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A novel property of propolis (bee glue): Anti-pathogenic activity by inhibition of *N*-acyl-homoserine lactone mediated signaling in bacteria

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ABSTRACT

Ethnopharmacological Relevance: An alternative approach to antibiotics is the development of anti-pathogenic agents to control the bacterial virulome. Such anti-pathogenic agents could target a phenomena known as quorum sensing (QS).
Materials and methods: Six bacterial *N*-acyl-homoserine lactone (AHL)-dependent bioreporter strains were used to evaluate if bee hive glue also known as propolis contains constituents capable of inhibiting QS-controlled AHL signaling. In addition, the effect of propolis on the QS-dependent swarming motility was evaluated with the opportunisitic pathogen, *Pseudomonas aeruginosa*.
Results: Differences in the propolis tincture samples were identified by physiochemical profiles and absorption spectra. Propolis tinctures at 0.0005% (v/v) that do not affect bacteria biosensor growth or the reporter system monitored were exposed to biosensors with and without the addition an AHL. No AHL signal mimics were found to be present in the propolis tinctures. However, when propolis and an inducer AHL signal were together exposed to five *Escherichia coli* and a *Chromobacterium violaceum* biosensor, propolis disrupted the QS bacterial signaling system in liquid- and agar-based bioassays and in C_{18} reverse-phase thin-layer plate assays. Swarming motility in the opportunistic pathogen, *Pseudomonas aeruginosa* PAO1 and its AHL-dependent LasR- and RhlR-based QS behaviors were also inhibited by propolis.
Conclusions: Together, we present evidence that propolis contain compounds that suppress QS responses. In this regard, anti-pathogenic compounds from bee harvested propolis could be identified and isolated and thus will be valuable for the further development of therapeutics to disrupt QS signaling systems which regulate the virulome in many pathogenic bacteria.

1. Introduction

The word propolis, used in Ancient Greece as: *pro* for infront of or at entrance to and *polis* for city or community. It is a chemically complex substance collected by honeybees (*Apis mellifera*) from cracks in bark and leaf buds of regional macroflora (Ghisalberti, 1979; Burdock, 1998). Propolis is a plant resinous substance masticated with bee salivary enzymes and mixed with beeswax. Bees use propolis for coating the hive to increase strength, i.e. it is also called bee-glue; in blocking holes and cracks in the hive and to adversely affect hive-invading microbes and insects. The chemical constitutents in propolis are related to bud and wound exudates collected and modified by bees from various flora (Burdock, 1998).

The propolis origin reflects the tree populations in any one region: popular (*Populus* spp.), alder (*Alnus glutinosa*), horse chestnut (*Aesculus hippocastanum*), birch (*Betula alba*), beech (*Fagus sylvatica*), and various conifer species (Bankova et al., 2000; Bankova, 2005). In Brazil, propolis samples have been classified into 13 types based on physiochemical characterization with certain propolis types containing profiles similar to the resinous secretions from certain regional flora (Silici and Kutluca, 2005; Daugsch et al., 2007). In general, bee glue is composed of resin and balsam (50%), wax (30%), essential and aromatic oils (10%), pollen (5%) and other substances such as organic material (Burdock, 1998). More than 200 compounds have been identified in different propolis samples (Casaldo and Capasso, 2002; Silici and Kutluca, 2005; Viuda-Martos et al., 2008). Flavonoids, aromatic acids, diterpenic acids and phenolic compounds are thought to be the principle constitutents responsible for the bioactivity of propolis (Viuda-Martos et al., 2008). Propolis has been used in traditional medicine due to its biological properties such as: antibacterial, anticavity, antitumor, antioxidant, antiviral, antiinflammatory, and immunomodulatory effects, among other beneficial attributes (Viuda-Martos et al.,

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2008). Thus, propolis is a rich natural product to explore for novel therapuetic molecules.

In bacteria, the expression of certain bacterial genes, including the virulome or total complement of virulence genes, frequently depends on the cell density of the population. This phenomenon, termed quorum-sensing (QS), is mediated by specific molecules called *N*-acyl-homoserine lactone (AHL) QS signals also known as autoinducers (White and Winans, 2007; Lowry et al., 2008; Atkinson and Williams, 2009). The specificity of AHLs is determined by the acyl side chain length, degree of its saturation and by the presence or lack of an oxo-, or hydroxy-group in the C3 position (Camilli and Bassler, 2006; Scott et al., 2006). AHLs are characterized as long- or short-chain AHLs depending on whether their acyl moiety consist of >8 or ≤8 carbons, respectively. Examples of bacterial phenotypes controlled by QS include biofilm formation, swarming motility, virulence factor expression, bioluminescence and other traits (Lowry et al., 2008; Atkinson and Williams, 2009). In this scenario, the QS mechanism in bacteria enable the regulation of gene expression and coordinated functions beneficial only when carried out by a large number of bacterial cells.

QS plays a intergral role in host-microbial interactions in animals, marine systems and in some organisms that cause plant diseases. Over 68 Gram-negative bacterial species contain at least one complete AHL QS circuit. This includes the QS core proteins known as LuxI-type protein (the AHL synthase), and a LuxR-type protein (the response regulator or receptor) (Case et al., 2008; Atkinson and Williams, 2009). The manipulation of the regulation of QS may provide alternative and novel therapeutic approaches against bacterial pathogens (Rasmussen and Givskov, 2006a,b; Dobretsov et al., 2009). Details on the biochemical and molecular mechanism underlying QS regulation and developing approaches to manipulate QS regulated behaviors in bacteria have recently been investigated (Rice et al., 2005; McDougald et al., 2007; Dobretsov et al., 2009).

One approach to treat antibiotic resistant bacteria is by the development of new mechanisms of antipathogenic treatments that act to attenuate the expression of virulence, which is less likely to impose a selection pressure for development of resistance (Rasmussen and Givskov, 2006a,b; Rasko and Sperandio, 2010). A strategy to develop novel antipathogenic treatments is by blocking the cell-to-cell communication mediated by QS systems. Over the last few years, many natural and synthetic compounds have been identified as QS disruptors (Rasmussen and Givskov, 2006a,b; Dobretsov et al., 2009; Rasko and Sperandio, 2010). The majority of the QS disruptor compounds have been identified and shown to inhibit QS signaling in screens using one response regulator (receptor) and thus may limit the application to a single QS receptor system. A preliminary screening of natural and synthetic libraries for inhibitors revealed that propolis displayed a qualitative inhibitor activity based on a LuxR-dependent system (Rasmussen et al., 2005).

In this work, we investigate the effects of propolis on its ability to agonize or antagonize QS-regulated responses in six AHL-signal-dependent reporter strains, each containing different LuxR-homolog receptor. We show using forward assays that the propolis at concentrations that are not inhibitory to bacterial biosensor(s) growth do not contain AHL signal mimics. However, using reverse assays, propolis inhibits the QS mechanism in agar- and liquid-based assays and in reverse-phase thin-layer chromatography coupled to bioreporter overlaid detection. We show that propolis that differ in their region of origin, physiochemical profile and absorption spectrum exhibit different QS inhibitory responses and that this response depends on the receptor protein.

Moreover, we shown that propolis inhibited swarming motility in the opportunistic pathogen *Pseudomonas aeruginosa* PA01. Propolis also inhibited the AHL-dependent *Pseudomonas aeruginosa* PAO1 LasR and RhlR receptors in biosensor assays paired with near-isogenic strains lacking the corresponding LuxR-type receptor.

2. Materials and methods

2.1. Commercial tincture and raw propolis

Five commercial sources of propolis tincture were used in this study. Propolis A, a 65% extract produced by Natural Factors and a product of Canada. Propolis B, a 50% extract produced by Soria Natural and a product of Spain. Propolis C, a 40% extract produced by Gol-Natur and a product of Italy. Propolis D, a 50% extract produced by Beehive Botanicals and Propolis E, a 70% produced by Y.S. Organic Bee Farms and both products of USA. HFP was extracted in 70% ethanol to produce a tincture (Isla et al., 2005).

2.2. Bacterial strains and growth conditions

Escherichia coli strains JM109 and JLD 271, *Chromobacterium violaceum* CV026, and *Pseudomonas aeruginosa* PA01 were grown in Luria-Bertani (LB) (Winson et al., 1998; Lindsay and Ahmer, 2005; Scott et al., 2006; Shrout et al., 2006; McClean et al., 1997). Each bacterial bioreporter strain used in this work is listed in Table 1 along with its AHL receptor protein and cognate AHL signal. All media were solidified with 16 g of Bacto Agar (Difco) per liter. For strain selection, media were supplemented with the following antibiotics: ampicillin (100 μg/ml), gentamicin (30 μg/ml), kanamycin (50 μg/ml), tetracycline (10 μg/ml) for *Escherichia coli*.

2.3. Effects of propolis on biosensor growth and the reporter (luciferase) system

An overnight liquid culture of JM109(pSB401) growing in LB was diluted 1:5 three hours before the start of the assay. Each experimental sample contained: 0.0005% (v/v) of the respective propolis (Tincture A–E) placed on sterile cellulose disks, 50 nM 3-O-C6 HSL, and JM109(pSB401) diluted to a starting $OD_{600\,nm}$ = 0.05. In the place of the propolis, the control group contained blank sterile disks. Data for the control and experimental groups represents the average of three replicates. After 0, 3, 6, 9, 12, 21, and 24 h incubation (grown at 30 °C while shaking at 150 rpm) $OD_{600\,nm}$ measurements were made. To evaluate the effects of propolis on the luciferase system, JM109 (pTIM2442), which constitutely produces luciferase at high levels (Alagely et al., 2010) was grown in the presence of the propolis tinctures at 0.0005% (v/v) and relative light units were measured at 0, 5 and 24 of growth using a Turner 20/20 Luminometer at a sensitivity setting of 39.9.

To test the effect of the propolis tinctures on the growth of biosensor *Chromobacterium violaceum* CV026, cellulose discs of 6 mm in diameter were impreganted with propolis tinctures A, B, C, D, and E and place on the surface of a soft-agar plate seeded with strain CV026. Ten microliter of propolis concentrations ranging from full strength to 10^{-4} dilutions of the five tinctures were prepared in 70% EtOH and applied to the discs. After 24 and 48 h of incubation the zone of inhibition as observed as a transparent zone directly adjacent to the disc was measured.

2.4. Inhibition and stimulation of bacterial QS by propolis tincture

We used bacterial reporter strains to test for compounds present in commercial propolis tincture samples. To determine if propolis contains molecules with varying specificity, we first used five different bacterial biosensors that differed by their LuxR homolog receptor (Table 1). The presence of molecules in propolis that stimulate or inhibit QS can be tested in forward and reverse assays using