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Combining atomic force microscopy and genetics to investigate the role of *KNR4* in *Saccharomyces Cerevisiae* sensitivity to K9 killer toxin

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ABSTRACT

K9 killer toxin is a small peptide secreted by the yeast *Williopsis saturnus var: mrakii* (previously known as *Hansenula mrakii*) with a strong cytocidal activity against sensitive yeast strains, including *Saccharomyces cerevisiae*. Treatment with this toxin results in the formation of pores at the surface of the cells, and more specifically at places where cell wall synthesis is the most active, namely at the tip of growing buds or mating projections. Yeast cells treated with K9 toxin then die by releasing cytoplasm and cellular materials from these pores. In the yeast *S. cerevisiae*, *KNR4* protein localizes at the sites of polarized growth (bud tips, shmoo tips), which are also the sites where the toxin forms pores in the cell wall. Mutants defective in *KNR4* gene are remarkably resistant to this toxin. In this study, we analyzed for the first time the biophysical effects of K9 on the yeast cell wall using Atomic Force Microscopy (AFM), a cutting edge technology that allows measuring the nanomechanical properties of living yeast cells, and their alterations by various drugs. To this end, we measured the effects of K9 toxin on the nanomechanical properties of the cell wall of *S. cerevisiae* wild-type cells and mutants deleted for *KNR4* gene, at the short (2 h) and long term (20 h). Our results reveal an important cell wall remodeling occurring in wild-type cells already after 2 hours and only visible in *knr4Δ* mutant after 20 hours of treatment. Moreover, we investigated the role of Knr4 protein in the cells sensitivity towards the toxin. We were able to show that the presence of the N-terminal domain of Knr4 protein, which is required for its correct cellular localization at the bud tip during cell cycle, is essential for the toxin K9 wild-type sensitivity. In addition, a series of deletion mutants from the YKO collection in which the Knr4 cellular localization is also lost display a reduced sensitivity to the K9 toxin. Taken together, these results shed light on the importance of the proper localization of Knr4 protein at sites of intensive cell wall growth for the wild-type cells sensitivity to K9 killer toxin.

Keywords: *K9 killer toxin, Saccharomyces cerevisiae, KNR4 gene deletion, Atomic Force Microscopy.*

1. INTRODUCTION

Yeast killer toxins are small peptides secreted by "killer" yeast to selectively inhibit the growth of other sensitive yeast species. Several of these killer toxins first bind to a yet unidentified receptor structure on the yeast cell wall [1-4]. Therefore they have been used as tools to investigate the biosynthesis pathways of specific cell wall components in the model budding yeast *Saccharomyces cerevisiae,* such as β -(1,6)-glucan[5, 6] and β -(1,3)-glucans [7]. K9 killer toxinproduced by the yeast *Williopsis saturnus var: mrakii*, previously known as *Hansenula mrakii*, is an 88 amino acids peptide which inhibits the β-(1,3)-glucan synthase of *S. cerevisiae in vivo,* on regenerating spheroplasts, and *in vitro* on purified cell membranes[8]. Exposure of whole living *S. cerevisiae* cells to the K9 killer toxin results in the formation of holes in the cell wall, at the tip of growing buds in exponentially growing cells or at the tip of mating projections (or shmoos) in cells exposed to mating pheromones. Stationary phase cells are not sensitive to the killer toxin [9]. The proposed mode of action of K9 killer toxin is the following: the toxin first binds to a complex cell wall receptor structure, progresses through the cell wall, then binds to an unknown protein located on the cell membrane, likely N-glycosylated, and finally inhibits the β(1,3)-glucan synthase [10,11–14]. The initial step, binding to the cell wall receptor structure, is essential to the cytocydal activity of the toxin, since the toxin does not kill sensitive yeast cells existing as spheroplasts, with no cell walls. Curiously, although spheroplasts survive K9 exposure, their morphology is however affected by the toxin, resulting in perfectly round shaped cells with an increased volume and apparition of huge cellular vacuoles [15].

In an attempt to characterize the molecular mechanisms of K9 toxin binding on *S. cerevisiae* cell wall, we choose to include in our study a mutant deleted in *KNR4* gene, which is remarkably resistant to this toxin. *KNR4* gene name stands for *"K*iller *N*ine *R*esistant *4"* and comes from its original isolation by a screen based on resistance to the Killer toxin K9 of *Hansenula mrakii* [7]. Mutants defective in *KNR4* gene display reduced β (1,3)-glucan synthase activity correlated with reduced levels of β (1,3)-glucan in their cell walls [16]. In the budding yeast *S. cerevisiae,* Knr4 protein localizes at the sites of polarized growth such as bud tips and shmoo tips [17, 18], which are also the places where the toxin forms pores in the cell wall. In most *S. cerevisiae* genetic backgrounds *KNR4* gene is not essential for growth under standard laboratory conditions, however its deletion leads to growth defects

under numerous stress conditions such as elevated temperature or presence of SDS, caffeine, antifungals (ex.: caspofungin) or cell wall affecting drugs like Calcofluor White and Congo Red. This wide range of phenotypes corroborates with the role of *KNR4* protein in transcriptional control of gene expression [17]. Indeed, *KNR4* is a conserved yeast hub protein [19], known to genetically and physically interact with several distinct partners with diverse cellular functions [20, 17, 21], (and *Saccharomyces Genome Database, http://www.yeastgenome.org*). Through these interactions, Knr4 notably influences the transcriptional control of a large number of genes among which numerous cell wall synthesis genes, including all three chitin synthase genes present in *S. cerevisiae* genome [22]. Both N-terminal and the C-terminal domains of *Knr4* protein are disordered, but only the N-terminus of Knr4 is required for the protein correct localization at the bud tip [18]. Moreover, *Knr4* participates in the transcriptional control of G1/S transition during cell cycle progression [18]. Finally, *KNR4* gene has homologs in the whole fungal genome, and functional and genomic data on homolog genes from *Candida albicans* [23], *Neurospora crassa* [24] and *Schizosaccharomyces pombe (www.pombase.org)* indicate that their products likely perform very similar cellular functions in these organisms.

In order to better understand the role of *Knr4* in the cells sensitivity to K9 killer toxin, we used Atomic Force Microscopy (AFM) to study the nanomechanical properties of the cell wall of *S. cerevisiae* and *knr4Δ* mutant upon exposure to K9 toxin. Since its invention in 1986 [25], AFM has emerged as a cutting-edge technology to study the biophysical properties of the cell wall of living cells, under aqueous controlled conditions [26, 27]. Moreover, AFM has been a useful technology to evaluate the effects of various stresses on the cell wall of living yeast cells [28] such as thermal stress [29], or antifungal drug treatment [30]. The results obtained show important modifications of the nanomechanical properties of the cell wall of both control and *knr4Δ* mutant strains after 20 h of K9 treatment, whereas 2 h of treatment caused modifications only in the case of the wild-type strain. We could therefore hypothesize on the role of Knr4 in the activation of the cell wall remodeling triggered by exposure to K9. Moreover, making use of variants of *Knr4* protein deleted for the N-terminal and C-terminal part and of a series of gene deletion mutants affected in *Knr4* cellular localization, we were able to show that the localization of *Knr4* protein at sites of active cell wall growth is a key element in the sensitivity of yeast cells to K9 killer toxin.

2. EXPERIMENTAL SECTION

2.1. K9 killer toxin production

Killer K9 toxin, also referred to as HM-1, is secreted in the culture supernatant of the strain *Hansenula mrakii* IFO0895 obtained from ATCC. This strain is cultured for 72 hours at 19°C in liquid YPD adjusted to pH 4.3 by the addition of 5mM Na citrate. Cells are pelleted by centrifugation, and the supernatant is filtered on 0.22 µm pores filter. Further concentration of the toxin is performed using Amicon ultra-15 centrifugal filter device at 4°C, for 40-60 minute, 5 times. At last about 60mL culture supernatant is concentrated in 1ml. The final toxin concentration is estimated by Bradford method. The concentrated K9 killer toxin has been checked by SDS-PAGE, resulting in one clear band of the killer toxin of 9.5 kDa size.

2.2. Genome integration of the genes encoding *Knr4* truncated variants.

We constructed the vectors containing the different truncated versions of *KNR4* gene with its own terminator, in which we inserted the KanMX4 marker which allows *S. cerevisiae* to grow in the presence of Geneticin (G418). KanMX4 marker was amplified by PCR using the primersKan Start - AGGTTTCAACCTTAAGACATGGAGGCCCAGAATAC and Kan End - GTATAAATTTCTTAAGCGACCAGCATTCACATACG. In-fusion cloning technology (Clontech) was used to insert this marker in the middle of *KNR4*terminator, on the vector pGEMT-*KNR4* digested by AflII, to generate vector pGEMT-*KNR4*-Terminator::KanMX4. KpnI and XhoI digestion was used to obtain the fragment of terminator with KanMX4, which was then ligated at the end of the 3 different truncated *KNR4* variants. All vectors used are presented in Table 2. Finally, digestion by EagI and KpnI allowed us to obtain the fragments with "Promoter-*KNR4* truncated variants-Terminator::KanMX4", which were integrated in the genome of the BY4741 by lithium acetate transformation method. Recombinants were selected on solid YPD medium containing 200ug/mL G418, and the correct intergration was verified by PCR using primers amont *KNR4*-CTTGGGATGCCGATCCGCC and Kan reverse-GCAACCGGCGCAGGAACAC.

2.3. KNR4-GFP construct integration in the mutants.

We integrated the gene encoding Knr4-GFP fusion into the genome of 10 mutants *bem1Δ, bem2Δ, bni1Δ, bud6Δ, pcl1Δ, pcl2Δ, spa2Δ, yck1Δ, rrd1Δ, tpd3Δ*, and the control strain BY4741. We took plasmid pHM53 as the original DNA to make the linear DNA. There are 2 AgeI digestion sites in *KNR4* in this vector. pHM53 was digested with AgeI enzyme, the fragment containing *KNR4-GFP* and *URA3* gene was purified and used to transform the yeast strains by classical lithium acetate transformation. The different mutants bearing *KNR4-GFP* genome integration were selected on minimal SD medium lacking uracil. The correct integration of *KNR4-GFP*in *KNR4* locus was further checked by PCR after genomic DNA extraction. All genomic DNA have been isolated using the MasterPure™ Yeast DNA Purification Kit (Epicentre).

2.4. Cellular localization of the Knr4-GFPprotein fusion

Fluorescence microscopy observation has been performed on each mutant displaying the correct integration and in the BY4741 control strain. At least 200 cells have been observed for each mutant, classified as displaying either correct or incorrect protein localization in comparison with the localization observed for the control strain BY4741 and counted. The mutants for which over

50% of the cells had a cellular localization of the fusion protein similar to the control strain were considered as not affected, and the ones for which over 50% of the cells displayed an incorrect localization were considered as affected in Knr4 protein localization. GFP fluorescence *in vivo* was monitored using a Leica DM4000B microscope, with excitation filters 421-450 nm and emission filter 466-500 nm.

2.5. Cultures conditions.

Saccharomyces cerevisiae strains used in this study are listed in Table 1. The control strain BY4741 was currently grown in YPD medium containing 2% (w/v) bactopeptone, 1% (w/v) yeast extract and 2% (w/v) glucose. The *knr4ΔN*, *knr4ΔN*+C, *knr4ΔC* and the YKO mutants were grown in YPD with 200µg/mL geneticin G418. For solid media, 2% (w/v) agar was added. *H. markii* IFO0895 strain was cultivated in liquid YPD adjusted at pH4.3 by addition of 5 mM Na citrate.

2.6. K9 killer toxin MIC determination.

Minimal Inhibitory Concentration (MIC) of K9 killer toxin against control *Saccharomyces cerevisiae* strain BY4741 was determined according to the protocol provided by the Clinical Laboratory Standard Institute (CLSI) [35]. Briefly, a yeast solution of OD_{600}=0.150 was prepared from fresh agar plate. This cell suspension was then diluted to obtain a concentration in cells 5×10^2-2.5×10^3 cells/mL. Different concentrations of K9 killer toxin (from 2.5 µg/mL to 80 µg/mL) were placed in tubes, containing 0.9 mL of the yeast suspension. For the control, 0.1mL of sterile water was used instead of K9. Cultures were incubated at 30ºC for 24h, before reading OD_{600} in order to determine the concentration of the K9 killer toxin needed to inhibit growth. This test was repeated 3 independent times with the same result. The MIC of K9 killer toxin was determined as 20 mg/L.

Table 1. List of *S. cerevisiae* strains used in this work.

Strain	Genotype	Reference
BY4741	Mat**a**; *his3Δ1 leu2Δ0; met15Δ0; ura3Δ0*	[31]
bem1Δ	BY4741; *YBR200W::kanMX4*	Open Biosystems (Thermo Scientific)
bem2Δ	BY4741; *YBR200W::kanMX4*	Open Biosystems
bni1Δ	BY4741; *YNL271C::kanMX4*	Open Biosystems
bud6Δ	BY4741; *YLR319C::kanMX4*	Open Biosystems
knr4Δ	BY4741; *YGR229C::kanMX4*	Open Biosystems
pcl1Δ	BY4741; *YNL289w::kanMX4*	Open Biosystems
pcl2Δ	BY4741; *YDL127w::kanMX4*	Open Biosystems
spa2Δ	BY4741; *YLL021W::kanMX4*	Open Biosystems
tpd3Δ	BY4741; *YAL016W::kanMX4*	Open Biosystems
yck1Δ	BY4741; *YHR135C::kanMX4*	Open Biosystems
knr4ΔN	BY4741; *KNR4Δ1-80- terminator ::kanMX4*	This study
knr4ΔN+C	BY4741; *KNR4Δ1-80, Δ340-505- terminator::kanMX4*	This study
knr4ΔC	BY4741; *KNR4Δ340-505- terminator::kanMX4*	This study

Table 2. Vectors used in this study.

Plasmid	Description	Origin
pGEMT-*KNR4*	*KNR4* gene with terminator in pGEM-T (Promega).	[32]
pRS426-*KNR4*	*KNR4*gene with promoter in pRS426[33](URA3 marker)	[32]
pRS426-*KNR4ΔN*	*KNR4Δ*N (80-505) with promoter in pRS426	[32]
pRS426-*KNR4ΔN+C*	*KNR4ΔN+C* (80-340) with promoter in pRS426	[32]
pRS426-*KNR4ΔC*	*KNR4ΔC* (1-340) with promoter in PRS426	[32]
pHM53	*KNR4* genefused to GFP, with its own promoter, in pRS306[33]integrative vector	[34]
pGEMT-*KNR4*-T-KanMX4	*KNR4* gene with its terminator with KanMX4 in pGEM-T (Promega)	This study
pRS426-*KNR4*-T::KanMX4	*KNR4*gene with its promoter and terminator::KanMX4 in PRS426	This study
pRS426-*KNR4ΔN*-T::KanMX4	*KNR4ΔN*(80-505)+ promoter and terminator::KanMX4 in PRS426	This study
PRS426-*KNR4ΔN+C*-T::KanMX4	*KNR4ΔN+C*(80-340)+ prom. and terminator::KanMX4 in PRS426	This study
PRS426-KNR4ΔC-T::KanMX4	*KNR4ΔC*(1-340)+promoter and terminator::KanMX4 in PRS426	This study

2.7. K9 halo tests.

Killer toxin halo tests are performed on solid YPD plates at 30ºC overnight. Yeasts clones to be tested are first grown in YPD liquid medium at 30°C overnight, then cell density is adjusted to OD_{600} = 4 by centrifugation and/or dilution in sterile YPD medium. 200µl of this yeast solution is sprayed on the agar plate (Petri dish containing solid YPD medium). After 1 h drying by incubation at 30ºC, K9 killer toxin solution at a concentration of 0.8 mg/mL is spotted on the plate, as independent dots of different volumes (10 and 20µL, see Figure 1). Alternatively, larger volumes (30µL) can be used, see Supplementary data. Petri dishes are incubated at 30ºC overnight.

2.9. AFM sample preparation and experiments.

Culture of yeast cells were done as described above. After collecting treated and non-treated cells by centrifugation, and washing two times with acetate buffer (18 mM Na Acetate, 1 mM $CaCl_2$, 1 mM $MnCl_2$, pH=5.2), cells were resuspended in the same buffer at OD_{600}=1.0, and immobilized in polydimethylsiloxane (PDMS) stamps prepared as described previously [36]. Briefly, freshly oxygen activated microstructured PDMS stamps were covered by a total of 100 µL of the cell suspension and allowed to stand for 15 minutes at room temperature. The cells were then deposited into the structures of the stamp by convective/capillary assembly. AFM Images were recorded in Quantitative Imaging™ [37] mode on a Nanowizard 3 AFM (JPK Instruments, Berlin, Germany), with MLCT AUWH cantilevers (nominal spring constant of 0.01 N/m, Bruker, USA). The cantilevers spring constants were determined by the thermal noise method [38] prior to each experiments. The applied force used in the AFM Imaging mode was kept at 1.0 nN. For nanomechanical properties measurements, force maps of 32 ×32 force curves were recorded on areas of 1 µm × 1 µm on top of the cells. The force-distance curves recorded were transformed into force-indentation curves by subtracting the cantilever deflection on a solid surface. The indentation curves were then fitted to the Hertz model, which links the force (F) as a function of the elastic modulus (E) and the square of the indentation (δ) for a conical indenter.

$$F=\frac{2E\tan\alpha}{\pi(1-\nu^2)}\,\delta^2 \quad (1)$$

In equation (1), α is the tip opening angle (17.5°) and ν the Poisson ratio assumed to be 0.5.

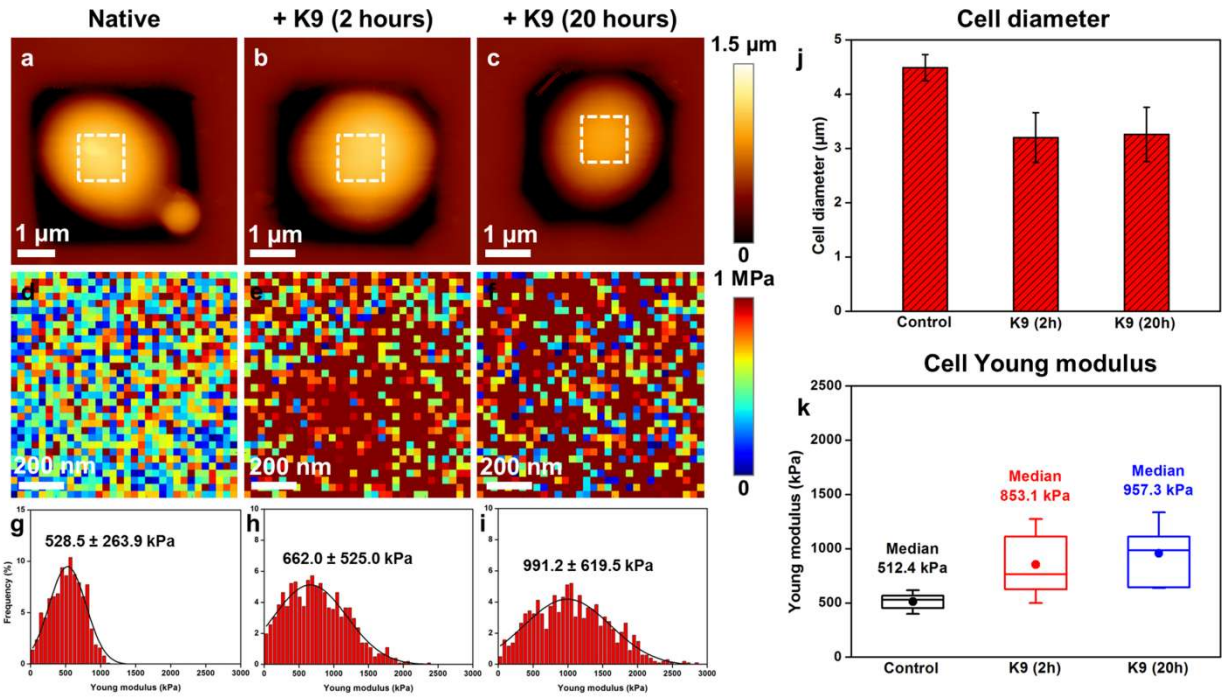

Figure 1. Imaging and nanomechanical properties of *Saccharomyces cerevisiae* strain BY4741 submitted to K9 treatment. (a) AFM Height image of a single cell immobilized in a PDMS stamp in native condition, (b) treated with K9 killer toxin during 2 hours and (c) treated with K9 killer toxin during 20 hours. (d, e, f) Elasticity maps recorded on areas of 1 µm × 1 µm on top of cells (white squares in a, b and c). (g, h, i) distributions of Young modulus values (n = 1024) corresponding to the elasticity maps. (j) Average diameter of cells (statistic on 10 cells coming from 2 independent cultures) and (k) average of Young modulus values (statistics on 10 cells coming from 2 independent cultures).

3. RESULTS SECTION

3.1. K9 killer toxin treatment modifies the cell wall nanomechanical properties of wild-type cells of *S. cerevisiae.*

To explore the K9 killer toxin effects on the nanomechanical properties of the cell wall of wild type BY4741, yeast cells were first cultivated at 30ºC to OD_{600} = 1. At this stage, K9 killer toxin was added at a concentration of 0.5 x MIC in order to be able to probe the effects of K9 without killing the cells. AFM analyses were carried out after 2 and 20 hours of incubation, and results were compared to those of control cultures without toxin addition. From the height images presented in Figure 1, average

diameters of cells treated with K9 or in native conditions were measured, on at least 5 cells from 2 independent experiments. Our results showed that in the case of the wild-type strain of *S. cerevisiae*, the average diameter of the cells treated with K9 toxin decreased from 4.5 ± 0.2 µm in native conditions to 3.2 ± 0.6 µm after 2 h of exposure to K9 toxin, but did not decrease further upon longer exposure (3.3 ± 0.5 µm after 20 h). Thus, the morphology of cells is dramatically and rapidly affected by K9 killer toxin. This result was confirmed by a flux cytometry analysis of the cell size among the whole population of K9 treated cultures (see Supplementary data 2). We then performed nanoindentation experiments in order to determine if this reduced size of the cells was correlated to changes in the nanomechanical properties of the cell wall. Results presented in Figure 1 show that K9 exposure of *S. cerevisiae* wild-type cells causes an increase of the cell wall Young modulus from 512.4 ± 87.5 kPa in native conditions, to 853.1±271.8 kPa after 2 h of K9 exposure. This increase was even more important after 20 h of exposure of the cells since the Young modulus of the cell wall reached 957.3±286.0 kPa. Therefore these data clearly show for the first time that K9 killer toxin causes an important cell wall remodeling, that might be associated to its known effect to inhibit the β -(1,3)-glucan synthase [1, 10, 11]. Indeed, in a recent work, we showed that treatment of yeast cells with caspofungin, a molecule that also inhibits the synthesis of the β-(1,3)-glucan synthase, results in a decrease in β -(1,3)-glucan compensated by an increase in the chitin content of the cell wall correlated to a higher cell wall Young modulus [30] in *S. cerevisiae* cells. The same year, another work showed that caspofungin exposure of *Candida albicans* cells resulted in a major remodeling of this yeast cell wall [39]. Therefore, similarly to the effects of this drug, one can suggest that the changes of the cell wall Young modulus of cells treated by K9 killer toxin are also due to a remodeling of the yeast cell wall.

3.2. K9 toxin treatment alters nanomechanical properties of the toxin resistant *knr4Δ* mutant.

In a further attempt to understand the effects of K9 killer toxin on the cell wall of *S. cerevisiae* cells, we decided to explore the nanomechanical properties of a mutant presenting a high resistance to the toxin caused by *KNR4* gene deletion [7]. Our aim was to establish whether the Δ*knr4* mutant resistance to the K9 killer toxin would also spare the cells from the modification of their nanomechanical properties. To this end, *Δknr4* cells were cultivated at 30ºC toOD_{600} = 1, and incubated with K9 killer toxin at 0.5 × MIC for 2 h and 20h before performing AFM experiments. Our data, presented in Figure 2, point out two distinct results: first, the deletion of *KNR4* gene induces a 30 % reduction of the diameter of the cellsin native conditions compared to the wild-type cells(3.1 ± 0.5 µm, compared to 4.5 ± 0.2 µm),which is not further modified by K9 toxin exposure (3.2 ± 0.5 µm after 2h, and 3.4 ± 0.4 µm after 20 hours, Figure 2).

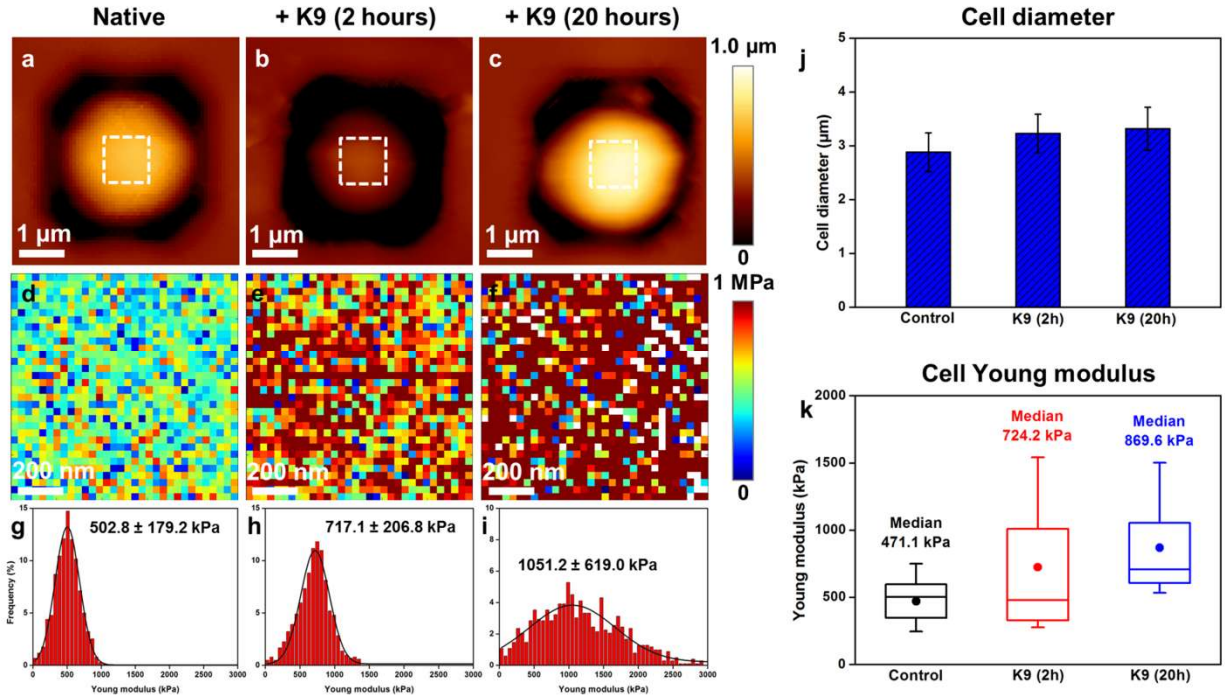

Figure 2. Imaging and nanomechanical properties of *knr4Δ* mutant cells submitted to K9 treatment. (a) AFM Height image of a single cell immobilized in a PDMS stamp in native condition, (b) treated with K9 killer toxin during 2 hours and (c) treated with K9 killer toxin during 20 hours. (d, e, f) Elasticity maps recorded on areas of 1 µm × 1 µm on top of cells (white squares in a, b and c). (g, h, i) distributions of Young modulus values (n = 1024) corresponding to the elasticity maps. (j) Average diameter of cells (statistic on 10 cells coming from 2 independent cultures) and (k) average of Young modulus values (statistics on 10 cells coming from 2 independent cultures).

Therefore, the toxin has no effects on the global morphology of *Δknr4* cells, which is in line with the fact that this strain is resistant to the toxin. Here again, this result was confirmed by flux cytometry analysis of the cell size among the whole culture population (see Supplementary data 2). However, when we investigated in more details the effects of the toxin on the cell wall nanomechanical properties, we obtained interesting results. Indeed, in native conditions, cells have a cell wall Young modulus of 471.1 ± 176.8 kPa whereas after 2 h of exposure to K9, the Young modulus is of 724.2 ± 485.9 kPa. The Young

modulus therefore appears increased, but these results, recorded on at least 10 cells coming from 2 independent cultures, mostly depict a high variability (standard deviation of 485.9) observed from cell to cell. After 20 h of exposure to K9, the Young modulus of the cell wall is increased to 869.6±346.8 kPa, which is similar to the results obtained on the control strain BY4741 after the same time of incubation. Therefore, K9 seems to also have effects on the cell wall of *Δknr4* mutant, but these effects are visible only after 20 h of treatment. Following our hypothesis that the increase of the Young modulus of the cell wall of *S. cerevisiae* upon K9 treatment is due to an increase in the cell wall chitin content, it seems that this might also happen at 20 hours, in the *knr4Δ* mutant. *KNR4* gene product is known, to repress the expression of genes responsible of chitin synthesis and *knr4Δ* mutant displays a high chitin content already without treatment [22]. So we expected the cell surface of *knr4Δ* mutant cells to be harder than the one of control cells. But conversely our results show that the *knr4Δ* (471 ± 176.8 1kPa in Figure2) has almost the same cell wall Young modulus as the wild type (512.4± 87.5 kPa in Figure 1). This is yet an indication that the Young modulus of the cell wall is not only related to the amount of specific components, but also to the complex molecular architecture of the yeast cell wall, as shown by our previous analysis [40].

3.3. Cellular localization of Knr4 protein is required for cell sensitivity to the toxin.

The full-length *Knr4* protein localizes at the presumptive bud site in G1 and then at the tip of small buds and at the mother-daughter neck during cytokinesis [22]. Since *Knr4* protein localization is reminiscent of the sites of pore formation by the K9 toxin, and since the absence of *Knr4* leads to resistance to the toxin, we decided to investigate whether *Knr4* localization was required for the cells sensitivity to the K9 killer toxin. *Knr4* is a hub protein [19], which interacts genetically and physically with numerous distinct partners [17, 20, 21]. Both N-terminal and the C-terminal parts of *Knr4* protein are unstructured, and we showed that the N-terminus is required for the protein correct localization at the bud tip [18]. Three mutant alleles of the gene were created by genomic recombination at the endogenous *KNR4* locus: *knr4ΔN* (encoding a *Knr4* protein without N-terminal domain), *knr4ΔC* (encoding *Knr4* without C-terminal) and *knr4ΔN+C* (encoding the protein deprived of both N-terminal and the C-terminal). To test the sensitivity of these three mutants to K9 killer toxin, we performed sensitivity halo tests on solid medium, with BY4741 and the *Δknr4* as respectively positive and negative controls. On the plate where the wild type BY4741 (sensitive to K9) was spread, empty halos formed by growth inhibition can be observed around the places where the K9 killer toxin was deposited (Figure 3A, white arrows). In contrast, no halo at all appeared on the plate on which was spread *knr4Δ* mutant resistant to K9toxin (Figure 3B). Remarkably, the mutant *knr4ΔN* was also found to be much more resistant to the K9 killer toxin than the control strain (Fig 3C). Hence, the N-terminal domain, which is necessary for *Knr4* correct localization is also required for the K9 cytocidal function, in agreement with our hypothesis that localization of the *Knr4*p is required for the cell sensitivity to the toxin. Unexpectedly, the C- terminal part of the protein may also be involved in this process, although to a lesser extent since*knr4ΔC* (Figure 3E) is more sensitive than the *knr4ΔN* (Figure 3C). This large unstructured C-terminal part of the protein is known to regulate *Knr4* protein-protein interaction [19]. Hence, it appears that the K9 killer cytocidal function requires the correct cellular localization of *Knr4* protein and mayalso involve *Knr4* interaction with other protein partners at the sites of intensive cell wall growth.

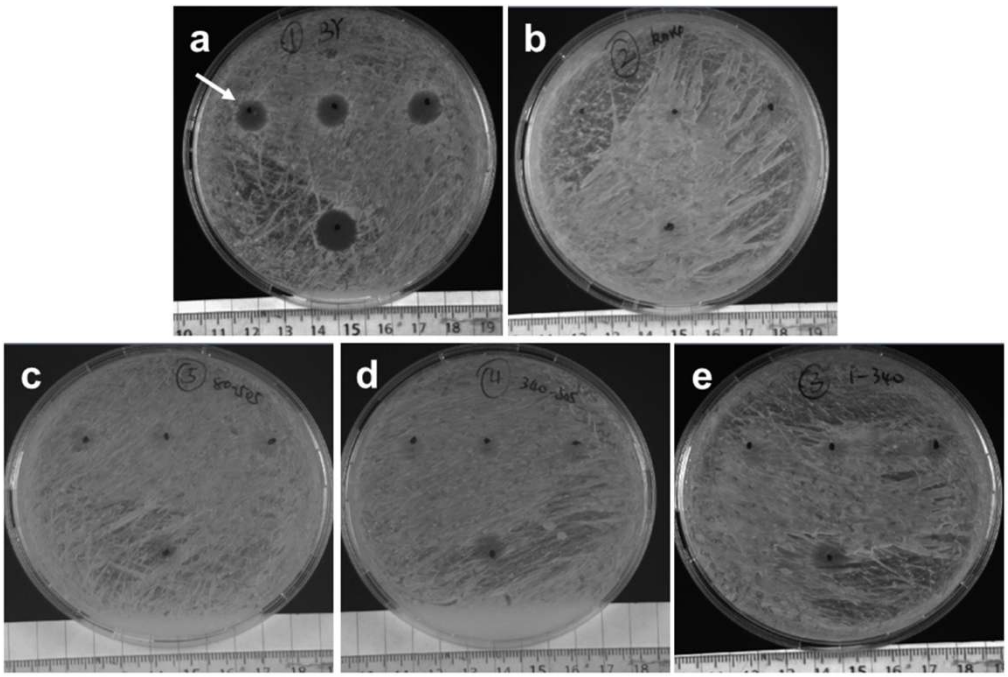

Figure 3. K9 sensitivity halo test of *Saccharomyces cerevisiae* control strain BY4741 and *Knr4* mutants. (a) B74741, (b) *knr4Δ*, (c) *knr4ΔN*, (d) *knr4ΔC*and (e) *knr4ΔN+C*. K9 killer toxin solution was deposited on the plates as 3 spots of 10µL (upper line) and one spot of 20µL (bottom).

3.4. K9 sensitivity of deletion mutants affected in *Knr4* protein localization.

To furthermore verify our hypothesis without modification of *Knr4* protein structure, we decide to make use of a series of genomic deletion mutants in which *Knr4* protein localization is lost. We first conducted a gene ontology search on the *Saccharomyces* Genome Database (www.yeastgenome.org), using the keywords "Morphogenesis'" and "Cell Polarity". Only the non-essential genes were retained, and the corresponding mutants were selected from the Yeast Knock Out collection (Open Biosystems, Thermo Scientific). A total of 30 of the selected mutants were transformed by a construct encoding a functional fusion of Knr4 with the Green Fluorescent Protein GFP. Fluorescence microscopy observation of these 30 mutants revealed an aberrant or perturbed cellular localization of the *Knr4*-GFP construct for 10 of them: *bem1Δ, bem2Δ, bni1Δ, bud6Δ, pcl1Δ, pcl2Δ, spa2Δ, yck1Δ, rrd1Δ, tpd3Δ*. This observations were first conducted with the *KNR4-GFP* fusion on a plasmid (as in [18]), and then confirmed after genome integration of the fusion construct at the endogenous *KNR4* gene locus (see material and methods). Then K9 sensitivity halo test were performed on these 10 mutants affected in Knr4 localization, similarly as above. These experiments showed that these mutants are indeed less sensitive to K9 than wild type cells (example on figure 4C). These results are in line with the ones obtained with the *knr4ΔN* mutant, and independently support the importance of the correct Knr4 protein localization at sites of intensive cell wall synthesis during cell cycle, such as the tips of small buds, which corresponds to the places where K9 toxin forms pores in the yeast cell walls [9].

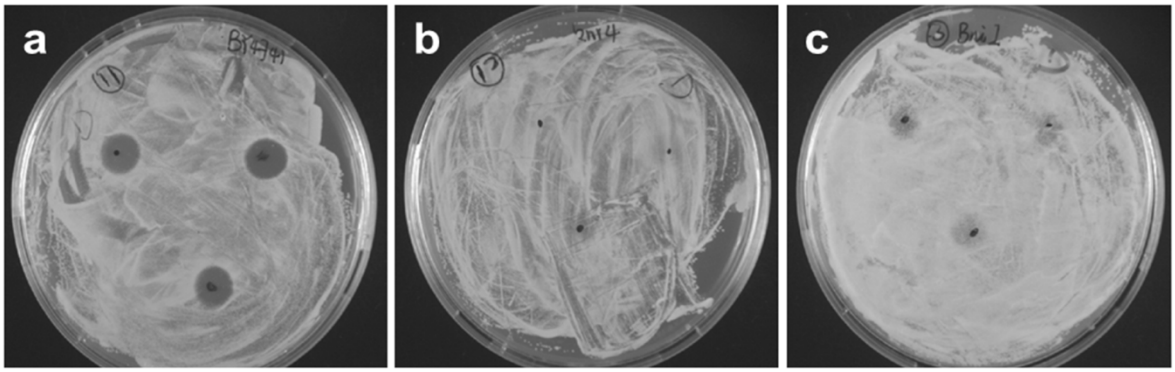

Figure 4. Example of K9 sensitivity halo test of one mutant affected in Knr4 localization, *bni1Δ*. (a) strain BY4741 (b) *knr4Δ*mutant, and (c) *bni1Δ* mutant.

4. CONCLUSIONS

Our results constitute the first study of the nanomechanical alterations of the yeast cell surface upon treatment by Killer toxin K9using Atomic Force Microscopy. Comitini et *al.* [41] used AFM to observe the cell wall surface of yeast treated by another killer toxin, Kpkt produced by *Tetrapisis poraphaffii,* but their study was performed on dead and dehydrated yeast cell walls fragments, while we conducted our work on living yeast cells using AFM in liquid conditions. Our results establish a quantitative measurement of the cell size reduction occurring upon K9 treatment already after 2 hours, as well as of the significant increase in the cell wall Young modulus, representative of a deep cell wall remodeling correlated notably with an increase in chitin content. Moreover, this study allowed us to gain further insights into the mechanism of action of K9 toxin and particularly on its progression from the cell surface towards its cellular target, the β(1,3) glucan synthase [14], which catalytic subunit Fks1 and Fks2 are integral membrane proteins. Even a genome wide search for K9 killer toxin targets did not allow the identification of the putative K9 toxin binding protein [13]. Thus, we decided to take a different approach and to directly make use of a mutant well known for its resistance to K9 killer toxin, the *knr4Δ* mutant. We provide here two independent results indicating that the presence of *Knr4* protein at sites of intensive cell wall synthesis (*i.e.* bud tips) is necessary for yeast cells to display wild-type sensitivity to the toxin. Hence, *Knr4* protein likely participates in the targeting of the toxin towards its target, the β(1,3) glucan synthase. In order to identify the precise receptor structure responsible for the binding of the K9 toxin at the surface of the cells, further works could involve functionalization of the AFM tip with purified toxin.

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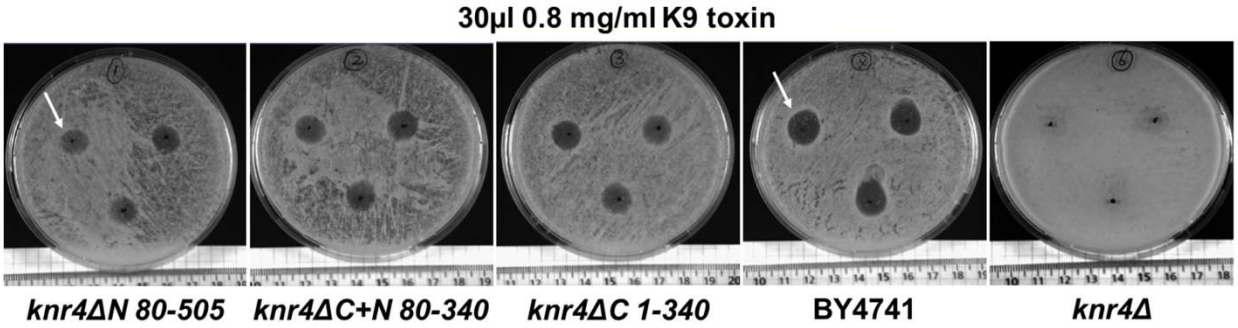
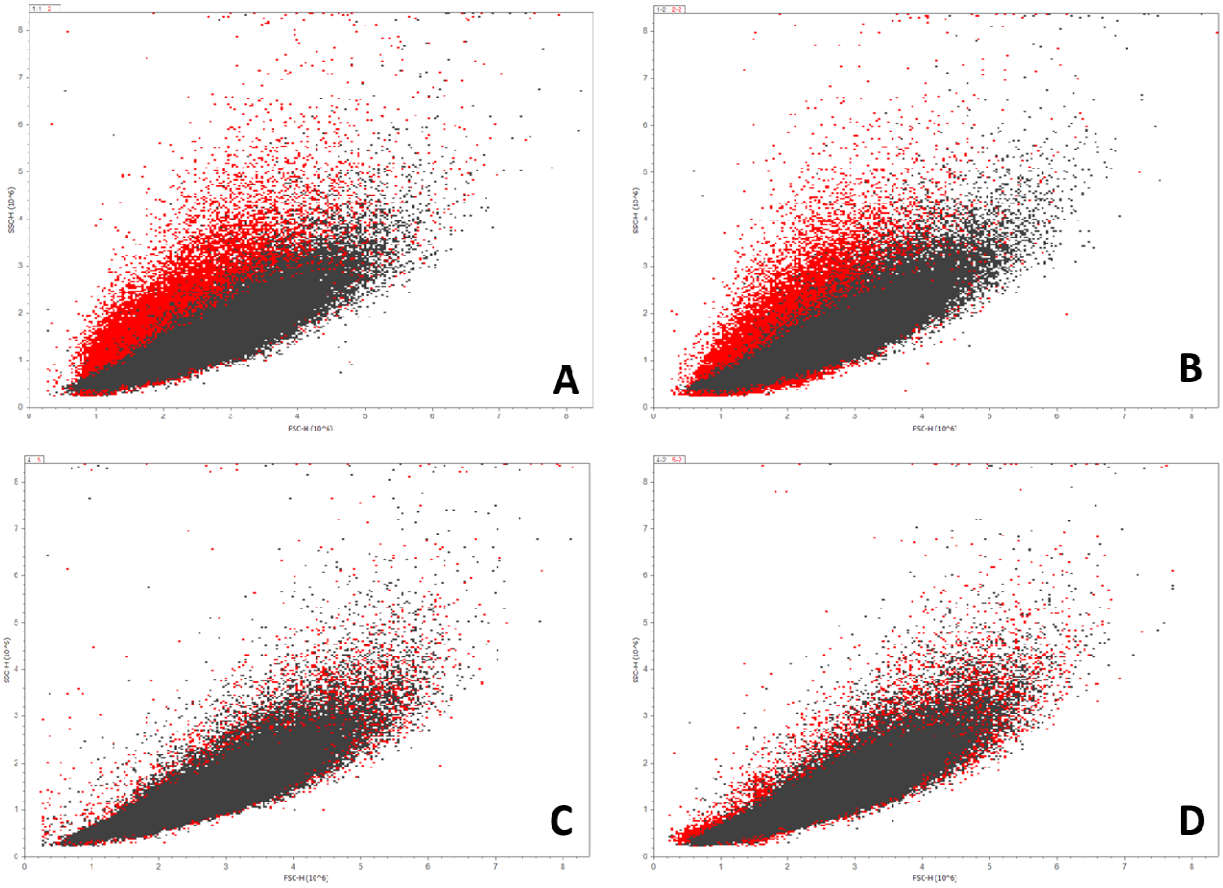
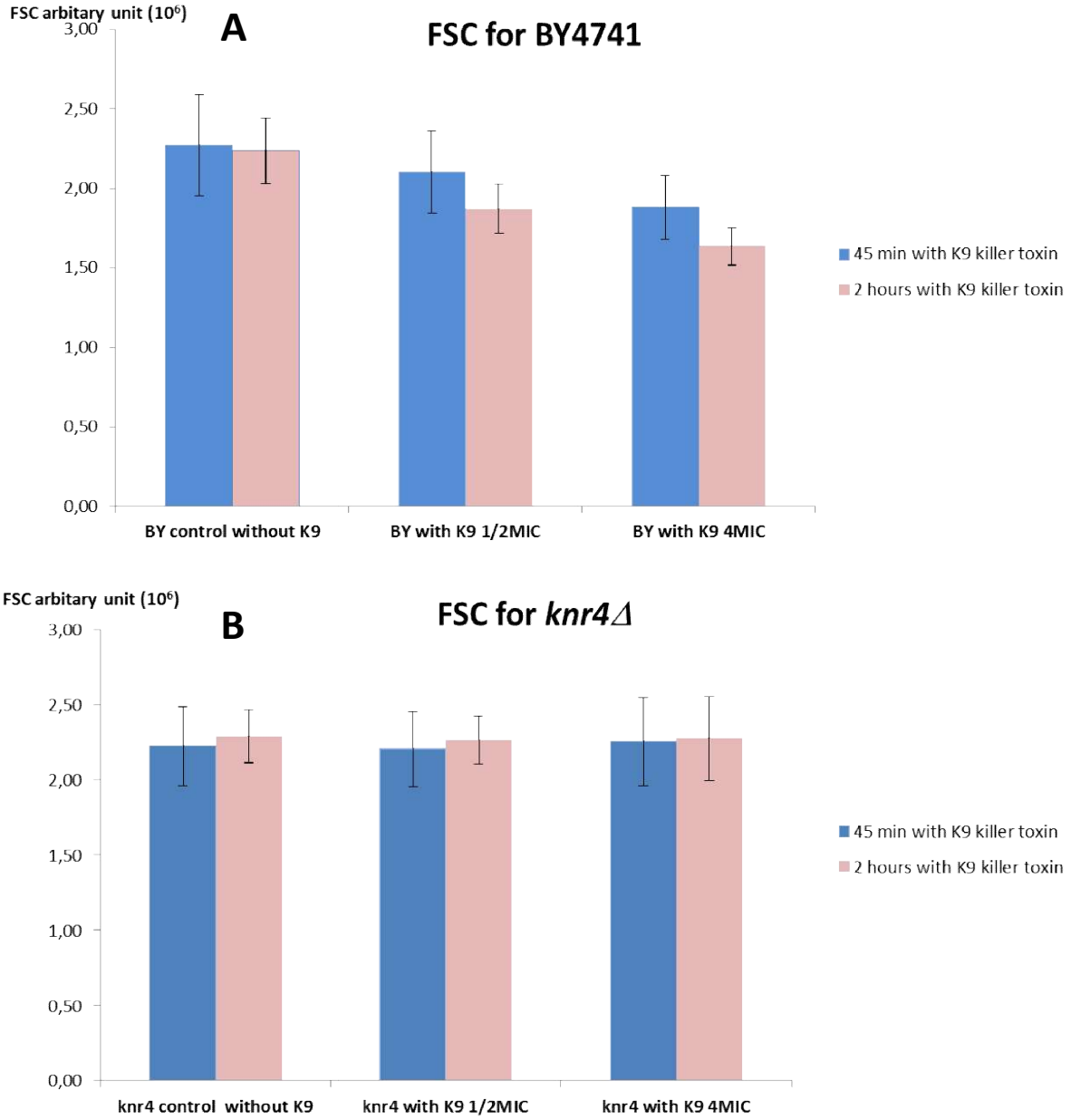
Supplementary Data



Supplementary data 1. K9 sensitivity halo test of *Saccharomyces cerevisiae* control strain BY4741 and knr4 mutants. (a) B74741, (b) *knr4Δ*, (c) *knr4ΔN*, (d) *knr4ΔC*and (e) *knr4ΔN+C*. K9 killer toxin solution was deposited on the plates as 3 spots of 30 µL.



Supplementary data 2. Cytometry Dot-plots. A-B: BY4741wt strain, C-D: *knr4Δ* mutant. Red color spots: cells treated by K9 1/2MIC; Black color: control cells without K9 treatment. A and C: obtained after 45min K9 treatment, B and D: obtained after 2 hours K9 treatment. The X-axe represents the FSC-H (10^6) whose value reflects the cells size. The Y-axe SSC-H (10^6) represents the granularity of the cells, correlated to their complexity.



Supplementary data 2. Cell size measurements (represented by FSC value). A is the FSC mean value of BY4741strain. B is the FSC mean value of the *knr4Δ*mutant. Means and errors bars were calculated from three independent experiments.