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Preliminary data on antibacterial activity of *Echinacea purpurea*-associated bacterial communities against *Burkholderia cepacia* complex strains, opportunistic pathogens of Cystic Fibrosis patients

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ABSTRACT

Burkholderia cepacia complex bacteria (Bcc) represent a serious threat for immune-compromised patient affected by Cystic Fibrosis (CF) since they are resistant to many substances and to most antibiotics. For this reason, the research of new natural compounds able to inhibit the growth of Bcc strains has raised new interest during the last years. A source of such natural compounds is represented by medicinal plants and, in particular, by bacterial communities associated with these plants able to produce molecules with antimicrobial activity. In this work, a panel of 151 (endophytic) bacteria isolated from three different compartments (rhizospheric soil, roots, and stem/leaves) of the medicinal plant *Echinacea purpurea* were tested (using the cross-streak method) for their ability to inhibit the growth of 10 Bcc strains. Data obtained revealed that bacteria isolated from the roots of *E. purpurea* are the most active in the inhibition of Bcc strains, followed by bacteria isolated from the rhizospheric soil, and endophytes from stem/leaf compartment. At the same time, Bcc strains of environmental origin showed a higher resistance toward inhibition than the Bcc strains with clinical (i.e. CF patients) origin. Differences in the inhibition activity of *E. purpurea*-associated bacteria are mainly linked to the environment –the plant compartment- rather than to their taxonomical position.

1. Introduction

The genus *Burkholderia* (Yabuuchi et al., 1992) comprises more than 60 species isolated from diverse ecological niches (Coenye and Vandamme, 2003). *Burkholderia* species are traditionally known as plant pathogens and soil bacteria, but this genus includes also human and animal pathogens such as *B. mallei* and *B. pseudomallei*. Several *Burkholderia* species may have both pathogenic and symbiotic interactions with plants, and some of them are human opportunistic pathogens. Some of *Burkholderia* sp., including members of the *Burkholderia cepacia* complex (Bcc), are serious threats for specific immune-compromised patient, especially those affected by Cystic Fibrosis (CF) and chronic granulomatous disease (CGD, Mahenthiralingam et al., 2008). The Bcc represents a heterogeneous group of bacteria since it includes microorganisms that are widespread in the environment and can colonize very different ecological niches, such as the respiratory tract of humans, soil, and the rhizosphere of many plants (e.g. Hallmann et al., 1999; Parke and Gurian-Sherman, 2001). Bcc bacteria are resistant to many clinically used antibiotics (e.g. Aaron et al., 2000; Nzula et al., 2002; Turner et al., 1998), and this is probably due to their ability to adapt to different environmental conditions, and to the fact that they can live in different ecological niches, often in beneficial or pathogenic association with other organisms. Studies on the model

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system *B. cenocepacia*, that is the first Bcc species that was isolated from CF patients, highlighted how the multi-drug resistant (MDR) Bcc bacteria are able to resist to different antibiotic classes (e.g. polymyxins, aminoglycosides and most β-lactams) and to all antibiotics belonging to at least two out of three classes (Saiman and Siegel, 2003). This is mainly due to different molecular mechanisms including enzymatic inactivation, alteration of drug targets, cell wall impermeability and active efflux pumps (Burns, 2007), with the latter representing a system for the extrusion of toxic compounds. In addition to this, the exposure of Bcc population to a given antibiotic may also result in the appearance of spontaneous resistant mutants. Therefore, in the last decades, the growing interest in discovering new antimicrobial compounds able to avoid the emergence of resistant mutants, has led many researchers to move in the field of natural compounds, and in particular, to find new antimicrobial compounds from unusual sources (bioprospecting) as marine metazoans (Blunt et al., 2004), or microorganisms from extreme environments, like Antarctica (Papaleo et al., 2012). However, one of the most interesting and promising source of antimicrobial compounds is represented by medicinal plants (e.g. Freires et al., 2015; Altintas et al., 2013; Martins et al., 2015). One of the most known and used medicinal plant is *Echinacea purpurea* (family Asteraceae), which is widely used since the last century to treat common diseases (for a review see Hudson, 2012), and whose essential oil (EO) shows antimicrobial activity (e.g. Sharma et al., 2010). The hypothesis that the medicinal properties, including the antimicrobial effect, of EOs might be due to the presence of plant-associated bacteria, has been recently proposed by Emiliani et al. (2014). Even though in the last decades many studies were performed on endophytic bacterial communities isolated from different plants, few information is available on endophytic bacteria inhabiting internal tissues of medicinal plants. Recently, studies of bacterial endophytes from medicinal plants representing a potential reservoir of important bioactive molecules have been performed (Emiliani et al., 2014; Chiellini et al., 2014; Mengoni et al., 2014; Maida et al., 2015). The antimicrobial activity of endophytes from medicinal plants has been widely studied on fungi (e.g. Vieira et al., 2014; Tolulope et al., 2015; Yadav et al., 2014). However, to the best of our knowledge, no reports are available about the antimicrobial activity of bacterial endophytes from medicinal plants and the possible contribution of bacterial endophytes to plant bioactive molecules. Even though fungal endophytes have been already studied in *Echinacea* sp. (Rosa et al., 2012), studies on antimicrobial activity of bacterial endophytes isolated from *E. purpurea* have not been reported up to now.

Previous studies on endophytic bacterial communities of different compartment of *E. purpurea* highlighted that bacteria isolated from different plant compartments belong to different taxa and have also a differential antibiotic resistance pattern and antagonistic activity (Chiellini et al., 2014; Mengoni et al., 2014; Maida et al., 2015). Consequently, *E. purpurea* bacterial endophytes may indeed produce substances which can be effective against human bacterial pathogens, including members of Bcc. Then, the aim of this work was to check whether the *E. purpurea*-associated bacteria might interfere with the growth of a panel of 10 Bcc strains, to pave the way to the possible identification of (new) natural antibiotics.

2. Materials and methods

2.1. Bacterial strains and growth conditions

E. purpurea-associated bacteria have been isolated and characterized from three different plant compartments –stem/leaves (S/L), roots (R) and rhizospheric soil (RS)- starting from a pool of 5 *E. purpurea* plants, as described in Chiellini et al. (2014). The anatomical part of the plants, (roots and stems/leaves) were considered as independent samples. Roots from five individual *E. purpurea* plants were grouped and pooled, and the same was performed for stems/leaves. Two grams of fresh tissue from each pool was surface-sterilized with 1% HClO and then washed three times with sterile water to remove the epiphytic bacteria. After that, samples were homogeneously pottered in a sterile mortar by adding 2 ml of 0.9% NaCl (Sigma Aldrich, USA). 100-μl samples of tissue extracts and their different dilutions were plated in triplicate. Rhizospheric soil (RS) from the five plants were treated separately at room temperature for 1 h with 20 ml of 10 mM Mg_2SO_4 to detach the bacteria from soil particles. After sedimentation, 100-μl samples of the supernatant at different dilutions were plated in triplicate. Endophytic and rhizospheric bacteria were grown on solid tryptone soya broth (TSB) medium (Biorad, CA, USA) at 30 °C for 48 h. From each sample, about 100 colonies were randomly selected, individually plated onto solid TSB Petri dishes and stored at −80 °C for the analysis.

A subset composed by 151 bacterial strains (52 from S/L, 54 from R and 45 from RS) was used in this work (Table 1S, Supporting information). The panel of Bcc strains used in this work with either environmental (4 strains) or clinical (6 strains) origin is shown in Table 1.

Table 1
List of bacterial strains belonging to the *Burkholderia cepacia complex* (Bcc) used in this work.

Species	Strain	Origin	Reference
B. cepacia	LMG 1222	Environmental (Plant-derived foodstuff, onion- *Allium cepa*, 1948)	Yabuuchi et al., 1993
B. cepacia	FCF 3	Cystic Fibrosis	Tabacchioni et al., 2008
B. ambifaria	LMG 19182	Environmental (Pea rhizosphere, Wisconsin, 1985)	Coenye et al., 2001
B. multivorans	LMG 17588	Environmental (Soil, USA)	Mahenthiralingam et al., 2000
B. multivorans	LMG 13010	Cystic Fibrosis	Vandamme et al., 1997
B. cenocepacia	LMG 19230	Environmental (Wheat root endophyte –Kapunda, Australia)	Balandreau et al., 2001
B. cenocepacia	LMG 16656	Cystic Fibrosis	Vandamme et al., 2003
B. cenocepacia	LMG 24506	Cystic Fibrosis	Vandamme et al., 2003
B. cenocepacia	FCF 23	Cystic Fibrosis	Tabacchioni et al., 2008
B. cenocepacia	LMG 21462	Cystic Fibrosis	Mahenthiralingam et al., 2000

Bacteria associated with the medicinal plants were grown on tryptone soy agar medium (TSA, Biorad, CA, U.S.A.) for 48 h at 30 °C. Bcc strains were grown on Luria Bertani (LB) medium for 48 h at 37 °C.

2.2. Analysis on strains belonging to genera Pseudomonas and Staphylococcus: species assignments and data comparison

The seqmatch tool of the Ribosomal Database Project (RDP) database (Cole et al., 2014) has been used to assign a species to each *Pseudomonas* and *Staphylococcus* strain.

The 12 strains attributed to *Staphylococcus* sp. genus (8%), the second most represented among all the analyzed bacterial strains, were also analyzed to assess the presence/absence of possible human pathogens inside the plant.

Sixty-seven sequences belonging to *Pseudomonas* sp. strains (44.4% of the total), have been assigned to Operational Taxonomic Units (OTUs) at 0.03 and 0.01 cutoff levels (respectively 1% and 3%