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ISOLATION AND CHARACTERIZATION OF *CAULOBACTER* MUTANTS IMPAIRED IN ADAPTATION TO STATIONARY PHASE

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ABSTRACT

The entry into stationary phase causes a change in the pattern of gene expression of bacteria, when the cells must express a whole set of genes involved mainly with resistance to starvation and to environmental stresses. As an attempt to identify genes important for the survival of *Caulobacter crescentus* in stationary phase, we have screened a library of 5,000 clones generated by random transposon mutagenesis for mutants that showed reduced viability after prolonged growth. Four clones were selected, which displayed either lower viability or a longer time of recovery from stationary phase. The genes disrupted were identified, and the gene products were found to be mainly involved with amino acid metabolism (glutamate N-acetyltransferase, 4-hydroxyphenylpyruvate dioxygenase and L-aspartate oxidase) or with recombination (exonuclease RecJ). Each mutant was tested for resistance to stresses, such as oxidative, saline, acidic, heat and UV exposure, showing different responses. Although the mutations obtained were not in genes involved specifically in stationary phase, our results suggest that amino acids metabolism may play an important role in keeping viability during this growth phase.

Key words: Stationary phase, stress, transposon, bacteria

INTRODUCTION

The onset of stationary phase in bacteria is one of the best examples of global gene regulation, when several key regulatory factors act coordinately to stablish a new pattern of gene expression. The proteins synthesized by the cell during the entry into stationary phase are involved in keeping viability throughout long periods of starvation. The total protein synthesis is reduced to about 20% of that found in exponentially growing cells in minimal medium (1), and this drops to 0.5% after 11 days in stationary phase (reviewed in 13).

In non-sporulating Gram negative bacteria, nutritional deprivation leads the cells to be more resistant to cold shock, oxidative stress and osmotic variations (13). Several proteins induced upon stationary phase are also induced by specific stresses, like high osmolarity, high temperature or acidic pH (9,10,15,18). *E. coli* cells submitted to these stresses show phenotypes typical of stationary phase cells, like smaller cells and multiple stress resistance (10,22).

In *E. coli* and other bacteria the main regulator of this phase is a sigma factor (σ^S in *E. coli*, σ^H in *Bacillus*), which is responsible for the induction of several genes required at this stage (2,10). The σ^S factor in *Pseudomonas*, σ^B in *Bacillus* and other transcription factors of stationary phase from *Rhizobium* and *Vibrio* are also regulated by quorum sensing, indicating an interconnection between quorum sensing and starvation-sensing pathways (14).

C. crescentus is an aquatic bacterium belonging to the alpha subfamily of the proteobacteria, and no homolog of σ^S was identified in the genome sequence (19), suggesting that the regulation of stationary phase genes could be carried out by a different system. A previous work showed that when in late stationary phase *Caulobacter* undergoes a morphological change and display increased resistance to stress (29). Very few

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proteins induced in this phase have been identified in *Caulobacter*, such as the FstH (7), and the ClpXP proteases (20), and some that are involved in stress response, as a catalase/peroxidase and a superoxide dismutase (23).

This work aims to identify genes from *Caulobacter* that are important for survival to stationary phase through transposon mutagenesis and isolation of mutants, and to characterize the stress resistance phenotype of the mutants isolated.

MATERIALS AND METHODS

Bacterial strains and media

C. crescentus NA1000 strain (6) was used as wild type for all experiments. Cells were grown at 30ºC in peptone yeast extract (PYE) or M2 minimal medium (5), supplemented with kanamycin (5 µg/ml) as necessary.

Isolation of stationary phase deficient mutants from a *C. crescentus* transposition library

Tn5 (Kan) transposon was inserted into *C. crescentus* strain NA1000 by conjugation with an *E. coli* S17-1 carrying plasmid pSUP2021 (25) as described (12). Screeening was performed as described previously (28), with the following modifications: the cells were grown in 96-well plates containing 0.3 ml of PYE/Kan medium at 30ºC for 14 days with agitation, followed by plating in PYE/Kan. Strains that showed reduced or no colonies in the plates were selected for further confirmation, now repeating the long term growth in larger culture volumes.

Identification of the Tn5 insertion sites

The determination of the Tn5 insertion sites was performed by reverse PCR as described (12). The PCR conditions were: 5 minutes 95ºC, 30 cycles of 1 minute 95ºC, 1 minute 42ºC or 52ºC and 1 minute 72ºC, and a final cycle of 7 minutes at 72ºC. The bands obtained were gel purified and cloned into the TOPO vector (Invitrogen). The recombinant plasmids were used for automatic DNA sequencing with BigDye terminators on a ABI Prism 377 sequencer (AP Biosystems). The insertion site was determined by comparison to the complete *C. crescentus* genome sequence (19).

Stress viability tests

Cell survival was measured by counting the C.F.U./ml after colonies were grown, using the *C. crescentus* NA1000 strain as a control to all experiments. Midlog phase cultures of each mutant were incubated under several stress conditions, aliquots were taken during several times after addition of the stress agent and then serial dilutions of the culture were plated in PYE/Kan. All the experiments were repeated at least three times.

To determine viability under oxidative stress, H_2O_2 was added to the culture at a final concentration of 10 mM. The tests for survival under acid (pH 4.0) conditions were as follows: 2 ml of each culture was centrifuged and the cells were ressuspended in the same volume of PYE medium adjusted to pH 4.0 with HCl, as described by Wortinger *et al.* (29). In the heat stress survival test, a midlog culture was transferred to 45ºC and samples were taken at several time points for plating. UV survival was determined by plating the same number of exponential growing cells of each strain in PYE medium, and exposing the plates in the dark to a UV lamp (220-300 nm, 2 J/m^2.s) for different times. Plates were incubated at 30ºC protected from light and the number of colonies formed after 5 days was counted. To test the response to high salt concentration, NaCl was added to the exponential phase cultures to a final concentration of 85 mM.

RESULTS AND DISCUSSION

Caulobacter undergoes a unique developmental program where two dissimilar daughter cells are generated at each cell division, which indicates that its adaptation to stationary phase must probably be a complex event. As an attempt to identify genes that are important to the survival of *C. crescentus* at stationary phase, we have screened a transposon library of 5,000 clones in search for mutants that showed a defective growth phenotype after prolonged time of incubation. The cultures were incubated for 14 days, when cells show the elongated morphology characteristic of late stationary phase (29), and then plated in rich medium. Four mutant strains were selected which showed either lower cell viability or a delay in the appearing of colonies, what could suggest a defect in recovery from stationary phase (Table 1).

In order to identify the genes that were disrupted by the transposon in each strain, the Tn5 insertion site was determined by DNA sequencing. The disrupted genes encode enzymes that are related mostly to metabolic functions (Table 1). In mutant 16-3A, the disrupted gene (*argJ*) encodes the glutamate N-acetyltransferase, which is required for the biosynthesis of arginine. This mutant is unable to grow in minimal medium, confirming that it is deficient in a biosynthetic pathway (not shown). In mutant 25-3C, the Tn5 integrated into the gene encoding the 4-hydroxyphenylpyruvate dioxygenase (HPPD), which is involved in the degradation of phenylalanine and tyrosine (17). In both cases, the integration of the Tn5 most likely did not afect the expression of other genes downstream, since one is in the opposite direction (16-3A) and the other is separated by 180 bp (25-3C) (Fig. 1).

In mutant 9-12D, the transposon inserted into the *nadB* gene, which encodes the enzyme L-aspartate oxidase. This gene is the first one in a probable operon (Fig. 1) in a gene locus for NAD biosynthesis, consisting of genes *nadA*, *nadB* and *nadC*, and the integration of the Tn5 likely has also an effect in the expression of the genes downstream of *nadB*.

Table 1. Identification and phenotype of the stationary phase mutants isolated.

Mutant	Disrupted ORF ID [a]	Putative product function	Stationary phase phenotype [b]
9-12D	AAK24875 (CC2913)	L-aspartate oxidase	longer recovery
16-3A	AAK25028 (CC3066)	glutamate N-acetyltransferase	longer recovery
25-3C	AAK24504 (CC2533)	4-hydroxyphenylpyruvate dioxygenase	lower viability
49-1C	AAK23367 (CC1386)	single-stranded-DNA-specific exonuclease RecJ	lower viability

[a] Numbers in parenthesis indicate the gene ID; [b] Viability was determined by counting the C.F.U. of each strain after incubation for 14 days in PYE medium. Time of recovery was determined as the amount of time required for colonies to reach a visible size, compared to the average time of the NA1000 strain, of serial dilutions plated after a 14 day incubation in PYE medium.

Mutations in the *nadB* gene from *E. coli, S. typhimurium* and *B. subtilis* turn them auxotrophic for nicotinic acid, therefore the *nadB* gene is essential for NAD biosynthesis in these bacteria (3,8,27). However, the *nadB* gene in *Caulobacter* is not essential, since mutant 9-12D does not need nicotinic acid for growth (not shown), indicating there must be an alternative biosynthetic route, as suggested for *Pseudomonas* (4).

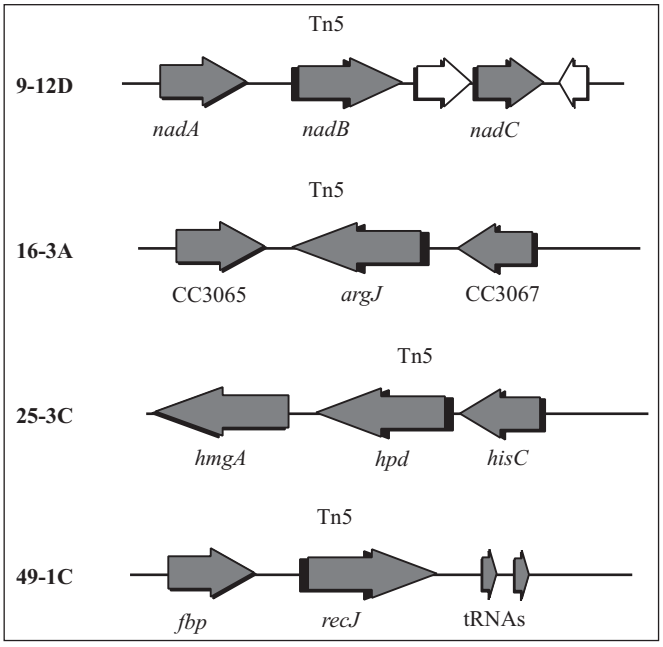

Figure 1. Schematic representation of the genomic regions containing the disrupted genes in each mutant strain. The gray arrows indicate open reading frames of proteins with known functions, and white arrows indicate open reading frames of hypothetical proteins. The directions of the arrows represent the direction of transcription. The Tn5 insertion site is indicated by the triangle. Gene nomenclature follows the annotated *C. crescentus* genome at the TIGR site (http://www.tigr.org).

In mutant 49-1C, the disrupted gene encodes the single-stranded-DNA-specific exonuclease RecJ, which is involved both in homologous and illegitimate recombination (24). *recJ* mutants show decreased DNA repair ability, and are therefore highly sensitive to low levels of UV irradiation and to exposure to DNA-alkylating agents (11,26). This gene is not transcribed with any other gene downstream (Fig. 1).

The mutant strains were then tested for their ability to withstand several environmental stresses, to determine whether their poor performance in stationary phase could be an effect of a more general stress-sensitive phenotype. The cells were tested for oxidative, saline, acidic, heat and UV exposure (Fig. 2), at conditions in which the wild type cells were not affected (12). As a second control, a mutant strain with the Tn5 inserted into the *rsaF* gene that encodes an outer membrane protein (OMP58) was tested for all stresses and responded like the wild type, indicating that the sensitivity to stress is not due to the presence of the Tn5 (not shown).

As it can be observed in Fig. 2, each strain showed a different pattern of response to individual stresses, but some general patterns can be pointed out. Mutant 16-3A did not show a particularly sensitive phenotype in any of the conditions. The deficiency in amino acid biosynthesis in 16-3A probably is responsible for a slow recovery from the nutrient starvation, causing the delay in colony formation observed in the screening proccess. Mutant 9-12D displayed decreased viability in acidic pH and UV light at longer time points, suggesting that despite the fact that the *nadB* gene is not essential for *Caulobacter*, its loss causes it to be more sensitive to some stresses.

Mutant 25-3C showed lower viability under oxidative stress, heat shock and UV at the 30 min time point, indicating that HPPD is important for the cell under stress conditions. In *Sinorhizobium meliloti*, a mutant disrupted in the *hmgA* gene, which encodes for another enzyme of the degradative pathway of tyrosine and phenylalanine, was described as showing deficiency in stationary phase survival (17).

Mutant 49-1C showed the most sensitive phenotype, with reduced viability under all the conditions tested. Oxidative stress generated by H_2O_2 can enhance DNA lesions, and it has been

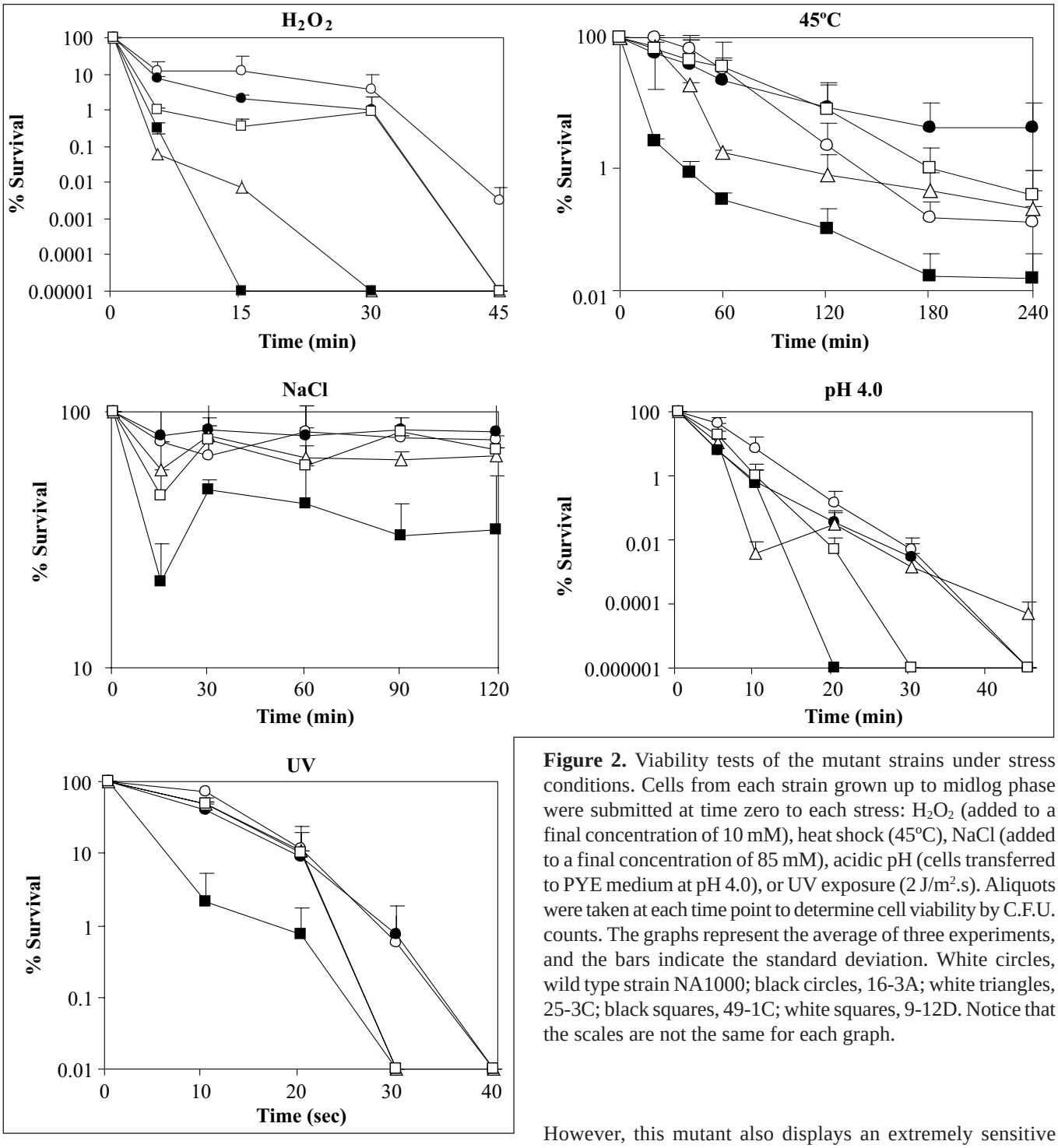

Figure 2. Viability tests of the mutant strains under stress conditions. Cells from each strain grown up to midlog phase were submitted at time zero to each stress: H_2O_2 (added to a final concentration of 10 mM), heat shock (45ºC), NaCl (added to a final concentration of 85 mM), acidic pH (cells transferred to PYE medium at pH 4.0), or UV exposure (2 J/m^2.s). Aliquots were taken at each time point to determine cell viability by C.F.U. counts. The graphs represent the average of three experiments, and the bars indicate the standard deviation. White circles, wild type strain NA1000; black circles, 16-3A; white triangles, 25-3C; black squares, 49-1C; white squares, 9-12D. Notice that the scales are not the same for each graph.

showed that a treatment with H_2O_2 induces the expression of RecA in *Xanthomonas campestris* (21). Therefore, a lower viability under UV irradiation and in the presence of H_2O_2 was expected, since the *recJ* gene is required for DNA repair. However, this mutant also displays an extremely sensitive phenotype under heat shock, and an intermediate survival rate in saline and acidic stress, indicating that a mutation in *recJ* has probably a pleiotropic effect.

In *E. coli*, it is well stablished that there is intense protein degradation to recycle amino acids during stationary phase (16). Our results suggest that amino acids metabolism may play

an important role in keeping viability during this phase. This hypothesis was also discussed in a work in *S. meliloti*, where a mutant deficient in degradation of leucine, valine and isoleucine had also a stationary phase phenotype (28). More recently, it was shown that three mutations in the *rpoS* gene from *E. coli* cause a growth advantage in stationary phase, and enhance amino acid catabolism (30). In this work the mutations selected were not in genes involved specifically in stationary phase, which indicates that the viability in this phase is dependent on many interdependent pathways, and probably no single mutation could have a phenotype. Another possibility is that disruption of such genes could be lethal for the cell, and therefore mutants could not be isolated.

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RESUMO

Isolamento e caracterização de mutantes de *Caulobacter* deficientes na adaptação à fase estacionária

A entrada em fase estacionária causa uma mudança no padrão de expressão gênica de bactérias, quando as células devem expressar um novo conjunto de genes envolvidos principalmente com resistência à carência alimentar e a estresses ambientais. Em uma tentativa de identificar genes importantes para a sobrevivência de *Caulobacter crescentus* em fase estacionária, nós varremos uma biblioteca de 5.000 clones gerados por transposição aleatória em busca de mutantes que mostrassem viabilidade reduzida após crescimento prolongado. Quatro clones foram selecionados, que mostraram menor viabilidade ou um maior tempo de recuperação da fase estacionária. Os genes interrompidos foram identificados, e os produtos gênicos mostraram-se estar envolvidos principalmente com o metabolismo de aminoácidos (glutamato N-acetiltransferase, 4-hidroxifenilpiruvato dioxigenase e L-aspartato oxidase) ou com recombinação (exonuclease RecJ). Cada mutante foi testado para resistência a estresses, como oxidativo, salino, ácido, calor e exposição à luz UV, mostrando respostas diferentes. Embora as mutações obtidas não tenham sido em genes envolvidos especificamente com fase estacionária, nossos resultados sugerem que o metabolismo de aminoácidos tem papel importante na manutenção da viabilidade durante esta fase do crescimento.

Palavras-chave: fase estacionária, estresse, transposon, bactéria.

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