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A proteome analysis of *Corynebacterium glutamicum* after exposure to the herbicide 2,4-dichlorophenoxy acetic acid (2,4-D)

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Abstract

The herbicide 2,4-dichlorophenoxy acetic acid (2,4-D) induces a wide spectrum of toxic responses in living organisms. In this study, we analyzed the stress-induced responses of *Corynebacterium glutamicum* cells on protein level upon treatment with 2,4-D. For this, growing *C. glutamicum* cells were exposed to sublethal concentrations of 2,4-D, and changes of the gene expression profiles in comparison to non-exposed organisms were analyzed by two-dimensional gel electrophoresis and mass spectrometry. 2,4-D induced the over-expression of at least six *C. glutamicum* proteins, four of which could be identified by MALDI-TOF-MS. One protein (Cg2521; long-chain acyl-CoA synthetase) was related to the energy metabolism, and two proteins were involved in cell envelope synthesis (Cg2410; glutamine-dependent amidotransferase, and Cg1672; glycosyltransferase). The last induced protein was the ABC type transport system (Cg2695, ATPase component). The newly observed proteins, except for the ABC transport system, were not in general stress-related proteins, but were specifically expressed upon 2,4-D exposure and, therefore, can be used as respective biomarkers. Moreover, since these proteins seem to play a pivotal role in the adaptation of the cell to 2,4-D, they may help to gain deeper insight into the damage mechanisms of 2,4-D induced in the living cell.

Keywords: *Corynebacterium glutamicum*; 2,4-dichlorophenoxy acetic acid; Stress response; Matrix assisted laser desorption; Ionization time-of-flight mass spectrometry; Two-dimensional gel electrophoresis

1. Introduction

The herbicide 2,4-dichlorophenoxy acetic acid (2,4-D) belongs to the chlorophenoxy group and became one of the most commonly used herbicides worldwide after it had been introduced in 1946 (www.24d.org). It can have negative effects on living organisms by damaging the DNA and plasma membrane. Toxic concentrations of 2,4-D can lead to apoptosis of human lymphocytes (Kaioumova et al., 2001), HepG2 cells (Tuschl and Schwab, 2003), and cerebellar granule cells (De Moliner et al., 2002). By entering the food chain of humans and animals, the herbicide can be associated with non-Hodkin's lymphoma and other cancers (Bradberry et al., 2000; McDuffie et al., 2001). Thus, a need exists to analyze and define the mode of action of this herbicide in living organisms. In general, biological tests are used to analyze the effectiveness of an environmental chemical, but may not provide sufficient information on identity and/or mode of action. Certain

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biomarkers may only cover a distinct area of application. For example, biomarkers for proteins observed by animal experiments can be considered specific only in dependence of the applied experimental parameters.

More promising for the evaluation of how a chemical behaves in the environment is the analysis of the global protein expression after exposure of living organisms to hazards (Hock and Seifert, 2002). It is long known that living cells respond to unfavorable environmental conditions with the expression of stress proteins. In most cases, such stress responses are specific and depend on the kind of evoked stress. The knowledge that specific protein patterns are induced in response to a defined stress has led to the suggestion that such proteins can be used in environmental analysis as indicators for the presence of certain chemicals (Möller et al., 2001; Bradley et al., 2002).

Studies in order to understand the toxic mechanism of 2,4-D in living cells have been performed using, e.g., *Saccharomyces cerevisiae* (Teixeira and Sa-Correia, 2002). The authors observed that the yeast produced many stress and heat-shock proteins during the adaptation period, and that eventually cell division occurred in the presence of 2,4-D. Another important work exposed *Delftia acidovorans* cells to 2,4-D (Benndorf and Babel, 2002). Surprisingly, the treatment did not induce heat-shock and oxidative stress proteins but others capable of degrading the herbicide and even to use it as a carbon source. Benndorf et al. (2006) worked with *Pseudomonas putida* KT2440 cells and observed numerous up- or down-regulated proteins and one new protein spot. The microorganism responded with several defense mechanisms, including energy conservation and catalyzation processes. In addition, some proteins (ABC transporters) participating in the transport mechanism were induced but no heat-shock related ones. Another microorganism used to investigate 2,4-D stress responses was *Burkholderia* sp. (Cho et al., 2000). The authors found the induction of two heat-shock proteins (Dnak, GroEL), and indicated the importance of them for the adaptation phase. Santos et al. (2004) applied pure phenol as stress factor to investigate its influence on *P. putida* KT2440. As demonstrated by this work, the microorganism effectively metabolized 2,4-D into phenol. It can therefore be assumed that the herbicide may induce similar effects in the microorganism targeted in our work, the *C. glutamicum*.

C. glutamicum is gram-positive and non-pathogenic, and is used for the industrial production of the amino acids lysine and glutamine (Leuchtenberger, 1996). The organism occurs in soil and, thus, serves well in environmental analysis to investigate, e.g., the influence of heavy metal residues on the ecosystem (Trajanovska et al., 1997; Hu et al., 2005). Besides of having a short generation time, the bacterium is safe to handle and its genome is known and available, which makes it very useful for conducting experiments in terms of hazardous exposure.

In this study, living *C. glutamicum* cells were used for the first time as test organisms to induce 2,4-D specific stress responses. For the purpose of identifying stress-related proteins that have the potential as putative biomarkers, *C. glutamicum* responses prior and after exposure to 2,4-D were investigated. The expressed proteins were analyzed with 2-dimensional gel electrophoresis (2-DE) and identified using matrix assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS) (Wildgruber et al., 2002; Drews et al., 2004). We focused on the expression of new protein spots and further on the stress mechanisms that these spots induced in living *C. glutamicum* cells.

2. Material and methods

2.1. Bacterial strain growth conditions

C. glutamicum cells were grown aerobically on a rotary shaker at 30 °C in brain heart infusion (BHI) medium, which was composed of D(+)-glucose, brain heart extract, peptone, sodium chloride and disodium hydrogen phosphate. To induce the herbicide stress, the BHI medium was supplemented with 0.45 mM of 2,4-D, and growth curves were followed by measuring the culture's optical density (OD) at 600 nm as shown in Fig. 1. Three independent experiments as biological replicates were performed to

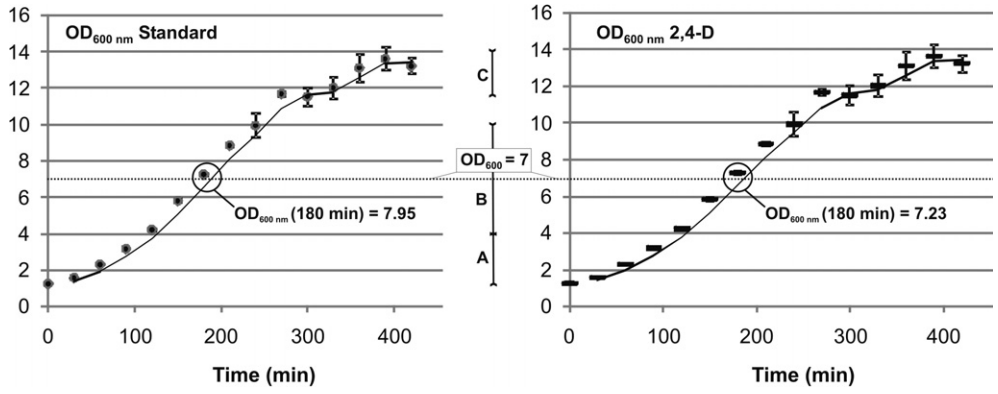

Fig. 1. Growth curves of *C. glutamicum* cells, comparing the standard without 2,4-D exposure and the population supplemented with 0.45 mM of the herbicide. (A) indicates to lag phase, (B) the log phase, and (C) the stationary phase. The dotted line shows the mid-log phase at which the cells were harvested. All $OD_{600\ nm}$ values include the standard deviation.