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A comprehensive proteomic analysis of totarol induced alterations in *Bacillus subtilis* by multipronged quantitative proteomics

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ABSTRACT

The rapid emergence of microbial drug resistance indicates the urgent need for development of new antimicrobial agents. Bacterial cell division machinery is considered as a promising antimicrobial target. Totarol is a naturally existing diterpenoid, which has the ability to restrain bacterial growth by perturbing the cell division. The present study was conducted to investigate the proteomic alterations in *Bacillus subtilis* as a consequence of totarol treatment to decipher its mechanism of action and possible molecular targets. Cellular proteome of the totarol treated *B. subtilis* AH75 strain was analyzed by using multiple complementary proteomic approaches. After the drug treatment, 12, 38 and 139 differentially expressed (1.5 fold change) proteins were identified using 2-DE, DIGE and iTRAQ analyses, respectively. *In silico* functional analysis of the identified differentially expressed proteins indicated a possible effect of totarol on the central metabolism for energy production, heme biosynthesis and chemotaxis. Interestingly, the primary dehydrogenases, which play a vital role in generating the reducing equivalent, were found to be repressed after totarol treatment indicating an apparent metabolic shutdown. Consequently, multiple cellular assays including resazurin assay and FACS analysis of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining confirmed the effect of totarol on respiratory activity and cellular metabolism.

Biological significance
The exact mechanism of action of totarol is still unclear and further investigations are essential to identify the molecular/cellular targets of this potential antimicrobial agent. The

Abbreviations: iTRAQ, isobaric tag for relative and absolute quantitation; DIGE, difference gel electrophoresis; CTC, 5-cyano-2,3-ditolyl tetrazolium chloride; FDR, false discovery rate; DAVID, Database for Annotation, Visualization and Integrated Discovery; KOBAS, KEGG Orthology Based Annotation System; MIC, minimum inhibitory concentration; DAPI, 4,6-diamidino-2-phenylindole; FACS, fluorescence-activated cell sorting.

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present study demonstrates the application of differential proteome to decipher the mechanism of action and molecular targets of totarol in *B. subtilis*. Our quantitative proteome analysis revealed that totarol induced alterations in the expression levels of 139 proteins (1.5 fold change and ≥2 peptides) in *B. subtilis*. Findings obtained from this study indicate that totarol treatment leads to metabolic shutdown by repressing the major central metabolic dehydrogenases in *B. subtilis*. In addition, expression levels of universal chaperone proteins, heme biosynthesis, and ribosomal proteins were found to be altered, which caused the filamentation of the bacteria. To the best of our knowledge, this is the foremost inclusive investigation describing totarol induced alterations in *B. subtilis* proteome and diverse physiological processes. We anticipate that this in depth proteomic study may contribute to a better understanding of the mode of action of totarol and its primary molecular and cellular targets.

1. Introduction

Infectious diseases are still the leading global health concerns; even though many synthetic as well as semi-synthetic effective drugs are in practice [1]. The journey of drug discovery started from the "natural products (NPs)" long ago during the ancient civilizations of India, China and other East Asian countries. During the last decade, the synthetic candidates have significantly replaced the NPs as antimicrobial drugs; at the same time excessive and indiscriminate usage of antibiotics has led to the unanticipated alterations in the microbial genome, which introduced a rapid spread of antibiotic-resistant strains. Consequently, the emergence of antibiotic-resistance highlighted again the need for comprehensive research on natural products for drug discovery due to their safe and secure applications [2]. Recently, regulators of cell division machinery have been studied extensively for identification of novel antimicrobial agents [3], and many natural compounds have been screened to evaluate their effects on bacterial cell division and growth.

Totarol is a natural diterpenoid extracted from the bark of *Podocarpus* family and possesses anti-microbial [4], anti-oxidant [5], cytotoxic [6], anti-fungal [7], anti-plasmodial [8] and anti-tumor activities [9]. Kubo et al. extracted six different diterpenoid products from the bark of *Podocarpus nagi*, investigated their effect against various microorganisms, and reported that totarol is the only active product with potential antimicrobial activity [10]. Subsequently, many researchers have actively focused on the elucidation of the mechanism of action of totarol and demonstrated multiple targets for this promising candidate including inhibition of the energy-coupled respiratory transport [11], prevention of peroxidation of unsaturated fatty acids in the lipid bilayer [5], and hampering the oxidative phosphorylation by acting either as an uncoupler or by inhibiting the crucial enzymes [12]. Recent studies indicated that totarol could also restrain the multidrug efflux pumps [13,14], disturb the phospholipid bilayer permeability [15], and inhibit bacterial cell division by perturbing the FtsZ polymerization [16]. However, the exact mechanism of action of totarol at the molecular level is still obscure, and further investigations are required to identify its molecular and cellular targets.

Quantitative analysis of bacterial proteome in the presence and absence of specific antimicrobial agents was found to be informative for identification of their molecular targets and unraveling their mechanism of action. In the present study, in order to evaluate the cellular effects of totarol, we have selected *Bacillus subtilis* as the model organism, since it is a widely studied non-pathogenic microorganism, whose genome sequence and physiological vegetative proteome in response to various stress conditions are already reported [17–24]. Here we aimed at deciphering the mechanism of action and possible molecular targets of totarol by using two dimensional electrophoresis (2-DE), 2D-differential in gel electrophoresis (2D-DIGE) and isobaric tags for relative and absolute quantitation (iTRAQ) analysis using LTQ-Orbitrap Velos, Q-TOF and MALDI-TOF/TOF mass spectrometers. Employing these complementary proteomic technologies, we were able to identify overall 1194 proteins (1% FDR). A total of 139 proteins were found to be differentially expressed (69 down-regulated and 70 up-regulated with a ≥1.5 fold change) and were considered for further analysis. *In silico* analysis involving the differentially expressed proteins indicated the modulation of glycolysis, TCA cycle and heme biosynthesis due to totarol treatment. Additionally, multiple cellular assays including resazurin-based metabolic activity assay and fluorescence-activated cell sorting (FACS) analysis of 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) staining for respiratory activity assay corroborated the effect of totarol on respiratory activity and cellular metabolism. To the best of our knowledge, this is the foremost inclusive investigation describing the totarol induced alterations in *B. subtilis* proteome.

2. Materials and methods

2.1. Growth curve analysis of *B. subtilis* under totarol treatment

The *B. subtilis* AH75 strain having a spectinomycin antibiotic marker was grown overnight at 37 °C in Luria Broth (LB) containing 100 μg/mL spectinomycin [25]. This culture was diluted with fresh LB media to maintain the OD_{600} at 0.05 and subsequently incubated again at 37 °C till the OD_{600} reached 0.1. *B. subtilis* growth was measured by monitoring the OD at 600 nm for the untreated control and IC_{50} (1.5 μM) and MIC (2 μM) totarol treated cultures at 37 °C. The growth curve was plotted with the mean values of triplicate experiments by measuring the OD at 600 nm at every 20 min intervals and continued till 300 min (mid-exponential phase).