INSTITUT PASTEUR

Research in Microbiology 162 (2011) 375–381

www.elsevier.com/locate/resmic

Effects of S-(3,4-dichlorobenzyl) isothiourea on different cellular events in the cyanobacterium *Anabaena* sp. strain PCC 7120

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Received 7 November 2010; accepted 14 January 2011
Available online 12 February 2011

Abstract

S-(3, 4-dichlorobenzyl) isothiourea (A22) has been reported to specifically inhibit the function of MreB, an actin-like protein in rod-shaped bacteria. This study investigated the role of A22 in cyanobacterium *Anabaena* sp. strain PCC 7120, which can form nitrogen-fixing heterocysts under combined-nitrogen deprivation. Results indicated that A22 could inhibit cell growth, cause abnormal cellular morphology and bring about asymmetric cell division and irregular DNA distribution. However, A22 has little effect on heterocyst formation. An A22-resistant mutant named C23 was isolated by growing cells on A22-containing plates. It had normal appearance of cell shape, division and DNA content when treated by A22. However, this mutant retained a wild-type allele of *mreB*.

Keywords: S-(3, 4-dichlorobenzyl) isothiourea; Cellular morphology; Cell division; DNA distribution; A22-Resistant mutant

1. Introduction

S-(3,4-dichlorobenzyl) isothiourea (A22), a novel S-benzylisothiourea derivative, can induce cell morphology changes, division defects and irregular chromosome segregation in *Escherichia coli* and *Caulobacter crescentus* (Iwai et al., 2002; Gitai et al., 2005). The A22-resistant mutants obtained in *C. crescentus* and *E. coli* always carry a mutation in the *mreB* gene, which encode a highly conserved actin-like protein, known as a shape-determining protein, in rod-shaped bacteria. Therefore, MreB has proven to be the direct target of A22 (Gitai et al., 2005; Kruse et al., 2006). There is some evidence to support the fact that A22 causes dose-dependent alteration of cellular morphology by changing cell shapes into spherical or lemon-like ones in *C. crescentus,* an alteration qualitatively similar to that caused by *mreB* depletion (Figge et al., 2004; Gitai et al., 2004, 2005). Furthermore, in *C. crescentus*, treatment by A22 mirrors the phenotypes of MreB depletion, such as blocked cell division and chromosome segregation (Gitai et al., 2005). Both *mreB* conditional lethal mutants and cells treated with A22 also show defects in the chromosome segregation of *E. coli* (Hiraga et al., 1989; Kruse et al., 2003, 2005, 2006; Madabhushi and Marians, 2009). In *E. coli* and *C. crescentus*, MreB forms dynamic helical filament structures that move along the cell longitudinal axis or condense into a ring mid-cell (Shih et al., 2003; Shih and Rothfield, 2006; Figge et al., 2004; Gitai et al., 2004, 2005; Margolin, 2009). This dynamic pattern, which correlates with the role of MreB in cell division, is also delocalized by A22 rapidly and reversibly in *C. crescentus* (Gitai et al., 2005). All these results indicate that A22 might specifically inhibit MreB function, rapidly and reversibly. Inhibition by A22 of MreB results from its competition with ATP for binding to MreB and preventing MreB from polymerization (Bean et al., 2009). The assays of S-benzylisothiourea derivatives demonstrate that the S-benzylisothiourea structure is essential and sufficient for A22 function and the 3-and/or 4-chloro substitution of the S-benzyl group enhances its activity in *E. coli* (Iwai et al., 2004).

MreB depletion is usually lethal for bacteria such as *C. crescentus*, making it difficult to obtain *mreB* mutants and observes the phenotype (Figge et al., 2004; Gitai et al., 2004,

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doi:10.1016/j.resmic.2011.02.001

2005). MreB is the only known target of A22, so it is often used as a powerful tool for investigating MreB function in vivo. Recent studies indicate that A22 also prevents sidewall synthesis by depressing glycan strand synthesis during elongation. However, it is uncertain whether this effect of A22 is mediated by MreB or by another unknown target of A22 (Uehara and Park, 2008). *Anabaena* PCC 7120 is a nitrogen-fixing filamentous cyanobacterium. When the combined-nitrogen source is limited in the environment, heterocysts are formed to fix nitrogen in the air (Zhang et al., 2006). In *Anabaena* PCC 7120, cell morphology is affected by deletion or overexpression of MreB (Hu et al., 2007). However, no changes in cell division, chromosome partition, cell growth or heterocyst pattern formation are found in the *mreB* depletion mutant compared with wild-type cells (Hu et al., 2007), suggesting that these characteristics are independent of MreB in *Anabaena* PCC 7120. These results differ significantly from those observed in other rod bacteria, although the reason behind the difference remains to be understood. They also raise the question of whether A22 could have any effect in this cyanobacterium. This study aims to determine the effect of A22 in *Anabaena* sp. strain PCC 7120.

2. Materials and methods

2.1. Strain, growth conditions, and mutant isolation

Anabaena PCC 7120 was grown in either BG11 medium (with nitrate) or BG110 medium (without nitrate) at 28 °C. A22 was dissolved in methanol and stored at 10 mg ml^{-1}. The final concentration in liquid and solid media after cells were cultured to $OD_{750} \approx 0.2$ and A22 was added were 0, 0.5, 1.0, 1.5, 2.0 and 2.5 μg ml^{-1}. Pure methanol was the control sample. The growth curve was measured by monitoring the optical density at 750 nm.

To isolate A22-resistant mutants, cells were cultured to $OD_{750} \approx 0.2$; then 10^9 cells were plated on BG11, with each plate containing 6 μg ml^{-1} A22, and incubated at 28 °C until resistant colonies appeared.

2.2. Measurement of cell size and septum position

When cultures were grown in liquid BG11 medium to $OD_{750} \approx 0.2$, different volumes of A22 stock were added to final concentrations of 0.5 and 1.0 μg ml^{-1}, and the phenotype was observed under an optical microscope (Zeiss Axio Imager A1 with a Zeiss AxioCam HRc digital camera). Using ImageJ (Abramoff et al., 2004), the relative length and width of 500 cells were measured; the length and width of the cells were in arbitrary units. The lengths of over 100 dividing cells were measured to determine the position of the septum.

2.3. DAPI staining and measurement of DNA contents

To measure the effect of A22 on DNA distribution, cells were treated with 0.5 or 1.0 μg ml^{-1} A22 and incubated for 30 min in the presence of lysozyme (3 μg ml^{-1}). Cells were washed twice with PBS buffer (0.85% NaCl, 10 mM $Na_2HPO_4.2H_2O$, pH7.2) and then stained by DAPI (4′-6-diamidino-2-phenylindole, 1 μg ml^{-1}), a fluorescent dye that binds strongly to DNA, for 20 min at 4 °C. Two successive washing steps with PBS followed. Finally, the cells were resuspended in a small volume and observed under a fluorescence microscope (Zeiss Axio Imager A1 with a Zeiss AxioCam HRc digital camera). The images were analyzed with Image-Pro Plus.

3. Results

3.1. A22-affected cell growth and morphology of Anabaena PCC 7120

Bacteria show different tolerances to A22 inhibition, with Gram-negative bacteria tending to be more susceptible: The MIC is 3.1 μg ml^{-1} for *E. coli*, 2.5 μg ml^{-1} for *C. crescentus*, 12.5 μg ml^{-1} for *Staphylococus aureus*, and >100 μg ml^{-1} for *Bacillus subtilis* (Iwai et al., 2002; Shebelut et al., 2009). To determine the resistance level of *Anabaena* PCC 7120 to A22, growth curves of *Anabaena* cells growing under various dosages of A22 were observed. At 0.5 and 1.0 μg ml^{-1}, A22 significantly slowed cell growth. At 2.0 μg ml^{-1} and higher concentrations, A22 completely inhibited cell growth, whereas at 0.1 μg ml^{-1} A22 or pure methanol control, the growth rate was unperturbed (data not shown). These results suggest that A22 slows the growth of *Anabaena* cells in a dose-dependent pattern and that the MIC of A22 for *Anabaena* PCC 7120 is around 2.0 μg ml^{-1}.

The morphologies of *Anabaena* cells treated by various concentrations of A22 were recorded using an optical microscope to study the effects of A22 addition to morphological changes over time. The relative length and width of more than 500 cells were further measured with the program ImageJ (Abramoff et al., 2004). Significant morphological changes in the cells occurred after addition of 0.5 μg ml^{-1} A22 for 72 h; many cells significantly increased or decreased in size and were transformed into very irregular shapes (Fig. 1A, middle). The relative length and width differed from each other, whereas dimensions were similar in all untreated *Anabaena* cells because of the regular cell shape (Fig. 1B). With higher concentrations of A22 (i.e., 1.0 μg ml^{-1}), the cells did not exhibit more severe deformation, as expected. Most cells simply became bigger than the untreated cells and the proportions of treated cell length remained similar to those of untreated cells (Fig. 1, right). Treatment with 2.0 μg ml^{-1} or higher concentrations of A22 caused no obvious alterations in cellular morphology; the phenotype was the same as with treatment by 0.1 μg ml^{-1} A22 or pure methanol (data not shown). Because A22 only influenced the growing cell, cellular morphology alterations caused by addition of A22 usually took several days to be easily observed.

3.2. A22 interferes with symmetric cell division

After addition of 0.5 μg ml^{-1} A22, sizes of the cells within one filament varied greatly (Fig. 1A). Typically, if a cell along