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Original Research

The roles of *SPBC409.08* and *SPAC9.02c* hypothetical genes in cell cycle and stress response, in *Schizosaccharomyces pombe*

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Abstract: Polyamine molecules are known to have important roles in the cell cycle control and fighting against stress in the cell. The mechanism and modification of polyamines are regulated by the cooperation of many proteins such as polyamine transporter proteins and polyamine acetyltransferases. In this study, our aim is to characterize two hypothetical *Schizosaccharomyces pombe* genes, *SPBC409.08* and *SPAC9.02c*, which show sequence similarity to spermine family transporters and polyamine N-acetyltransferases, respectively. To this end, we generated deletion mutants of *SPBC409.08* and *SPAC9.02c* genes using Bahler method and checked the cell cycle progression and stress responses of these mutants. Our results showed that *SPBC409.08Δ* cells showed some defects in the cell size, while *SPAC9.02cΔ* cells showed some sensitivity to UV irradiation. These data support their potential roles in the cell cycle and stress response. To our knowledge our results are the first experimental characterization of these genes.

Key words: Polyamines, Spermine, *S. pombe*, Cell cycle, Stress response.

Introduction

The cell cycle is a series of events that lead to cell division. There are many checkpoints that work in the cell cycle to ensure that cell division is proceeding in a well-controlled environment, which otherwise lead to cell death or cancer (uncontrolled cell division) development. A major determinant in the cell cycle progression is the intracellular and extracellular stress conditions such as DNA damage, environmental osmotic stress and lack of nutrition. These stress conditions are recognized and responded by specific stress response pathways such as MAPK pathway. These pathways should be communicating with the cell cycle machinery to halt the cell cycle and induce repair mechanisms upon stress conditions. This concept leads to certain cancer therapies, in which cancer cells are exposed to stress inducers such as DNA damaging agents to stop division and bring cancer growth into an end. Consequently, loss of interaction between the stress response pathway and the cell cycle can render these kinds of therapies ineffective. Our ultimate aim in this project is to uncover novel stress response mechanisms that contribute to the cell cycle control.

Polyamines have multiple roles in cell survival and cell death pathways. They can act as modulators or protective agents in apoptosis (1). In rat lymphocytes, for instance, it was shown that upon apoptotic induction (by heat shock or gamma irradiation), the mRNA levels of polyamine biosynthesis enzyme ornithine decarboxylase (ODC) increased, which was followed by a drop in polyamine levels just before DNA laddering (2). Polyamine mechanism is well characterized in plants and it is known to be involved in combat to stress conditions such as drought or UV irradiation (3,4). The polyamines are also known to play important roles in the cell cycle. Polyamine biosynthesis inhibition in Chinese hamster ovary was shown to increase the length of S phase (5). Polyamine-depletion in T lymphoblastic leukemia cells was also reported to increase $p27^{Kip}$ level and the induce G1 arrest (6). In IEC-6 cells polyamine biosynthesis inhibitor inhibited growth and arrested cell cycle in G1 phase, which was accompanied by an increase in 27^{Kip1}, $p21^{Cip/WAF}$ and p53 levels (7). In mammals, in the absence of polyamines, DNA synthesis level was reported to decrease in G1/S transition (8). Moreover, another study showed that lack of spermine and spermidine leads to cell cycle defects in *Schizosaccharomyces pombe* (9). This encouraged us to identify and characterize polyamine pathway genes in *S.pombe* and try to reveal their roles in the stress response and the cell cycle control.

Although the entire genome of the *S.pombe* has been completely sequenced, there are a number of genes that wait for characterization. These uncharacterized genes are classified according to their sequence similarities in different organisms. Two of these genes are *SPBC409.08* and *SPAC9.02c* that are classified as spermine family transporter and polyamine N-acetyltransferase, respectively, based on sequence similarity. In this study, we aim to experimentally characterize these two genes using reverse genetics. In the first step, *SPBC409.08* and *SPAC9.02c* deletion mutants were prepared. Next, the phenotype of these mutants were compared with the wild-type cells in terms of stress response.

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Materials and Methods

Model organisms

Schizosaccharomyces pombe strains 975 (*h+ ade*) and 972 *(h- ade)* were used as control wild-type strains. The deletion mutants of *SPBC409.08* and *SPAC9.02c* genes were generated using the strains 975 and 972, respectively.

Cell culture

The strains were grown in YEA culture (5g/l difco yeast extract, 30g/l glucose and 75mg/l adenine, pH 5.6), which contains 2% agar (w/v) in agar cultures. For the induction of sporulation, cells were planted onto SPA agar (10g/l glucose, 1g/l KH2PO4, 1ml/l 1000x vitamin stock (1g/l panthothenate, 10g/l nicotinic acid, 10g/l inositol, 10mg/l biotin))

Generating deletion mutants

A PCR-based gene targeting method (10) was used for constructing gene deletion. Hygromycin and Kanamycin cassettes were used for the deletion of the *SPBC409.08* and *SPAC9.02c* genes, respectively. PFA6a-kanMX6 (10) and pFA6a-hphMX6 (11) plasmids carrying kanamycin and hygromycin resistance genes (respectively) were used to replace the gene that would be deleted. The cassettes including the antibiotic resistence genes were transformed into *S.pombe* cells using LiOAc Method of Transformation.The successful deletion mutants were selected by a two step procedure (1) the cells were incubated in a medium containing 200mg/l G418 to select for kanamycin resistance gene and 300µg/ml hygromycin to select for hygromycin resistance gene (2) the cells on the selective media were checked with colony PCR whether they had the antibiotic resistance cassette in the right place.

Lithium Acetate (LiOAc) method of transformation in *S.pombe*

S. pombe cells were inoculated in 100ml YEA medium and grown O/N at 30°C until they reach the density of 0.5-1x10^7 cells/ml. First, the cells were centrifuged down at 4000rpm for 2 minutes. Next, the pellet was washed with 50 ml ddH_2O and then with 1ml of 0.1M LiOAc (pH4.9). After washing, cells were incubated in 2ml of 0.1M LiAc (pH4.9) at RT for 1 hour. After the incubation, cells were centrifuged at 4000rpm for 2 minutes. The pellet was resuspended in 150µl of 0.1M LiOAc (pH4.9) and then incubated with 100ng DNA (cassettes) and 350µl of 50% PEG for 1 hour at RT. Following incubation, 58µl DMSO was added and the cells were heat shocked at 42°C for 5 minutes. Then, cells were allowed to cool to RT for 10 minutes and centrifuged at 4000rpm for 2 minutes. Pellet was washed with 1ml ddH_2O and re-centrifuged. Finally, pellet was resuspended in 100µl ddH_2O and plated on the agar medium with the correct nutritional supplement and/or antibiotic. The cells were first plated onto YEA and then replica-plated onto selective medium after an incubation time of 24 hours at 30°C.

Confirmation of the mutants by colony PCR

The deletion mutants were confirmed using colony PCR after antibiotic selection. Two sets of colony PCR were performed to make sure the deletion cassette was integrated into the correct location. In the first set, forward primer was designed for the upstream region of the gene and reverse primer was designed for the deletion cassette. In the second set, forward primer was designed to match the cassette and reverse primer to match the downstream region of the gene. The colonies were initially boiled with the dNTP mix, PCR buffer and primers, at 98°C for 10 minutes and Taq Polymerase was added after the mixture had been cooled down. PCR program was 30 cycles at 94°C for 20 sec, 50°C for 40 sec, and 72°C for 1min/kb.

Spot tests

For spot tests, 8 µl of 10-fold serial dilutions (starting from 5x10^4 to 5 cells) were spotted out on the media, and incubated at the indicated temperatures for 3~5 days.

FACS analysis

S. pombe cells were grown O/N at 30°C until they reach the density of 0.5-1x10^7 cells/ml. 1ml culture was washed with 1ml ddH_2O and resuspended in 400µl ddH_2O. For fixation, 400µl of 100% Ethanol was added gradually (100µl at a time) and vortexed after every addition. After 24 hours incubation at 4°C, 200µl of the sample was washed 2 times with 1ml of 50mM sodium citrate buffer. After washing, the sample was resuspended in 500µl 50mM sodium citrate buffer and incubated at 37°C with 1mg/ml RNase for 3 hours. The sample was then briefly sonicated for 20 seconds and stained with Sytox Green (Invitrogen).

Results

Cell size comparison

In *S. pombe* cells, any defect in the cell cycle could be reflected in the cell size. Hence, we compared the cell size of *SPBC409.08Δ* and *SPAC9.02cΔ* cells with the wild-type cells. The wt and mutant cells were grown under optimum conditions until they reach 5x10^6 cells/ml and observed under the microscope. The cell lengths of the cells were measured at their fast growing log phase (before the culture reaches the saturation). The average wt cell size was measured to be 13.4µm±1.83 (average size±STD), *SPBC409.08Δ* average size was 11.9µm±1.63 and *SPAC9.02cΔ* average size was measured to be 13.2µm±2.94. 40 cells were analyzed for each cell type. Two tailed t-test was used to compare the mutants' and wild-type cells' length. *SPBC409.08Δ* mutants were found to be significantly shorter than wt cells (p<1.86x10^{-4}), while *SPAC9.02cΔ* mutants showed no significant difference when compared with the wild-type cells (p<0.75) (Figure 1(a)).

Growth rate analysis

As a first step in the characterization of *SPBC409.08Δ* and *SPAC9.02cΔ* mutant cells, growth rate of these mutants under optimum conditions (30°C, YEA rich media) was compared with the wild-type (wt) strains. The wild-type and the mutant cell cultures were initiated with a density of 10^6 cells/ml and their growth was followed for 10 hours. The cell densities were checked every 2 hours to see the growth rate. Growth of wt and

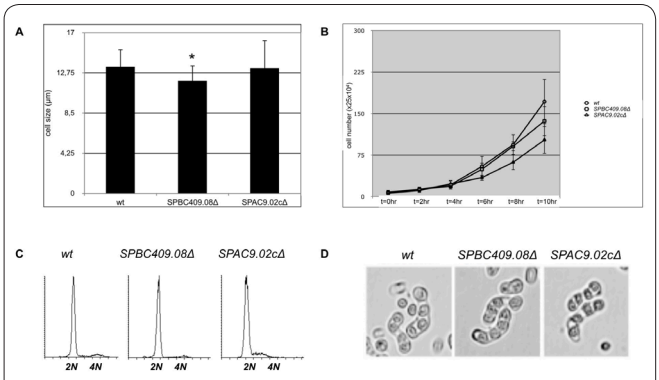

Figure 1. Analysis of the mutants at the optimum growth conditions. (A) Growth rate analysis of the wild-type, *SPBC409.08*Δ and *SPAC9.02cΔ* cells at the optimum conditions (B) Cell size analysis of the wild-type, *SPBC409.08*Δ and *SPAC9.02cΔ* cells (C) Flow cytometry analysis of the wild-type, *SPBC409.08*Δ and *SPAC9.02cΔ* cells (D) Spore formation in the wild-type, *SPBC409.08*Δ and *SPAC9.02cΔ* cells.

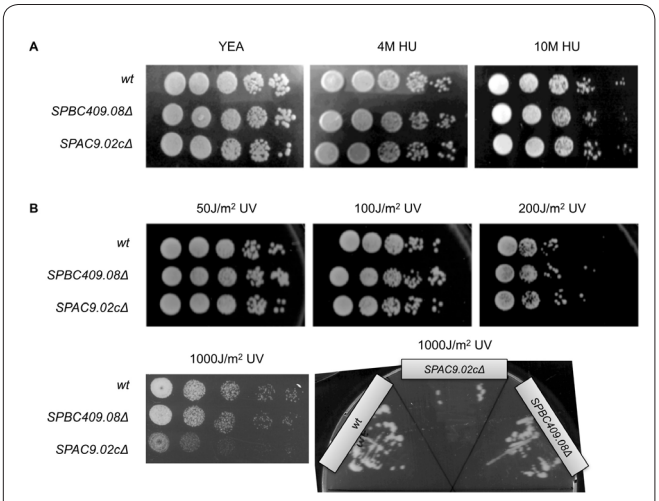

Figure 2. DNA damage response of the mutants compared to wild-type cells. DNA damage response of the mutants upon (A) different concentrations of hydroxyurea (B) different exposures of UV irradiation.

mutant cultures are shown in Figure1. The results were compared using two tailed t-test and no significant difference was observed between the growth rates of wt and mutant cells (Figure 1(b)). The same result was observed when the wild-type and mutant cells were plated onto YEA agar plates and kept at 30°C for 3 days. As expected, no difference was detected on the agar plates (Figure 2(a), control experiment) indicating that under optimum conditions, the mutants grow as well as the wt cells.

FACS analysis

In order to detect any deficiencies in the cell cycle progression, the wild-type and mutant cells were also analyzed by flow cytometry. As shown in Figure 1(c), wild-type cells were mostly accumulated at the 2N region, which is a characteristic results for *S.pombe* cells. The 4N bump represents cells, which are completing S phase with incomplete cytokinesis (binucleate cells). *SPBC409.08Δ* cells showed very similar patterns with wild-type cells, while *SPAC9.02cΔ* cells FACS profile showed a small but consistent difference. More *SPAC9.02cΔ* cells were detected in the region between 2N and 4N compared to the wild-type cells.

Sporulation analysis

In the next step, the mutants' ability to form spores were checked. Mutant and wt *S.pombe* cells were kept at the SPA sporulation medium for 3 days with wild-type cells of the opposite mating type. Nitrogen depleted SPA medium is expected to induce G1 arrest in wild type cells. These cells are then expected to mate with the opposite mating type and form spores after successful meiotic division. h^+ *SPBC409.08Δ* cells were mixed with h^- wild-type cells, while *h- SPAC9.02cΔ* cells were mixed with h^+ wild-type cells on SPA plate. Both mutants could successfully form spores, which indicates proper G1 arrest, mating and meiosis upon nutrient starvation stress (Figure 1(d)).

Stress Response analysis

In an attempt to understand the roles of *SPBC409.08* and *SPAC9.02c* genes in the stress response, the deletion mutants were exposed to DNA damaging agents and osmotic stress. The wt and mutant cells were exposed to DNA damaging agents such as hydroxyurea and UV irradiation. To determine the effect of hydroxyurea, it was added to the YEA agar plates in varying increasing amounts until wild-type cells stop growing. In the presence of 4mM or 10mM hydroxyurea no difference was detected between the wild-type and mutant cells (Figure 2(a)). The mutant and the wild-type cells were then plated onto YEA agar and exposed to different amounts of UV irradiation (50J/m^2 to 1000J/m^2). The growth rate of the mutants was not different from the wild-type cells in cases of no UV light, 50J/m^2, 100J/m^2, 200J/m^2 UV (Figure 2(b)). We then increased UV irradiation to 1000J/m^2, which has a significant effect in even wt cell growth. Since the cells grew very slow for spot test, we also streaked the cells on the plate. In case of 1000J/m^2, we detected less cell growth in *SPBC409.08Δ* cells (Figure 2(b)), which indicates sensitivity to high amount of UV light.

The mutants were then plated onto YEA agar plates containing $CaCl_2$, KCl, NaCl or sorbitol, which induce osmotic stress response. Different concentrations of these salts were tried for this experiment. The concentrations were increased until wild-type cells stop growing (similar to DNA damage experiment). Figure3 shows 120mM $CaCl_2$, 0.5M KCl, 0.2M NaCl or 2M Sorbitol containing YEA agar plates. In each condition, the cells were incubated at 30°C and their growth were compared to the wild-type cells. In the presence of osmotic stress conditions, no difference was detected between the mutants' and the wild-type cells' growth rate (Figure 3).

Double mutants

As shown in Figure 1(c), single mutation of *SPBC409.08* and *SPAC9.02c* are not sterile and can form spores successfully. Hence we decided to cross these single mutants and create double mutants of *SPBC409.08Δ SPAC9.02cΔ*. In the final step, we checked the stress response of this double mutant, which has no *SPBC409.08* and *SPAC9.02c* gene. No difference was detected between the growth of wild-type and double

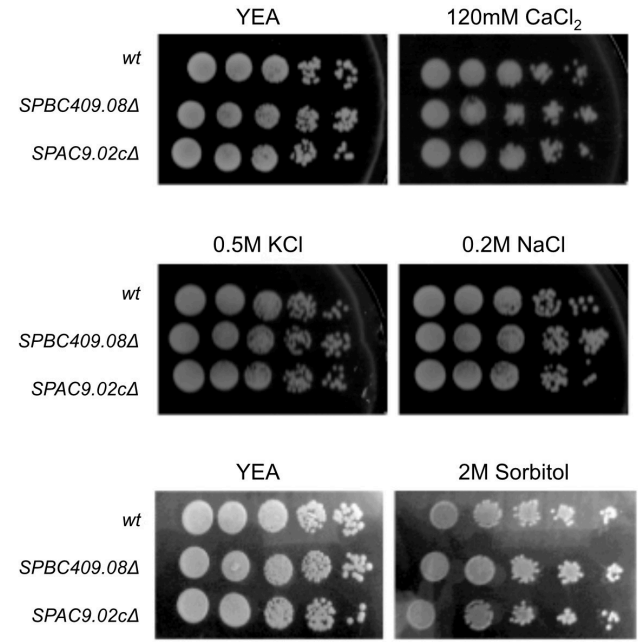

Figure 3. Osmotic stress response of the mutants compared to wild-type cells. Stress response of the mutants upon different concentrations of (A) $CaCl_2$, KCl, NaCl salts or (B) 2M Sorbitol.

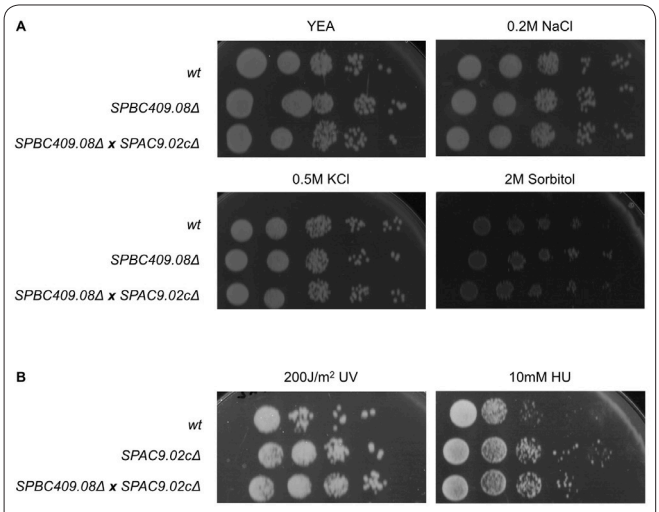

Figure 4. Analysis of double mutants: (A) DNA damage and (B) osmotic stress responses of *SPBC409.08*Δ *SPAC9.02cΔ* double mutant cells.

mutants on YEA agar plates, at the optimum conditions (Figure 4(a)). Osmotic stress responses of the double mutants were checked in the presence of 120mM $CaCl_2$, 0.5M KCl, 0.2M NaCl or 2M Sorbitol. There was no difference between the growth rates of wild-type cells, single or double mutants (Figure 4(a)). When the cells were exposed to DNA damaging agents, *SPBC409.08Δ SPAC9.02cΔ* double mutant showed no additive effect on the sensitivity to UV irradiation or hydroxyurea (Figure 4(b)). To conclude, no additive effect was observed in the double mutants.

Discussion

Fission yeast is a very suitable organism to investigate the cell cycle, hence it has been used extensively (12). It is a haploid organism, which also makes it easy to create deletion mutants of nonessential genes. In our previous work we had found that a well characterized gene, Atf1, provides a link between cell cycle and stress response pathway (13). So we tried to find novel candidate genes that could constitute a bridge between these two in *S. pombe* and focused on polyamine metabolism genes to identify new genes that may be important in the cell cycle control and stress response. In our study we focused on two candidate genes, *SPBC409.08* and *SPAC9.02c,* that may be involved in the polyamine metabolism and not yet been experimentally characterized. We generated deletion mutants of these nonessential genes and checked the stress response upon DNA damaging agents and environmental osmotic stress.

Fission yeast is a rod shape organism, whose cell size is an important indicator of cell cycle progression, which is directly affected by the cell cycle progression. Any defect in the cell cycle could be reflected in cell size. For instance, *cdc25* gene deletion results in elongated *S. pombe* cells, while *wee1* gene mutation leads to shorter cells. In our analysis, we found that *SPBC409.08Δ* mutants were also shorter than wt cells, which indicates that this gene is important in cell size regulation.

We also checked the stress response of the mutants and tried to find any sensitivity against osmotic or DNA damage stress. Deletion of genes that are involved in stress response pathways such as MAPK pathway had been previously shown to be sensitive to osmotic stress (14).

In our study we induced osmotic stress by adding different concentrations of $CaCl_2$, KCl, NaCl or sorbitol to YEA medium. In neither of these conditions, our mutants showed any sensitivity. In case of DNA damaging agents, however, *SPAC9.02cΔ* cells showed sensitivity to UV irradiation but not to hydroxyurea. UV irradiation induces DNA damage by forming dimers in the DNA molecule, which halts cell cycle in G1 phase or G2/M transition. Hydroxyurea, on the other hand, depletes dNTPs and halts DNA replication in S phase and the cells cannot pass through G2/M transition. This indicates a specific role of *SPAC9.02c* gene in UV induced-DNA damage response. *SPAC9.02c* gene was previously shown to be sensitive to HDAC inhibitors valpronic acid (VPA) and sodium butyrate (SB) (15). These agents were supposed to inhibit cell cycle progression and induce apoptosis (16,17).

Although the cell cycle and stress response defects were detected in *SPBC409.08Δ* and *SPAC9.02cΔ* cells, meiotic division seems to progress properly in these mutants. Both of the mutants could successfully produce four spores upon meiotic induction by nitrogen starvation.

Finally, double mutants of the genes of interest were generated to check any additive phenotypes. However, there were no additive effects in either cell size or stress sensitivity.

Polyamines are involved in very important processes in the cell such as apoptosis, transcription, translation, cell cycle progression. Hence, polyamine metabolism (uptake, release, de novo synthesis, interconversion and degradation) is kept under strict regulation in cells (18). Thus, redundant mechanisms are evolved to ensure polyamine homeostasis. This, we believe, could be one of the reasons we did not see extreme phenotypes in our deletion mutants. *SPBC409.08Δ* was slightly shorter than the wild-type cells and in *SPAC9.02cΔ* cells DNA damage sensitivity could only be detected at 1000J/m^2

UV irradiation. *SPAC9.02c* is a hypothetical Polyamine N-acetyltransferase gene, the product of which is a rate limiting enzyme in polyamine catabolism. Overexpression of this enzyme was previously reported to increase DNA lesions, which finally arrests cell cycle by inducing checkpoints (19). On the other hand, Polyamine N-acetyltransferase deficient mouse embryonic cells were reported to proliferate normally and have similar polyamine pools as the control cells, which indicated that this enzyme was not the major player in polyamine homeostasis and these cells were believed to have an additional system for the polyamine interconversion (20). It has been shown that increased polyamine levels leads to an increase in antienzyme 1 level, which negatively regulates the synthesis and uptake of polyamines (21, 22). This negative feedback loop keeps the polyamine levels constant in cells. A similar negative feedback loop exists for *P.polycephalum, S.cerevisiae, N.crassa* and *S.pombe* (23, 24, 25, 26, 27, 28). In *S.pombe*, increase in polyamine levels could only be observed in the complete absence of the antienzyme 1 (28), which is intact in our mutant cells, thus it is expected to keep the polyamine levels relatively stable. This, we believe is the reason for the mild phenotypes we observed in our mutants. In parallel with our results, another research on polyamines and their effect on DNA damage showed that spermine exposure lead to increased spontaneous mutations in *mut 1-4 S.pombe* mutants and enhanced UV-induced mutations in *rad13* mutants as well as normal pigmented cells (29). This indicates the mutagenic effect of increased amount of spermine in *S.pombe* and an important reason to keep the polyamine amounts stable in the cells.

Our results showed that hypothetical gene *SPBC409.08* might be involved in cell cycle and *SPAC9.02c* gene product might be involved in stress response. To our knowledge our study is the first experimental characterization of these genes.

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