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Original article

Cold-active antibacterial and antifungal activities and antibiotic resistance of bacteria isolated from an alpine hydrocarbon-contaminated industrial site

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Abstract

Selection pressure in hydrocarbon-contaminated soils may lead not only to increased microbial resistance to antibiotics, but also to increased capacity of the soil indigenous population to produce antimicrobial compounds. Therefore, we studied the antibiotic resistance pattern and antibacterial and/or antifungal activities of 47 bacterial strains isolated from an industrial alpine site heavily polluted with petroleum hydrocarbons. Resistance to penicillin was more widespread (49%) than resistance to chloramphenicol or rifampicin (28%) or streptomycin (26%). Only 9% of the strains were resistant to tetracycline. The ability to produce cold-active (10 °C) antimicrobial compounds was tested by using human pathogenic bacteria (*Escherichia coli*, *Shigella flexneri*, *Salmonella enterica*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*) and yeasts (*Candida albicans*, *Cryptococcus neoformans*) as indicator microorganisms. About two-thirds of the 47 tested strains produced compounds that inhibited growth of at least one indicator microorganism. Six strains inhibited growth of both bacteria and yeast indicators; 12 and 16 strains showed either antibacterial or antifungal activity, respectively. The most versatile bacteria with regard to multiple antibiotic resistance and antimicrobial activity belonged to Actinobacteria or Gammaproteobacteria. The antimicrobial compounds produced by three *Pseudomonas* spp. and two *Serratia* spp. strains were characterized in more detail by TLC and HPLC. Depending on the sensitivity of growth inhibition to enzymes, the compounds produced by the three pseudomonads contained a proteinaceous component.

Keywords: Antibiotics; Antibacterial; Antifungal; Cold-active; Hydrocarbons

1. Introduction

Due to the continuous increase in antibiotic-resistant bacterial infections, development of multiple drug resistance among pathogens and the emergence of new infections, there is a need for novel and efficient antimicrobial compounds [1,2].

Microorganisms represent one of the largest resources for the discovery of novel medically useful components [3]. The exploitation of underexplored environments as a resource for isolation of useful novel bioactive compounds has been recognized as promising. Extremophiles which produce biomolecules under unusual conditions represent a valuable source of novel bioproducts including antimicrobial compounds [4–7]. The ability of psychrophiles to produce secondary metabolites with potential pharmaceutical interest has been well documented [8]. The development of novel antibacterial and antifungal compounds against drug-resistant pathogenic microorganisms has been claimed as one of the core foci of research and development interest related to Arctic and Antarctic genetic resources [9]. However, cold-active antagonistic properties of microorganisms able to thrive in cold environments have not been investigated as extensively as those of mesophiles [10]. Microbial producers of cold-active antimicrobial compounds have been isolated from various aquatic and terrestrial environments in

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Argentina, the Arctic and the Antarctic [6,11–13], but have not yet been studied in alpine areas. Most of the described antimicrobial compounds are active against bacteria; cold-active activity against yeasts is rarely reported [12,13].

In the natural environment, antibiotics and other secondary metabolites have multiple functions related to the survival of microorganisms, e.g. conferring a competitive survival advantage [14–16]. The production of antimicrobial compounds under severe environmental conditions could be a particular advantage for reducing interspecies competition [10]. Little is known on the role of microorganisms from hydrocarbon-polluted environments as producers of antagonistic compounds. Máthé et al. [17] described a significant correlation between antibiotic resistance and the hydrocarbon biodegradation potential of bacteria isolated from long-term-contaminated soils. Hydrocarbon-tolerant bacteria isolated from hydrocarbon-contaminated sites were characterized by multidrug resistance [18,19]. Since genes responsible for the degradation of hydrocarbons (catabolic genes) and for antibiotic and heavy metal resistance are located on plasmids, horizontal gene transfer may promote multiple resistance. In the absence of selection pressure, such as exposure to hydrocarbon contamination, the indigenous microbial population is characterized by a lower biodegradation potential and tolerance to antibiotics [17] and antibiotic resistance is primarily exhibited toward only a single antibiotic [20].

We hypothesized that the selection pressure present in hydrocarbon-contaminated soils may lead to increased microbial resistance to antibiotics, but also to increased ability of the soil indigenous population to produce antimicrobial compounds. In order to confirm this hypothesis, we evaluated the antibiotic resistance pattern and antibacterial and/or antifungal activities of 47 culturable bacterial strains isolated from an industrial alpine site strongly polluted with petroleum hydrocarbons (from a hydrocarbon-contaminated industrial alpine site). These strains are subjected to multiple extremes, i.e. the presence of contamination and low temperatures in their natural environment. To the best of our knowledge, microorganisms from such environments have not yet been studied for their potential to produce antagonistic compounds.

2. Materials and methods

2.1. Bacterial strains: isolation, identification and cultivation

The bacterial strains used in this study were isolated from hydrocarbon-contaminated alpine soil collected from a former industrial district in Bozen, South Tyrol, Italy. Leakage from heavy oil storage tanks was the main reason for contamination. The soil contained 13,300 mg hydrocarbons kg^{-1} dry soil; 40% and 60% of this contamination consisted of C_{10}–C_{20} and C_{20}–C_{40} hydrocarbons, respectively, which points to a high content in heavy oil. At the time of sampling, the mean soil temperature in the sampling area was 8–10 °C [21].

The isolation and identification of the culturable bacterial strains used in this study are described in detail in Zhang et al. [21]. Among 73 isolates, 48 strains were identified as unique based on phenotypic characteristics (colony morphology, pigmentation, growth properties) and 16S rRNA gene sequencing. GenBank accession numbers of these strains are GQ161991, FJ948107, GQ240227, GQ246952, GQ246953, GQ240228, GQ131578, FJ972171, GQ131577, GQ131579, GQ161990 and HQ588828–HQ588864 [21].

In this study, 47 of these strains (one *Bacillus* strain became inviable at the beginning of the study) were tested for their antibiotic resistance pattern and for their antibacterial and/or antifungal activities at low temperatures. Strains were routinely cultured on R2A agar (yeast extract 0.05%, glucose 0.05%, starch 0.05%, bacto tryptone 0.05%, sodium pyruvate 0.03%, K_2HPO_4 0.03%, $MgSO_4 \times 7H_2O$ 0.005%, pH 7) and maintained as a suspension in skimmed milk (10%, w/v) at −80 °C.

2.2. Resistance to antibiotics

Susceptibility of the 47 strains to antibiotics was determined on R2A agar supplemented with penicillin, streptomycin, rifampicin, tetracycline and chloramphenicol (30 μg ml^{-1}). These antibiotics belong to different classes according to their structures. Resistance or susceptibility to antibiotics was evaluated after 7–10 days of incubation at 10 °C. Growth on R2A agar without antibiotics was used as control. Two replicates were prepared per strain and antibiotic.

2.3. Indicator microorganisms for screening for production of antimicrobial compounds

Five bacteria (*Escherichia coli* DSM30083, *Shigella flexneri* DSM4782, *Salmonella enterica* DSM9274, *Pseudomonas aeruginosa* DSM1117, *Staphylococcus aureus* DSM683) and two yeasts (*Candida albicans* DSM1386 and *Cryptococcus neoformans* DSM11959) were used as human pathogenic indicator microorganisms. They represented different taxa and different types of infections. The bacteria were grown in LB medium (bacto tryptone 1%, yeast extract.5%, NaCl 0.5%, pH 7.0) at 30 °C for 24 h. The yeasts were cultured in Sabouraud-2 %-glucose medium (peptone from meat 0.5%, peptone from casein 0.5%, glucose 20%, pH 5.6) at 25 °C for 48 h.

2.4. Screening for antimicrobial activities

The 47 strains were grown in R2A broth at 10 °C and 150 rpm until the stationary growth phase was reached. Growth was monitored by measuring OD600. Production of antimicrobial compounds was evaluated by an agar diffusion assay. Two replicates per test strain and indicator microorganism were used and sterile R2A broth was used as control. The indicator microorganisms (10^6 cells, i.e. 0.1 ml of a cell suspension containing 10^7 cells ml^{-1}) were spread on LB plates (bacterial indicator strains) or Sabouraud plates (yeast indicator strains). After 15 min, 4–6 sterile cellulose filter discs (diameter 12.7 mm; Rotilabo, Carl Roth) were placed on each plate, and 50 μl of producer cells (pre-grown in R2A

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