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In Search of the *E. coli* Compounds that Change the Antibiotic Production Pattern of *Streptomyces coelicolor* During Inter-species Interaction

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ABSTRACT

The aim of this work was to investigate the interaction between *E.coli* and *Streptomyces coelicolor* A3 (2) for the increased production of undecylprodigiosin and identify the *E. coli* actives mediating this inter-species interaction. The antibiotics of interest were the red-pigmented undecylprodigiosin and blue-pigmented actinorhodin. Pure cultures of *S. coelicolor* in a defined medium produced higher concentrations of actinorhodin compared to those of undecylprodigiosin. The latter however, is more important due to its immunosuppressive and antitumor properties. As a strategy to increase undecylprodigiosin production, we added separately, live cells and heat-killed cells of *E. coli* C600, and the cell-free supernatant of *E. coli* culture to *S. coelicolor* cultures in shake flasks. The interaction with live cells of *E. coli* altered the antibiotic production pattern and undecylprodigiosin production was enhanced by 3.5-fold compared to the pure cultures of *S. coelicolor* and actinorhodin decreased by 15-fold. The heat-killed cells of *E. coli* however, had no effect on antibiotic production. In all cases, growth and glucose consumption of *S. coelicolor* remained almost the same as those observed in the pure culture indicating that the changes in antibiotic production were not due to nutritional stress. Results with cell-free supernatant of *E. coli* culture indicated that the interaction between *S. coelicolor* and *E. coli* was mediated via diffusible molecule(s). Using a set of extraction procedures and agar-well diffusion bioassays, we isolated and preliminarily identified a class of compounds. For the preliminary verification, we added the compound which was the common chemical structural moiety in this class of compounds to the pure *S. coelicolor* cultures. We observed similar effects on antibiotic production as with the live *E. coli* cells and their supernatant indicating that this class of compounds secreted by *E. coli* indeed could act as actives during interspecies interaction and increase the production of undecylprodigiosin.

1. Introduction

One of the healthcare challenges of the 21st century is the urgent need for the discovery and large-scale production of new antibiotics. Along with these, there is the continual search for other pharmaceuticals such as vaccines, antitumor, antiparasitic and antifungal compounds. Most laboratory research and all industrial production of antibiotics involve the use of pure cultures of the producing strains. Yet, in nature, microorganisms exist in populations of mixed cultures where complex inter-species interactions have evolved. Antibiotics are believed to be the result of such interactions inferring the producing species a competitive advantage over others sharing the same nutritional resources. The biosynthetic potential of microorganisms can therefore be masked in pure cultures. Inter-species interactions therefore, should be explored not only in the quest for the new pharmaceuticals but also in their large scale production.

Two-thirds of the clinically used antibiotics of natural origin are derived from streptomycetes which are Gram-positive, soil-dwelling bacteria. *Streptomyces coelicolor* A3(2) with a completely sequenced genome has long been established as a model research organism [1]. Two of the antibiotics it produces are pigmented; actinorhodin blue and undecylprodigiosin red, facilitating visual observation of their production. Actinorhodin is a weak antibiotic. Undecylprodigiosin on the other hand, is known to have immunosuppressive and anticancer properties in addition to the antimicrobial activities [2]. *S. coelicolor* however, produces mainly actinorhodin and low levels of undecylprodigiosin in defined

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media. Undecylprodigiosin is chemically similar to prodigiosin produced by *Serratia marcescens*. Strategies used in order to increase prodigiosin production included the addition of amino acids [3], sodium dodecyl sulfate [4] or some vegetable oils [5] to the media and optimisation of culture conditions [6]. As a departure from these, we wanted to investigate if any interaction of *S. coelicolor* with another soil-dwelling microorganism would elicit more undecylprodigiosin production.

E. coli is a Gram-negative, non-sporulating, facultatively anaerobic bacterium with a completely sequenced genome that is also widely used as a model organism. Although *E. coli*'s primary habitat is the gastrointestinal tract of mammals, its secondary habitat is soil [7,8]. It is highly likely that through their shared habitat, both *S. coelicolor* and *E. coli* would have developed interaction mechanisms.

Previously, we reported that in a bioreactor, when *S. coelicolor* was co-cultured with live *E. coli*, it changed its production pattern of these two antibiotics; production of undecylprodigiosin was increased and actinorhodin suppressed [9]. In this follow-up work, our aim was to investigate the nature of this interaction in a systematic manner. Specifically, one of the most important objectives was to identify the compound(s) mediating this interaction that led to the change in *S. coelicolor*'s antibiotic production pattern.

Inter-species interactions can induce the unexpressed biosynthetic pathways for new compounds as well as improve the productivity of the antibiotic-producing strains [10]. In this context, a new antibiotic, pestalone, was produced by a marine fungus *Pestalotia* sp. when challenged with some marine bacteria in a mixed fermentation [11]. Induction of the production of the blue pigment, pyocyanin, by *Pseudomonas aeroginosa* was observed in a co-culture with *Enterobacter* sp. [12]. In addition, interaction with some actinomycetes triggered the biosynthesis of secondary metabolites in *Aspergillus nidulans* [13]. Other researchers used biotic elicitors in order to increase secondary metabolite production [14].

The mechanisms of such interactions are still not known. An important step in the elucidation of the mechanism of any interspecies interaction is the identification of the elements such as the signalling or eliciting compound(s), the receptors, transcriptional factors and metabolic reaction pathways that take part in the interaction system. The starting point for such investigations can be the identification of the elicitor or signalling compound(s). In this work, we report for the first time that we isolated and identified a diffusible compound produced by *E. coli* which caused the observed changes in *S. coelicolor*'s antibiotic production.

2. Materials and methods

2.1. Microorganisms and cultivation

S. coelicolor A3 (2) MT 1110 was grown in a chemically defined liquid medium designed by Hobbs et al. [15]. The same medium was also used for the cultivation of *E. coli* C 600. Mannitol/soya flour (MS) [16] solid medium was used for the maintenance and spore stocks of *S. coelicolor*. LB agar was used for the maintenance of *E. coli*. In all experiments, the inocula of *S. coelicolor* and *E. coli* were used and prepared as described before [17,18]. Vegetative inoculum of *S. coelicolor* at 5% (v/v) level was inoculated in to 100 mL defined liquid medium with 4 glass beads of 0.4 cm diameter in a 500 mL flask. The shake flask experiments were then conducted using an orbital shaker at 200 rpm, at 30 °C over 7 days.

Live and heat-killed cells and cell-free culture supernatant of *E. coli* were then prepared as follows: a volume that would correspond to 2.5% (v/v) of the *S. coelicolor* culture was taken from the *E. coli* culture and centrifuged at 13000 rpm for 10 min. The supernatant was used for those experiments that tested the effect of the *E. coli* culture supernatant on *S. coelicolor*. The dead *E. coli* cells were obtained by heating the culture in boiling water for 30 min before separating by centrifugation at 13000 rpm for 10 min. Live and heat-killed cells of *E. coli* were then washed and re-suspended in normal saline with the adjusted cell counts of 1×10^7 cells/mL before addition to *S. coelicolor* cultures immediately after inoculation.

In the experiment where the effect of inter-species interaction on *E. coli* growth was investigated, for both pure and co-culture of *E. coli,* 2.5% (v/v) of an overnight grown culture, which contained approximately 1×10^5 cells/mL, was used as inoculum for 200 mL of the defined liquid medium in 500 mL flasks.

All experiments were accompanied with a pure culture of *S. coelicolor* which was referred to as the control, and each run was conducted either in triplicate or duplicate and the results were presented as the arithmetic average.

2.2. Analyses

Concentrations of actinorhodin and undecylprodigiosin were determined according to the previously described methods [19,20]. The growth of *S. coelicolor* in pure and interacted cultures was measured as the dry weight of cell material according to the method described before [21]. Growth of *E. coli* in the defined liquid medium was estimated as viable counts in both pure and mixed culture with *S. coelicolor* using LB agar. Analox glucose analysis machine (Beckman-RIIC Ltd.) was used to determine the glucose concentration.

2.3. Agar-well diffusion bioassay

The agar-well diffusion bioassay was used in order to monitor and track the compounds from the *E. coli* culture that elicited undecylprodigiosin production, as explained in the next section. MS agar plates were inoculated with *S. coelicolor* spore suspension and incubated at 30 °C for 30–35 h. Thereafter, a sterile cork-borer was used to make wells of 7 mm diameter on the surface of the agar plates. The bottom of each well was sealed with sterile 10%w/v agar. 200 μL of either cell-free supernatant of *E. coli* culture or samples from the isolation process of the elicitor was introduced into each well. If the samples from the isolation process were in ethyl acetate or methanol, they were then diluted with 50 mM tris–HCl, pH 7.2, 1 mM EDTA (sample to buffer ratio of 1:8) in order to reduce the toxicity of these solvents on *S. coelicolor*. The plates were then incubated at 30 °C for 2-3 days. The occurrence of the red pigment around the wells indicated the production of undecylprodigiosin and hence the presence of the elicitor molecule in the sample. The isolation of the elicitor from the *E. coli* culture is explained in the results and discussion section.

3. Results and discussion

3.1. Interaction of S. coelicolor with E. coli on solid medium

A preliminary investigation on the effect of *E. coli* on the antibiotic production by *S. coelicolor* was carried out by growing the two bacteria smeared adjacently on the same MS agar medium in a Petri dish. A significant stimulatory effect on the production of red-pigmented undecylprodigiosin by *S. coelicolor* was seen at the interface between *E. coli* and *S. coelicolor* biofilms as the dark border shown in Fig. 1, within three days of inoculation. This red-pigmentation was not observed on the rest of the *S. coelicolor* biofilm which was not in direct contact with *E. coli*. Furthermore, in the pure cultures of *S. coelicolor* on MS agar, there was no production of undecylprodigiosin even after ten days. Instead, with the