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Original article

Alternative scenarios of starvation-induced adaptation in *Pectobacterium atrosepticum*

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Abstract

Bacteria have high adaptive potential that ensures their survival during various environmental challenges. To adapt, bacteria activate a physiological program of stress response that makes them able to persist under adverse conditions. The present study sought to examine the ability of a particular bacterial species to induce a stress response in alternative scenarios. Cells of the phytopathogenic microorganism *Pectobacterium atrosepticum* were taken as a model. The cells were exposed to starvation in different physiological states (actively growing exponential phase and stationary phase cells), and the resulting starving cultures were monitored using CFU counting, quantitative PCR and electron microscopy. When exponential phase cells were subjected to starvation, the nucleoids of the cells became condensed and their DNA was detected by qPCR less effectively than that of cells growing in nutrient-rich medium, or stationary phase cells after starvation. Exponential phase cells subjected to starvation showed increased expression of genes encoding DNA binding histone-like proteins, whereas, in cultures inoculated by stationary phase cells, cell-wall-deficient forms that were inefficient at colony forming and that had a non-culturable phenotype were formed. The cell-wall-deficient forms displayed reduced expression of genes encoding synthases of cell wall components.

Keywords: Adaptation; Stress response; Nucleoid condensation; Cell-wall-deficient forms; Viable but non-culturable cells

1. Introduction

Since bacteria are among the most simply organized organisms, they possess high adaptive potential that enables them to endure a variety of environmental challenges. Adaptive responses are often related to dramatic physiological and morphological changes in the cells. It is generally accepted that a bacterial cell must be specifically pre-prepared physiologically in order to realize its adaptive potential. First, the cell must "feel" itself as part of the population, since the stress response is under the control of cell-to-cell communication, and thus adaptation is a function of the population, but not of the particular cell [3,39,44]. Second, the cell must be targeted toward performing one or at least a limited set of the physiological programs. It is known, for instance, that actively proliferating cells are deficient in their stress resistance [43,47].

However, bacterial cells in a natural ecosystem may be exposed to a stress effect without being pre-prepared physiologically to adapt–for example, when existing at low population density or using the strategy of intensive reproduction. Our previous works on the plant pathogenic bacterium *Pectobacterium atrosepticum* [10,31] and other microorganisms (unpublished data) showed that additional steps within the framework of the stress response resulted in overcoming some restrictions that limited formation of the adaptive response in

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bacterial cells. When the density of the bacterial population is below the communication level, the starvation-induced stress response starts from cell division even in the absence of the exogenous growth substrate. Division proceeds until the population density reaches the level necessary for communication; afterwards, the adaptive response is activated [10,31]. In our present work, we sought to determine whether bacteria utilize specific stress reactions so as to resist exposure to stress factors during the period of intensive reproduction that negatively affects their resistance.

The present study aimed at comparing the adaptive strategies of actively growing cells in logarithmic growth phase and the cells pre-prepared for the stress response in stationary phase when growth processes were substantially terminated. The process of bacterial adaptation to unfavorable conditions is known to be coupled with optimization of population density, acquiring the specific physiological features of stressed cells, modification of cell morphology and alterations in gene expression. These parameters were monitored in the present study for *P. atrosepticum* cells that were subjected to starvation in different physiological states. The dynamics of cell number assessed by CFU and genome copy counting, cell ultrastructure and gene expression showed that exponential and stationary phase cells survive under starvation conditions, employing distinct adaptive strategies.

2. Materials and methods

2.1. Bacterial strains, media and culture conditions

P. atrosepticum SCRI1043 (formerly *Erwinia carotovora* ssp. *atroseptica* SCRI1043) [4] was grown in Luria–Bertani (LB) medium [32] with aeration (200 r.p.m.) at 28 °C. Cells were harvested (14,000 g, 10 °C, 10 min) at the middle logarithmic (0.8 OD, 5×10^7 CFU ml^{-1}) or stationary (1.9 OD, 4×10^9 CFU ml^{-1}) growth phase, then washed twice in a carbon-free AB medium (1 g $L^{-1}NH_4Cl$; 0.62 g $L^{-1}MgSO_4 \times 7H_2O$; 0.15 g $L^{-1}KCl$; 0.013 g $L^{-1}CaCl_2 \times 2H_2O$ and 0.005 g $L^{-1}FeSO_4 \times 7H_2O$, pH 7.5). After that, cells were resuspended in AB medium with the initial population density of $1.0 \times 10^9 \pm 4.4 \times 10^8$ CFU ml^{-1}. The resulting starving cultures were incubated in glass vials without aeration at 28 °C. The number of CFUs was determined by plating serial 10-fold dilutions of the cell suspensions in 0.5% NaCl onto 1.5% LB agar after 0, 1, 4, 15, 20, 30, 45 and 60 days of incubation. Media used in this study were autoclaved at 121 °C for 40 min.

2.2. Determination of *P. atrosepticum* genome copy numbers

Genome copy (GC) numbers were determined by the quantitative real-time polymerase chain reaction (qPCR) with respect to calibration curves, as described previously [10,11]. qPCR was performed using the TaqMan detection method. The primers and probes used (Supplementary Table) were designed using Vector-NTI version 9 software (Invitrogen) and synthesized by Evrogen (Moscow, Russia). Primers for genes *rpoD*, ECA4070 and *expR* were used for genome copy counting. The reaction mixture contained 1 × PCR buffer (67 mM Tris–HCl, pH 8.8, 17 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, and 0.1% Tween 20), 200 μM of each dNTP, 0.04 U of Hot-start DNA polymerase (Evrogen), 0.1 μM of each primer and TaqMan probe. The thermal cycling conditions used for PCR involved heating to 94 °C for 2 min, followed by 45 cycles at 94 °C for 10 s and 60 °C for 1 min. Changes in fluorescence emission were detected using an CFX96 quantitative PCR system (Bio-Rad). The amount of fluorescence was plotted as a function of the PCR cycle using CFX Manager Software Version 2.1 (Bio-Rad). The amplification efficiency (E) for all primers was determined using a dilution series of a DNA pool. A value of 5.5 fg of *P. atrosepticum* DNA (5064 kb) was accepted as one GC. The DNA concentration was determined using a Qubit fluorometer (Invitrogen, USA).

Genome copy numbers were analyzed in raw or purified cell lysates that had been diluted 10-fold in deionized water. To obtain lysates, *P. atrosepticum* cells were harvested by centrifugation (14,000 g, 10 °C, 10 min), resuspended in 50 μL TE buffer (Tris-HCl 10 mM, pH 8.0; EDTA 1 mM, pH 8.0) and treated with 2U of Turbo-DNAse (Ambion, USA) for 30 min at 37 °C. Then 1% Triton X-100 (w/v) was added and suspensions were incubated at 100 °C for 10 min. Four volumes of deionized water were then added to the samples and the cell debris was harvested by centrifugation (14,000 g, 10 °C, 10 min) and discarded. Five μl of the obtained raw lysates were used to perform qPCR.

The raw lysates were purified by phenol-chloroform and subsequent chloroform extraction [8]. Five μl of the aqueous phase obtained (purified cell lysates) were used for qPCR. The GC numbers were determined after 0, 1, 4, 15, 20, 30, 45 and 60 days of cell incubation under starvation. Cell lysates obtained from exponential and stationary phase cultures were used as positive controls. Genome copy numbers in the PCR reaction mixture ranged from 10^2 to 10^7 GC, and the calibration curve was linear in this range.

The amplification inhibition values were measured as ratios of GC titers determined in purified and raw cell lysates. The non-culturability values were calculated as ratios of CFU titers and GC titers determined in purified cell lysates.

2.3. Transmission electron microscopy

P. atrosepticum cells were harvested by centrifugation at 10,000 g for 10 min at 5 °C during the exponential or stationary growth phase in full LB medium and after 30 days of incubation in carbon-deficient AB medium. The samples were fixed in 2.5% glutaraldehyde prepared in 0.1 M phosphate buffer (pH 7.2) at 4 °C overnight, washed three times (15 min each) with 0.1 M phosphate buffer containing sucrose (6.8%) and post-fixed by incubation in 1% (w/v) osmium tetroxide in the same buffer with sucrose (25 mg/ml) at 4 °C for 4 h. The samples were dehydrated by passage through a graded ethanol series (30%, 40%, 50%, 60%, 70%, 80%, 90% and then 96% ethanol), before being transferred

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to 100% acetone and propylene oxide. Thereafter, the samples were immersed in Epon resin (Fluka) containing propylene oxide added at the proportions (v/v) 1:2, 1:1, 2:1, with each step involving a 12-h incubation. The samples were then embedded in pure Epon resin. Ultrathin sections (ca. 300 nm) were prepared using a glass knife with an ultramicrotome (LKB-8800, Sweden), mounted on copper grids and stained with 2% aqueous uranyl acetate (w/v) for 20 min and Reynolds' lead citrate for 7 min. The sections were examined using a transmission electron microscope (JEM-1200 EX; Jeol; Japan) operated at an accelerating voltage of 80 kV.

For determination of the cell sizes, 80–100 cells of each culture were measured. When longitudinal dimensions of cells were analyzed, only those cells that were in longitudinal orientation were measured.

2.4. Gene expression analysis

Samples for bacterial RNA extraction were collected after four days of incubation under starvation conditions. Total RNA was isolated from bacterial cells using the RNeasy Protect Bacteria Mini Kit (Qiagen, USA) according to the manufacturer's instructions. Total RNA was treated with 2 U of Turbo-DNAse, quantified using a Qubit fluorometer (Invitrogen, USA) and used for cDNA synthesis. The reaction mixture for reverse transcription contained 100 pmol random hexamers, 1 mM dNTPs, 200 U RevertAid reverse transcriptase (Thermo Scientific, USA) with the corresponding 1 × reaction buffer. First, water and random hexamers were mixed with RNA and incubated for 5 min at 70 °C and cooled immediately on ice. The other components were then added. Incubation was performed using a DNA Engine thermocycler (Bio-Rad, Hercules, CA, USA). Reverse transcription was performed as follows: 10 min at 25 °C, followed by 1 h at 42 °C and 10 min at 70 °C. Two μl of cDNA were used as the template for qPCR. qPCR was performed as described above. The amplification efficiency (E) for all primers was determined using a dilution series of a pool of cDNAs. Additional controls included the omission of reverse transcriptase to measure the extent of residual genomic DNA contamination and omission of template. The expression level of target genes was calculated relative to reference genes. The geNorm software was used to choose genes that displayed stability of expression under the experimental conditions. Among the candidate reference genes (*gyrB*, *tufAB*, *rpoD*, *groES, recA* and *ffh*), the latter two had the most stable expression levels; therefore these genes were used as reference ones. The genes that were analyzed in this study and primer sequences are listed in the Supplementary Table.

2.5. Statistical analysis

The experiments were performed in three biological replicates, each one assayed in technical triplicate. The significance of differences in results of each test and relative control values was determined using Student's t-test.

3. Results

3.1. The dynamics of colony forming unit (CFU) and genome copy (GC) titers in starving cultures of *P. atrosepticum* inoculated by exponentially growing or stationary phase cells

To characterize the adaptive reactions of *P. atrosepticum* cells having different initial physiological status, bacteria grown to logarithmic or stationary phase were transferred to carbon-deficient medium with the initial population density of $1.0 \times 10^9 \pm 4.4 \times 10^8$ CFU mL^{-1}. The alterations in cell titers of the starving cultures were assessed by CFU values and genome copy (GC) counting using qPCR. DNA quantification was carried out in raw cell lysates and lysates purified by phenol-chloroform extraction (see Materials and methods). The lysates were purified in order to prevent inaccessibility of target DNA for in vitro amplification due to compaction by DNA binding proteins or other metabolites that may influence nucleoid architecture [8,35,45] and may lead to a false reduction in the GC titer. Additionally, comparison of the quantities of target DNA revealed in raw and purified cell lysates may enable assessment of modifications in nucleoid organization during the stress response.

CFU and GC titers were sharply reduced in both types of cultures during the first 15 days of starvation (Fig. 1A,B); this reduction was more pronounced in the starving cultures inoculated by exponential phase cells. Around 1.5 and 2.5% of cells of their initial number remained alive in the starving cultures inoculated by exponential or stationary phase cells, respectively. Thereafter, the CFU titer decreased gradually to different degrees in both types of cultures. In cultures inoculated by exponential phase cells, almost 15% of bacteria retained culturability after 60 days of starvation relative to the 15-day time point; for cultures inoculated by stationary phase cells, the same parameter had a value of 3%. In turn, genome copy titers determined in purified cell lysates decreased slightly (relative to the 15 day time point) in cultures inoculated by exponential phase cells and did not change in the stationary cell-inoculated cultures (Fig. 1A,B). In the latter, purification of cell lysates did not influence the quantity of detected genome copies (Fig. 1B). However, when lysates obtained from the cultures inoculated by exponentially growing cells were purified, the number of genome copies increased compared to the raw lysates (Fig. 1A).

The obtained results indicated that dynamics of the ratios of CFU vs. GC values and of GC values determined in purified vs. raw lysates changed asynchronously in two types of starving cultures investigated. These parameters were used to determine the ratio of non-culturable and culturable cells in bacterial cultures (non-culturability value) and to assess the presence of inhibitors that may influence nucleic acid metabolism in bacterial cells (amplification inhibition value), respectively.

In our experiments, amplification inhibition was close in value to one for starving cultures inoculated by stationary phase cells (Fig. 2A), similarly to cultures grown under

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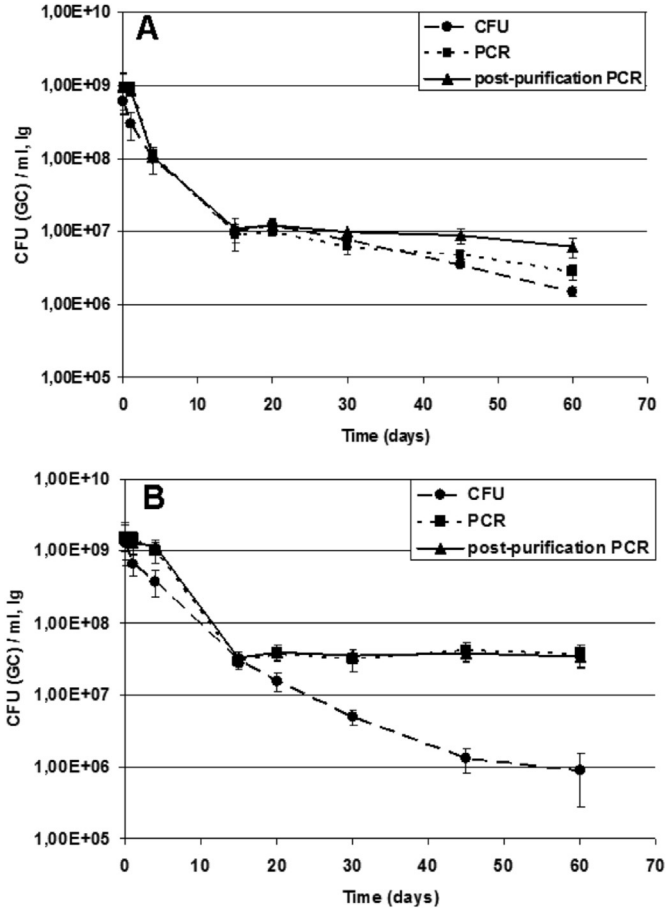

Fig. 1. The dynamics of the P. atrosepticum cell number determined by CFU counting (circles) and qPCR in raw (squares) or purified (triangles) cell lysates in the starving cultures inoculated by exponentially growing (A) or stationary phase (B) cells. The values represent means ± SD for three biological replicates, each analyzed in technical triplicates.

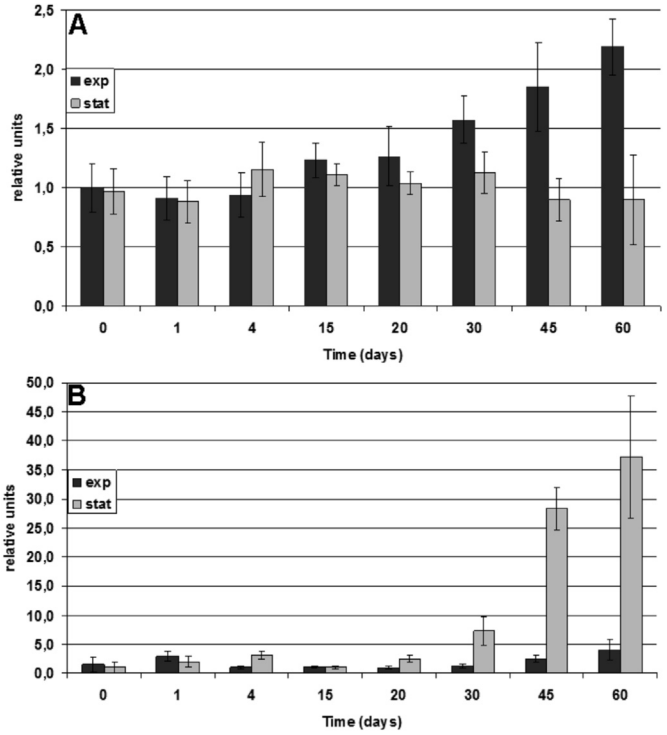

Fig. 2. The dynamics of amplification inhibition values (A) and of non-culturability values (B) in starving P. atrosepticum cultures inoculated by exponentially growing (dark gray) or stationary phase (light gray) cells. The values represent means ± SD for three biological replicates, each analyzed in technical triplicates. The amplification inhibition values were calculated as ratios of genome copy values determined in raw and purified cell lysates. The non-culturability values were calculated as ratios of genome copy values determined in purified cell lysates and CFU values.

nutrient-rich conditions (data not shown). In contrast, this value increased during incubation for cultures inoculated by exponential phase cells and reached 2.2 ± 0.2 at 60 days of the experiment (Fig. 2A).

The non-culturability values increased in both types of starving cultures. However, this increase was much more pronounced in cultures inoculated by stationary phase cells (Fig. 2B). After 60 days of incubation, non-culturability values reached 4.1 ± 1.8 and 37.2 ± 10.4 for cultures inoculated by exponential and stationary phase cells, respectively. Non-culturability values for cultures growing under nutrient-rich conditions were close to one (data not shown).

qPCR-detected DNA of *P. atrosepticum* in starving cultures was unlikely to be that released from cells damaged during starvation, since the harvested cells were treated with DNAse prior to the lysate preparation (see Materials and methods). Additionally, no significant amounts of GC were detected in cell-free supernatants of the starving cultures (data not shown).

The obtained data indicate that there was intensive formation of non-culturable forms in starving cultures inoculated by stationary phase cells. In the starving cultures inoculated by exponential phase cells, DNA became less easily available for in vitro amplification, pointing to changes in nucleoid organization.

3.2. Ultrastructure of exponential and stationary phase cells of *P. atrosepticum* after their exposure to starvation

Exponential and stationary phase *P. atrosepticum* cells were used in our experiments as inocula. These cells were rods (exponential phase cells 1.52 ± 0.15 × 0.54 ± 0.06 μm and stationary phase cells 1.91 ± 0.17 × 0.56 ± 0.05 μm) with easily distinguishable outer and cytoplasmic membranes and an electron-dense fine granular cytoplasm with evenly distributed nucleoid (Fig. 3A,B). The dividing cells represented a considerable fraction during the period of active growth (exponential phase) (Fig. 3A); in the stationary phase, only few dividing cells were observed. The cell cytoplasm became more dense and cloggy upon entry into stationary growth phase compared to those of exponentially growing cells. Electron-transparent inclusions that resembled polyalkanoates – the described storage compounds [40] – were observed in stationary phase cells (Fig. 3B, hatched arrow).

The ultrastructure of both exponential and stationary phase *P. atrosepticum* cells altered significantly after their exposure to starvation. In the starving cultures inoculated by exponential phase cells, bacteria became smaller (1.13 ± 0.2 × 0.52 ± 0.17 μm) and

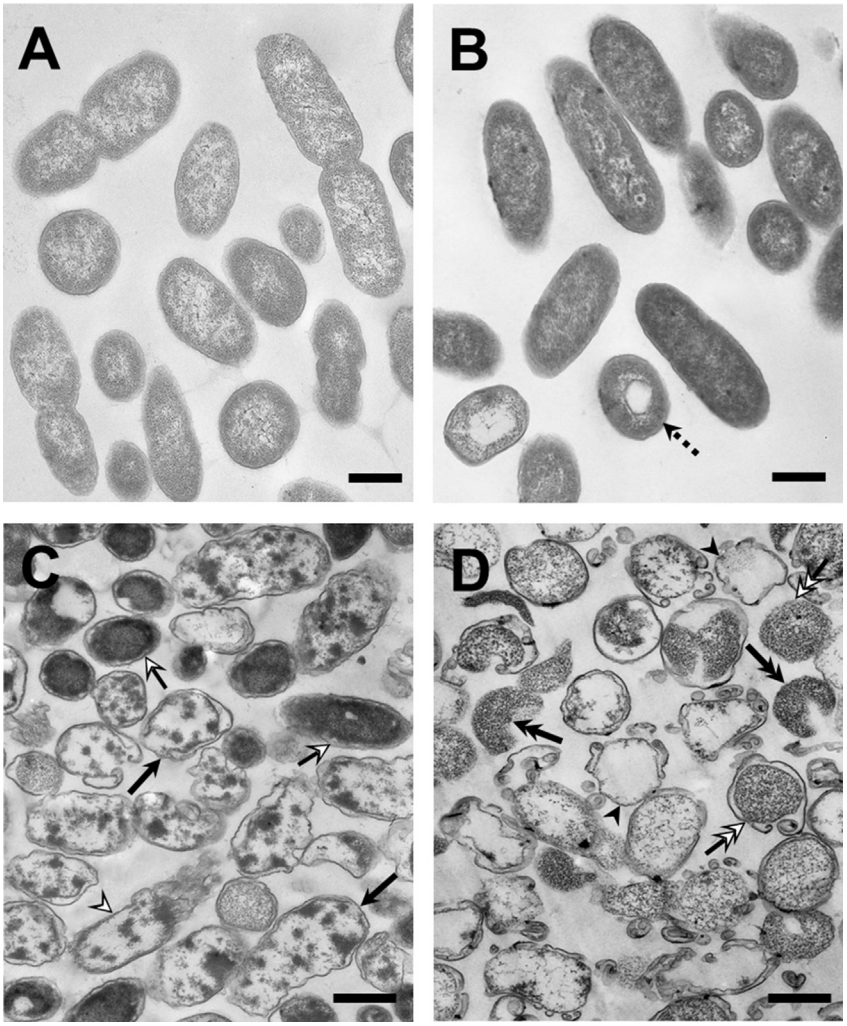

Fig. 3. TEM of P. atrosepticum SCRI1043 cells. A, B: Exponential and stationary phase cells, respectively. C, D: Cells of starving cultures (30 days of incubation in carbon-deficient medium) inoculated by exponential or stationary phase cells, respectively. Marks on the photos: B, polyalkanoate-like granules (hatched arrow); C, cells with cytoplasm of high electron density and enlarged periplasmic space (white arrows); plasmolyzed cells with electron-transparent cytoplasm with highly condensed zones (black arrows); lysed cells (white arrowhead); D, cell-wall-deficient forms (white double arrows); crescent-shaped cells without cell wall (black double arrows); ghost-cells (black arrowhead); note the desquamated remnants of cell wall. Scale bars = 0.5 μm.

more rounded than the unstressed cells, and were represented by two morphotypes (Fig. 3C). The first was characterized by a cytoplasm of high electron-dense enlarged periplasmic space (Fig. 3C, white arrows). The cells of the second morphotype were plasmolyzed and had an electron-transparent cytoplasm with highly condensed zones (Fig. 3C, black arrows). Analogous electron-dense zones were previously described in bacteria and were attributed to DNA fibrils that were formed as a result of nucleoid condensation [5]. The cell walls of both morphotypes displayed integrity. Lysed cells were also found in starving cultures inoculated by exponential phase *P. atrosepticum* cells (Fig. 3C, white arrowhead).

In the starving cultures inoculated by stationary phase cells, bacteria had an ovoid form and reduced size ($0.84 \pm 0.21 \times 0.49 \pm 0.22$ μm) compared to unstressed cells (Fig. 3D). The outer membranes and cell walls of those cells broke down and cells displayed cell wall-deficiency (Fig. 3D, white double arrows). The inner membranes remained intact. Some cells that fully freed themselves from the cell wall became crescent-shaped (Fig. 3D, black double arrows). The cell cytoplasm had evenly granular structure. Ghost-cells (Fig. 3D, black arrowhead) that lacked the cytoplasm, and desquamated remnants of cell walls were observed on photos of starving cultures inoculated by stationary phase cells.

Thus, cells of different physiological status (exponential and stationary phase cells) gave rise to distinct cell morphotypes after the stress effect. Nucleoid preservation was characteristic for the cells present in the starving cultures inoculated by exponential phase cells; in turn, cell wall deficiency was a typical ultrastructural modification of stationary phase cells under starvation conditions.

3.3. Differential gene expression in exponential and stationary phase cells of *P. atrosepticum* after their exposure to starvation

The above mentioned data indicate that the strategies of adaptation of exponential and stationary phase cells of *P.*

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atrosepticum include preservation of the nucleoid or deliverance from the cell wall, respectively. Therefore we compared expression levels of genes encoding histone-like DNA binding proteins and enzymes necessary for synthesis or degradation of cell wall polymers in the cells of tested cultures (Supplementary Table). Gene expression analysis was carried out after four days of incubation; the later time points were characterized by severe RNA degradation.

In order to correctly analyze the relative expression levels of the target genes, reference genes that had high stability of expression under experimental conditions used in our study were experimentally selected using geNorm software (https://genorm.cmgg.be/). *recA*, *ffh*, *tufAB*, *rpoD*, *groES* and *gyrB* were analyzed as candidate reference genes; the stability of their expression had been previously shown on *Pectobacterium* and other bacteria [7,26,38]. The expression of *recA* and *ffh* was the most stable among six candidates in the cells of both types of starving cultures and unstressed exponentially and stationary growing cells. Therefore, these two genes were used for normalization of the expression level of target genes in our experiments.

Expression of several genes encoding DNA binding proteins (*hns1*, *hns2*, *hns3*, *hupA*, *hupB*) was upregulated in bacteria of starving cultures inoculated by exponential phase cells compared to unstressed cells of the exponential phase (Fig. 4). In contrast, transcript abundance of these genes was reduced or did not differ when stationary phase cells were exposed to starvation. As a result, expression levels of *hns1*, *hns2*, *hns3*, *hupA* and *hupB* genes were higher in starving cultures inoculated by exponential phase cells rather than by stationary phase cells (Fig. 4). Expression levels of other analyzed genes encoding DNA binding proteins (*ihfA*, *fis*, *dps* and *crp*) did not differ in two types of starving cultures (data not shown). The increased relative expression level of a range of genes of DNA binding proteins in bacteria of starving cultures inoculated by exponential phase cells is consistent with condensation of their nucleoids (Fig. 3 C) and reduced detectability of DNA in these cells by qPCR (Figs. 1A, 2A).

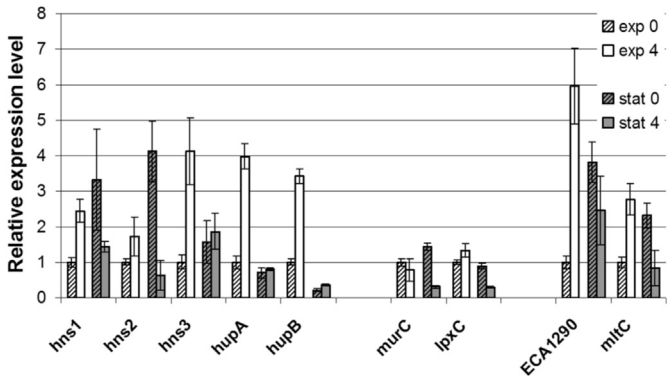

Fig. 4. Expression levels of genes encoding DNA binding proteins (hns1, hns2, hns3, hupA, hupB), synthases of cell wall components (murC, lpxC) and hydrolases of cell wall polymers (ECA1290, mltC) in exponentially growing (white columns) or stationary phase (gray columns) P. atrosepticum cells prior to starvation (hatched columns) and after four days of incubation under starvation conditions (non-hatched columns). The values represent means ± SD for three biological replicates each analyzed in technical triplicates. The expression levels of all genes in unstressed exponentially growing cells were equal to one.

Two of six genes encoding hydrolases of cell wall polymers (*eca1290*, *mltC*) were upregulated in bacteria of four-day starving cultures inoculated by exponential phase cells (Fig. 4), as compared to those of stationary phase cells; the expression level of four other genes (*eca4161*, *amiC*, *ampD*, *slt*) did not differ in two types of starving cultures (data not shown). These data are inconsistent with formation of cell-wall-deficient forms in starving cultures inoculated by stationary phase cells (Fig. 3D).

This discrepancy could be explained as follow. First, upregulated hydrolases (*eca1290*, *mltC*) may provide cell lysis, that occurs in cultures inoculated by exponential phase cells more intensively than in those of stationary cells, but not the cell wall deficiency of stationary phase cells exposed to starvation; and other hydrolases that were not analyzed in our study may be necessary for delivery from the envelope in the case of cell-wall-deficient forms. Second, the expression of genes encoding hydrolases of cell wall polymers might have been induced later than four days after stress exposure in order to cause cell wall deficiency; however, we were unable to check this possibility due to high-level RNA degradation after four days of cell incubation. And third, the formation of cell-wall-deficient forms may be a result of the reduced synthesis of cell wall components, rather than their increased degradation alone. We assessed the expression levels of genes encoding enzymes necessary for synthesis of cell wall components: UDP-N-acetylmuramate-L-alanine ligase (*murC*) – a peptidoglycan biosynthetic enzyme and UDP-3-O-[3-hydroxymyristoyl] N-acetylglucosamine deacetylase (*lpxC*) – an outer membrane lipid A biosynthetic enzyme in the cells of investigated cultures (Fig. 4). Both of these genes were downregulated in stationary phase cells upon starvation. The transcript levels of these genes remained constant during the stress response of exponentially growing cells, resulting in different expression levels of these genes in two types of starving cultures. This suggests that de novo synthesis of cell wall components occurs less intensively during the stress response of stationary phase cells than during that of exponential phase cells. This is consistent with the cell wall deficiency of stationary phase cells resulting from their adaptation to starvation conditions.

4. Discussion

This investigation shows that *P. atrosepticum* may use alternative scenarios to adapt to starvation conditions, depending on the physiological status of the cells at the onset of the stress effect. When the actively growing cells encountered the stress effect, adaptation was related to the preservation of genetic material. Bacterial genomic DNA became less easily available for in vitro amplification (Figs. 1A, 2A), and nucleoids displayed condensation (Fig. 3C). Genes encoding several DNA binding proteins (*hns1*, *hns2*, *hns3*,

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hupA, *hupB*) in *P. atrosepticum* SCRI1043 were upregulated in cells of starving cultures inoculated by exponentially growing cells compared to those of stationary growing cells (Fig. 4). Overproduction of these proteins was previously shown to result in nucleoid compaction [12,15,17]. These facts may speak in favor of alterations in nucleic acid metabolism in the cells. Our results are in accordance with those that demonstrated changes in DNA architecture as a result of stress response in other bacterial species [19,28,35,45].

In turn, adaptation to starvation of cells that had already stopped their active reproduction and turned to the stationary growth phase, thus being pre-prepared for the stress response, consisted of delivery from the cell walls (Fig. 3D). These cell-wall-deficient forms had reduced colony-forming capacity and could not be detected by CFU counting (Figs. 1B, 2B). The rescue from the cell wall is known to be the mechanism that enables bacteria to become "invisible" to host defense systems due to loss of a range of surface antigens [1,33,42]. However, cell-wall-deficient bacteria might also be adaptive forms arising in response to a variety of stress factors [20,21,34]. These forms often lose colony-forming capacity, but may revert to proliferating forms [22,37]. In our study, we were unable to find hydrolases that may be in charge of polymer destruction during formation of cell-wall-deficient forms in *P. atrosepticum*. However, we found that cell wall deficiency was coupled with downregulation of genes encoding enzymes necessary for biosynthesis of cell wall components (Fig. 4). This suggests that loss of the cell wall may be a result of reduced cell wall renovation, rather than simply increased cell wall degradation.

The peculiarities of the nucleoid architecture and structure of the cell wall is often assessed by staining combined with analysis by epifluorescent microscopy [6,13]. However, sizes of starving *P. atrosepticum* cells described in our study lay beyond the resolution limits of these approaches [36]. In our experiments, use of DNA staining dyes enabled only detection of DNA within the cells, but did not enable distinguishing peculiarities of nucleoid architecture; cell wall staining did not permit differentiation of intact and damaged cells (data not shown). As an alternative, we used a combination of complementary approaches, all of which indicated nucleoid condensation in one of the tested cultures and reduced colony-forming ability coupled with rescue from the cell wall in another.

Adaptation to stress conditions is known to be related to formation of different types of "peculiar cells": viable but non-culturable cells [29,30,41], cell-wall-deficient forms [20,21], cells with condensed nucleoids [2,27], cyst-like resting cells [24] and persisters [16,18]. The former three were detected in this study and the first two of them were also previously observed in the genus *Pectobacterium* (formerly *Erwinia*) [9,11,46]. Cyst-like resting cells have very specific ultrastructural characteristics [25] that were not identified in our study. Persister cells are often considered to be the main reserve of microbial populations that survive during unfavorable conditions. These cells, that are present in populations throughout the entire life cycle, have very weak proliferative activity that confers a high level of resistance [16,18]. The marker for persisters is their ability to survive after antibiotic treatment.

The characteristic features of different adaptive forms of microorganisms may overlap. For example, cell-wall-deficient forms often reversibly lose their colony-forming capacity and thus may be classified as both cell-wall-deficient forms and viable-but-non-culturable cells [14,23]. Cyst-like resting cells may also be non-culturable [25]. Therefore, since no persister ultrastructural features, like "persister-specific" genes, have been identified, we cannot exclude that the adaptive forms of *P. atrosepticum* observed in our study were persisters, in addition to being cell-wall-deficient forms or cells with condensed nucleoids. However, regardless of whether the observed cells were true persisters or not, they share different physiological and morphological characteristics in two types of starving cultures investigated.

Taken together, our results show that *P. atrosepticum* is capable of different types of starvation-induced stress responses. The scenario used for adaptation depends on the physiological status of the cells at the time of exposure to the stress factor.

Conflict of interest

There is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.resmic.2016.01.009.

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