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Antioxidant response of a novel *Streptomyces* sp. M3004 isolated from legume rhizosphere to H_2O_2 and paraquat

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Abstract

This paper aims to investigate the effect of H_2O_2 and paraquat on the activities of superoxide dismutase (SOD) and catalase (CAT), and membrane lipid peroxidation (LPO) levels in newly isolated *Streptomyces* sp. M3004. SOD activities of *Streptomyces* sp. M3004, grown in 10 mM and 30 mM H_2O_2, were significantly lower than the control cultures. On the other hand, as an antioxidant enzyme, CAT activity in both H_2O_2 treatment conditions increased significantly compared with the control. These activity values in 10 mM and 30 mM H_2O_2 treatment on the 48th hour of incubation were 3.8- and 6.6-fold higher than the control, respectively. SOD activity decreased significantly with respect to paraquat concentration, which was added at the start of the incubation. CAT activities increased significantly in 1.0 mM and 3.0 mM paraquat treatments compared to control. As an indicative marker of membrane damage, LPO levels of the novel isolate *Streptomyces* sp. M3004 treated with H_2O_2, and paraquat stress conditions were significantly higher than the control. Nevertheless, compared with the 30 mM H_2O_2 in both treatment conditions, LPO levels in 10 mM H_2O_2 were significantly higher. The decreases in SOD activities in paraquat and H_2O_2 treatment conditions resulted in the increases in the LPO levels although it increases in CAT activities.
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Keywords: *Streptomyces* sp.; Superoxide dismutase (SOD); Catalase (CAT); Lipid peroxidation (LPO); H_2O_2; Paraquat

1. Introduction

Filamentous soil bacteria, which belongs to the genus *Streptomyces*, are widely recognized as industrially important microorganisms because of their ability to produce many kinds of novel secondary metabolites, including antibiotics. In the course of screening for new metabolites, several studies were carried out in order to isolate new *Streptomyces* species from different habitats. Recent studies are focusing on the response of antioxidant system of bacteria, which is important in terms of biotechnology, such as *Streptomyces* growth in various oxidative stress conditions. The production of reactive oxygen species (ROS) in bacteria attack, almost, all cell components, including DNA, protein and lipid membrane [1,2]. This ROS, which includes the superoxide anion, hydrogen peroxide and the hydroxyl radical in bacteria, is mainly related to respiratory chain activity [3,4]. Organisms defend themselves against destructive ROS by non-enzymatic (Vitamins E and C or flavonoids and metal chelators) and enzymatic means (antioxidant enzymes). As an antioxidant enzyme, superoxide dismutase (SOD) catalyzes the dismutation of the superoxide radical (O_2^-) into hydrogen peroxide, which is subsequently metabolized by antioxidant enzymes catalase (CAT) or glutathione peroxidase (GPX). H_2O_2 is a small molecule, which is capable of diffusing through bacterial membranes—unlike the superoxide anion. In case any of these processes fails, O_2^- can interact with H_2O_2 to generate the highly reactive and destructive hydroxyl radical ($OH^{\bullet}$) by the Haber–Weiss reaction [5]. Newly reported researches, which show that oxidative stress leads to the appearance of at least 10 polypeptides which may be responsible for the adaptation to stress and cell responses to oxidative stress, have been investigated in detail [6–9]. Furthermore, a substantial overlap of different stresses has been detected in gene expression upon heat shock, oxidative stress and other stress conditions [10]. Most of the enzymes in bacteria are transcriptionally activated first by superoxide and subsequently by hydrogen peroxide. The study on the oxidative stress in bacteria is important for

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identification of antioxidative response mechanisms. In recent years, besides hydrogen peroxide interest in paraquat, as an oxidant, has increased. Paraquat is extremely toxic to living cells [11,12]. It is generally agreed that paraquat undergoes a redox cycling reaction whereby it is metabolized to the reduced paraquat radical intermediate, which reduces molecular oxygen to reactive oxygen species, specifically superoxide and peroxide radicals [13]. This, then, leads to the destruction of the cell wall and causes systemic toxicity [14,15]. Reported studies show that further research is necessary to explain the mechanisms of oxidative stress.

In the study, antioxidant response of *Streptomyces* sp., which is widely used in the productions of antibiotics as secondary metabolites, was investigated. To get insight into the role of the antioxidant response against H_2O_2 and paraquat stresses, SOD and CAT activities and LPO levels in relation to incubation time were studied on *Streptomyces* sp. M3004 isolated from Turkish soil.

2. Materials and methods

2.1. Isolation and numerical taxonomy

The soil samples were collected from the rhizosphere of nine different legumes in Turkey. The samples (3–5 g) were dried at room temperature to a constant weight and then heated at 105 °C for 5 min. A 10-fold dilution series was prepared for each sample and aliquots of each dilution plated onto starch casein agars [16] supplemented with filter-sterilized cycloheximide (50 μg ml^{-1}), nystatine (50 μg ml^{-1}) and rifampicin (0.5 μg ml^{-1}). The inoculated plates were incubated at 25 °C for 14 days. Three hundred representative isolates, putatively assigned to the genus *Streptomyces* on the basis of colony morphology, were subcultured onto modified Bennett's agar [17], incubated at 25 °C for 14 days and checked for purity by microscopic examination of Gram-stained smears. All purified isolates were transferred onto oatmeal (ISP3), inorganic salt starch (ISP4) and peptone-yeast extract-iron agar plates (ISP6), which were incubated at 25 °C for 14 days [18]. The ISP3 and ISP4 plates were examined by eye and aerial spore mass colour, substrate mycelium pigmentation and colour of any diffusible pigments were recorded using the National Bureau of Standards (NBS) Colour Name Charts [19,20]. The ISP6 plates were used to determine whether the isolates produced melanin pigments. The isolates were assigned to 21 multi-member groups and 37 isolates were chosen from the colour groups for numerical study.

Selected isolates were tested for numerical analysis using a total of 102 unit characters of morphological, pigmentation, physiological, nutrient, biochemical, degradation and antimicrobial activity tests together with seven type strains of *Streptomyces violaceusniger* clade members [21], 25 neutrophilic–mesophilic type strains, 37 rhizosphere isolates and seven randomly selected duplicate cultures. The test results were evaluated using simple matching coefficients [22] on the X-Taxon program, and clustering was achieved by using the UPGMA [23] algorithm by NTSys-pc program. The 69 test strains were assigned to three major (6 and 7 strains), five minor (2–4 strains) and 35 single-member clusters (SMC) at or above 85% similarity level. Members of the major cluster 3, which included *Streptomyces* sp. M3004, showed significant antimicrobial and enzymatic activities and could be differentiated from known type and reference strains at 86% similarity level. Therefore, *Streptomyces* sp. M3004 was chosen for molecular characterization [24].

2.2. Strains, maintenance and cultivation

The M3004 strain was maintained as a glycerol suspension (20%, v/v) at −20 °C. Single colonies of *Streptomyces* sp. M3004 were grown on dried starch-casein agar plates [25] supplemented with cycloheximide (50 μg ml^{-1}) nystatine (50 μg ml^{-1}) and filter sterilized rifampicin (0.5 μg ml^{-1}) for 7 days at 30 °C and the resultant biomass used to inoculate 50 ml of glucose–malt yeast extract (GMYE) broth in 100 ml conical flasks. The inoculated flask was shaken at 180 rpm at 25 °C or 30 °C for 7 days and an aliquot was also subcultured on GMYE agar plates to check culture purity. Biomass was harvested by centrifugation at 10,000 rpm for 10 min, washed twice with sterile TE buffer (Tris–HCl, pH 8.0; 10 mM; EDTA, 1 mM) [26] and stored at −20 °C. Approximately 100 mg wet weight biomass of organism was used for DNA extraction.

2.3. Extraction and purification of DNA

The guanidine thiocyanate DNA extraction procedure of Pitcher was used to isolate DNA from test strains [27]. Pretreatment of cells with proteinase K (100 μg/ml^{-1}) and sodium dodecyl sulphate (SDS; 2%, w/v) greatly facilitated the susceptibility of cells to the extraction procedure.

2.4. PCR amplification and sequencing of 16S rDNA

The 16S rRNA gene was amplified by PCR using a prokaryotic universal primers: p27f (5′-AGA GTT TGA TCC TGG CTC AG 3′) and p1525r (5′–AAG GAG GTG WTC CAR CC 3′) [28] and HotStar *Taq*® DNA polymerase (Qiagen). PCR amplification conditions used for thermal cycling (Thermo-Hybaid) were as follows: initial denaturation at 98 °C for 3 min, followed by 35 cycles consisting of denaturation at 94 °C for 1 min, primer annealing at 55 °C for 1 min and primer extension at 72 °C for 3 min. At the end of the cycles, the reaction mixture was kept at 72 °C for 10 min and then cooled to 4 °C.

Amplified 16S rRNA product was checked from 0.8% agarose gels by using the method of Heery et al. [21]. The PCR product was also purified with Ultrafree-MC column (Millipore). The purified product was directly sequenced by using a *Taq* DyeDeoxy Terminator cycle sequencing kit (PE Applied Biosystems) and analysed with an automatic sequence analyser (ABI PRISM™ 310 Genetic Analyser, PE Applied Biosystems). Sequencing primers were also used which included 125F (5′-GAACGGGTGAGTAACACGT-3′); 520F (5′-CAGCAGCCGCGGTAATAC-3′); 692F (5′-AATTCCTGGTGTAGCGGT-3′) and 926F (5′-AAACTCAAAGGAATTGACGG-3′) [29,30].

2.5. Phylogenetic analysis

A BLAST analysis (http://www.ncbi.nlm.nih.gov/BLAST/) against various sequence databases was used to identify strains related to strain *Streptomyces* sp. M3004. Sequence data for related species were retrieved from GenBank, and aligned manually using the AL16S program [31]. The evolutionary distance matrix was calculated according to the Jukes and Cantor method [32], and the phylogenetic tree was generated using the neighbour-joining method [33]. The PHYLIP package [34] was used for all the analyses. The TreeView program was also used to display, edit and print phylogenetic tree [35]. Analysed partial 16S rRNA sequence data were deposited to GenBank using SEQUIN program. The *Streptomyces* sp. M3004 sequence data was submitted to the GenBank public database and was assigned the accession number AY987376.

2.6. Media and growth conditions

Spore cultures of *Streptomyces* sp. M3004 were prepared by inoculating solid medium M65 which contained 4 g glucose, 4 g yeast extract, 10 g malt extract, 2 g $CaCO_3$, 12 g Agar, 20 g starch in 1 liter of ultra-pure water [36]. The basal chemically defined fermentation medium contained 0.6 g $MgSO_4{\cdot}7H_2O$, 3.5 g KH_2PO_4, 2.0 g asparagine, 10 g glycerol, 21.0 g 3-(*N*-morpholino) propanesulfonic acid (MOPS) buffer, and 1 ml trace salt solution (1.0 g $FeSO_4{\cdot}7H_2O$, 1.0 g $MnCl_2{\cdot}4H_2O$, 1.0 g $ZnSO_4{\cdot}H_2O$, 1.0 g $CaCl_2$) in 1 liter of ultra-pure water. The pH was adjusted to 7.0 before autoclaving. The cultures were inoculated with 2 ml spore suspensions and they were incubated with agitation at 150 rpm at 28 °C in 500 ml shaking flasks containing 50 ml of culture for 96 h. After the cultivation process, the cells were harvested by centrifugation followed by washing twice with distilled water and kept at −20 °C.

2.7. Preparation of cell-free extracts

For preparation of cell extracts, wet *Streptomyces* sp. M3004 cells were harvested by centrifugation, washed twice with 10 mM potassium-phosphate

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