allowing us to separate a culture entering stationary phase (10 h) into two subpopulations (found in the low-density fraction and the high-density fraction), both containing only culturable bacteria (Fig. 5A) (6). Evaluation of the relative amounts of aggregate protein in the two subpopulations (Fig. 5B and C) revealed more than a twofold increase within the subpopulation found in the high-density fraction compared to that in the low-density fraction (Fig. 5B and C). This result demonstrates a heterogeneous distribution of various relative amounts of aggregate protein among cells, even in cultures containing only culturable cells. Interestingly, we have previously observed that the subpopulation localized in the high-density fraction is composed mostly of those cells predisposed to die (6). Taken together, our results indicate that at least part of the observed increase in the relative amount of aggregate protein occurs before cell death.

The initial amount of aggregate protein conditions the die-off rate during phosphate buffer starvation. We then asked if protein aggregates were involved in the chain of events leading to the loss of culturability during senescence. Indeed, a higher relative level of aggregate protein in the future dead cells could be prerequisite to cell death. We investigated whether the initial amount of aggregate protein in a bacterial culture influences the die-off rate of this culture during starvation. For this purpose, mutants with mutations in genes involved in defenses against heat shock

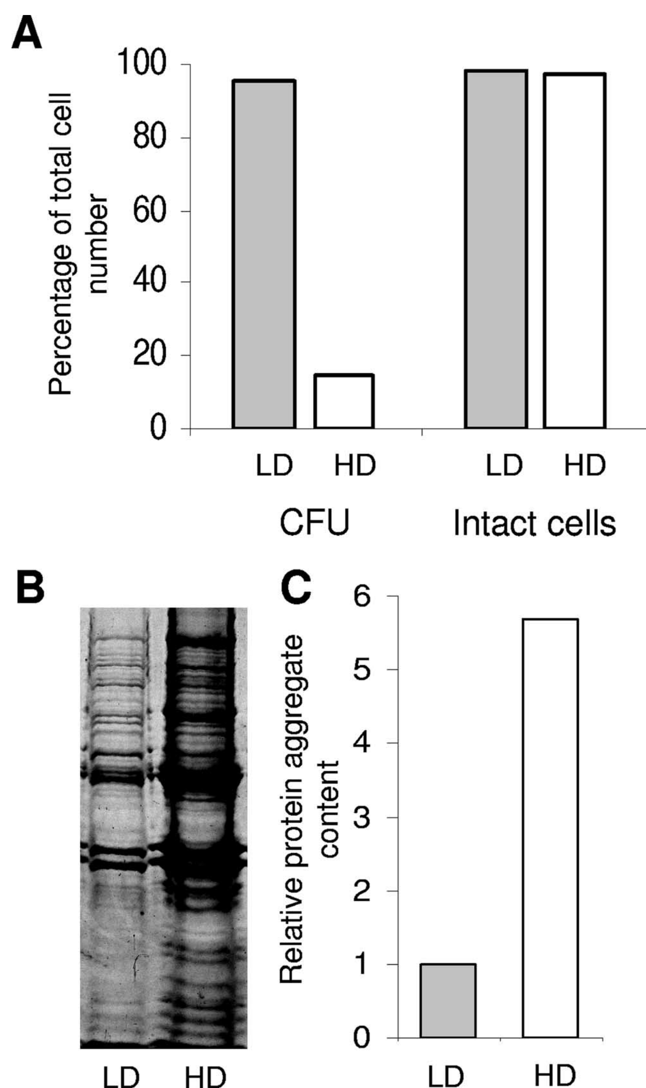

FIG. 4. Dead cells show larger relative amounts of aggregate protein. (A) Determination of reproductive ability (CFU) and cell integrity in the low-density (LD) (shaded bars) and high-density (HD) (unshaded bars) cell populations from a 48-h stationary-phase culture. (B) Coomassie blue-stained SDS-polyacrylamide gel showing the relative amounts of aggregate protein (per mg of soluble protein) from LD and HD cells prepared from equal amounts of cells as determined by OD. (C) Relative aggregate amounts from LD an HD cells quantified using Quantity One software (Bio-Rad), with analyses repeated three times to confirm reproducibility.

(Δ*clpX*, Δ*dnaK*, and Δ*clpB*) or oxidative stress (*skx* and *hpx*) and the wild type were grown to an OD_{600} of 0.5. Cells were then washed and starved in phosphate buffer. We evaluated the percent survival after 24 h of phosphate buffer starvation and the initial amount of aggregate protein for each strain, as described in Materials and Methods. As depicted in Fig. 6, the initial relative amount of aggregate protein was inversely proportional to the percent survival after 24 h of phosphate buffer starvation. More interestingly, overproduction of Mn-superoxide dismutase provoked a decrease in the initial relative amount of aggregate protein and an increase in the survival percentage in wild-type cells, a phe-

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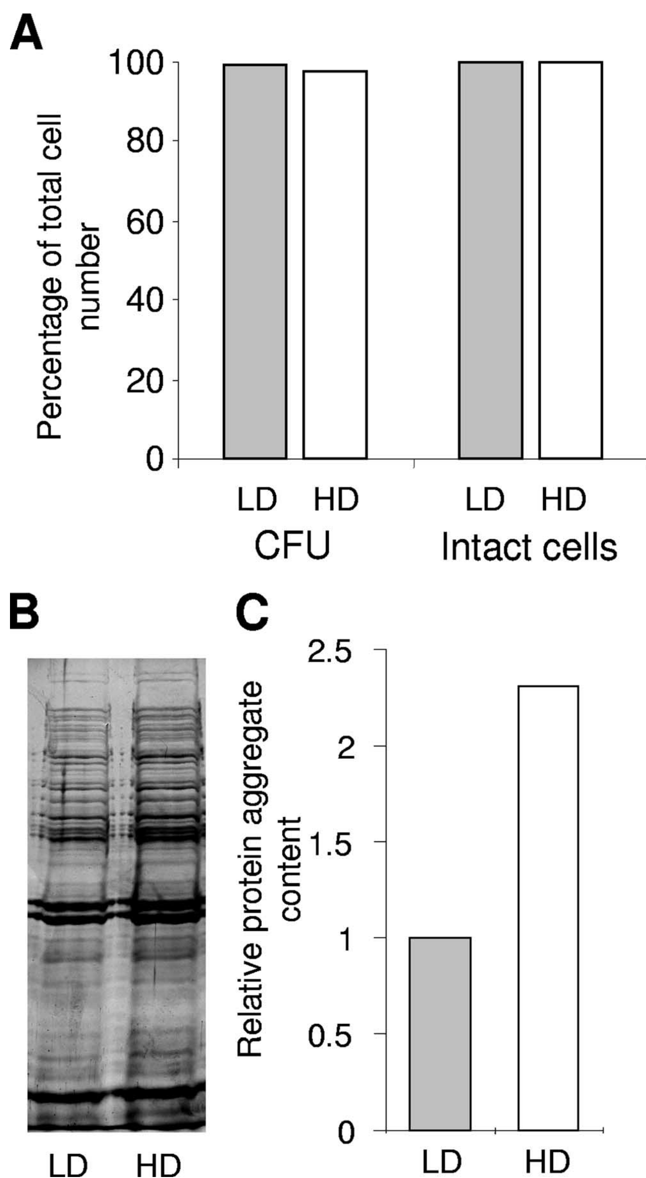

FIG. 5. Future dead cells have larger relative amounts of aggregate protein. (A) Determination of reproductive ability (CFU) and cell integrity in the low-density (LD) (shaded bars) and high-density (HD) (unshaded bars) cell populations from a 10-h culture. (B) Coomassie blue-stained SDS-polyacrylamide gel showing the relative amounts of aggregate protein (per mg of soluble protein) from LD and HD cells prepared from equal amounts of cells as determined by OD. (C) Relative aggregate amounts from LD and HD cells quantified using Quantity One software (Bio-Rad), with analyses repeated three times to confirm reproducibility.

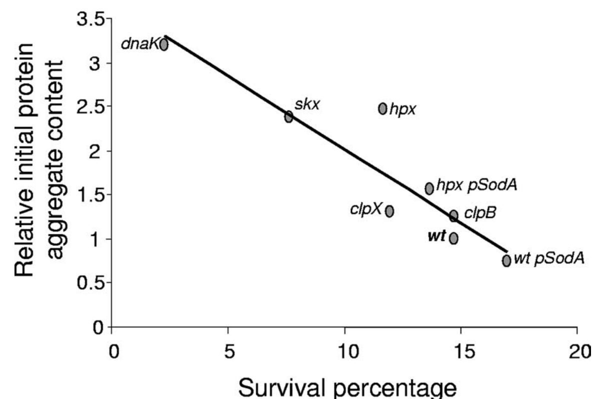

FIG. 6. The initial relative amount of aggregate protein conditions the die-off rate during phosphate buffer starvation. The initial relative amount of aggregate protein as a function of percent survival after 24 h of phosphate buffer starvation is shown. The initial relative amount of aggregate protein was taken from the cell population in logarithmic phase (OD_{600} = 0.5). At the same time, cells were starved in phosphate buffer. wt, wild type.

nomenon that was again most prominent in the *hpx* mutant (Fig. 6). Together, our results strongly indicate that the relative amount of aggregate protein appears to be a key element conditioning the fate of bacterial cultures during starvation.

DISCUSSION

In a previous study (14), we demonstrated the presence of protein aggregates overrepresenting abnormal proteins in an exponentially grown *E. coli* culture. In light of these observations, protein aggregates could be considered damage able to pass from one generation to the next, thus accumulating over time or senescence. Considering the small number of protein aggregates in a cell, mathematical modeling predicted that an asymmetrical distribution of this damage is beneficial for the population (1, 12, 17). One direct consequence of an asymmetrical distribution would be the formation of two distinct cell subpopulations dependent on damage levels. Interestingly, Lindner et al. (13) have recently shown that indeed two distinct *E. coli* subpopulations dependent on the amount of aggregate protein exist under unstressed growth conditions. Moreover, an association between protein aggregate segregation and replicative aging has been proposed (13). This suggests an asymmetric strategy whereby dividing cells segregate the damage at the expense of aging individuals, resulting in the perpetuation of the population (13). The association between protein aggregates and signs of replicative aging in *E. coli* does not allow us to also definitively associate protein aggregates with bacterial cell death. However, based on earlier observations and results presented in this study, we propose that the relative amount of protein aggregates which overrepresent abnormal proteins represent one aging factor leading to bacterial cell death.

We first measured the relative amount of aggregate protein (per mg of soluble protein) in the global cell population and observed a significant increase during population senescence. Interestingly, this increase occurred concomitantly with cell death, suggesting a first link between these two parameters. We can confirm that the increase came mainly from the dead cell fraction and not the culturable cell fraction. Indeed, using several mutants known to lose cell culturability in stationary phase more rapidly than the wild type, we first demonstrated that the relative amount of aggregate protein is directly associated with the concentration of dead cells inside a cell population in stasis. Next, using the same experimental procedure that allowed Desnues et al. (8) to separate culturable and dead cells within the same global population from stationary phase (48 h), we have provided evidence for a heterogeneity in terms

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of the relative amount of aggregate protein among cells, with dead cells containing increased levels compared to culturable cells. Interestingly, Desnues et al. (8) reported higher levels of oxidized proteins (carbonylated proteins) in dead cells than in culturable cells, and we have observed that these carbonylated proteins relate mainly to aggregate proteins (E. Maisonneuve et al., unpublished results). Taking the results together, we propose that the accumulation of oxidized proteins is only a part of a much more general phenomenon based on the accumulation and segregation of all damaged proteins (abnormal and/or oxidized proteins) in the form of a protein aggregate(s) during senescence.

Finally, our results indicate that at least a part of the increase in the relative amount of aggregate protein observed in dead cells occurs before the loss of culturability and conditions bacterial cell death. Indeed, using the separating procedure at 10 h of growth where 100% of cells were culturable, we observed a larger relative amount of aggregate protein in the subpopulation of cells previously demonstrated to be predisposed to die (6). This observation is in good agreement with our results obtained with mutants, showing that the initial relative amount of aggregate protein influences the percent survival of a culture during starvation.

Together, the results presented in this study suggest that protein aggregates which overrepresent abnormal proteins represent one aging factor affecting not only sibling-specific fitness but also bacterial cell death.

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