Video Article

Thin-layer Chromatographic (TLC) Separations and Bioassays of Plant Extracts to Identify Antimicrobial Compounds

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Abstract

A common screen for plant antimicrobial compounds consists of separating plant extracts by paper or thin-layer chromatography (PC or TLC), exposing the chromatograms to microbial suspensions (*e.g.* fungi or bacteria in broth or agar), allowing time for the microbes to grow in a humid environment, and visualizing zones with no microbial growth. The effectiveness of this screening method, known as bioautography, depends on both the quality of the chromatographic separation and the care taken with microbial culture conditions. This paper describes standard protocols for TLC and contact bioautography with a novel application to amino acid-fermenting bacteria. The extract is separated on flexible (aluminum-backed) silica TLC plates, and bands are visualized under ultraviolet (UV) light. Zones are cut out and incubated face down onto agar inoculated with the test microorganism. Inhibitory bands are visualized by staining the agar plates with tetrazolium red. The method is applied to the separation of red clover (*Trifolium pratense* cv. Kenland) phenolic compounds and their screening for activity against *Clostridium sticklandii*, a hyper ammonia-producing bacterium (HAB) that is native to the bovine rumen. The TLC methods apply to many types of plant extracts and other bacterial species (aerobic or anaerobic), as well as fungi, can be used as test organisms if culture conditions are modified to fit the growth requirements of the species.

Video Link

The video component of this article can be found at http://www.jove.com/video/51411/

Introduction

Assaying for antimicrobial compounds in plants requires separating the components of a plant extract, exposing a test microorganism to those components, and determining whether the microorganism's growth is inhibited by any of the compounds. Separations by paper or thin-layer chromatography (PC or TLC) are convenient because many compounds can be separated on a planar surface. Separation is based on polarity, with some compounds binding tightly to the adsorbent (cellulose in the case of PC, and a variety of adsorbents in the case of TLC) and migrating less than others[1]. **Figure 1** provides an example of the relative positions of polar and nonpolar phenolic compounds after separation on a silica TLC plate.

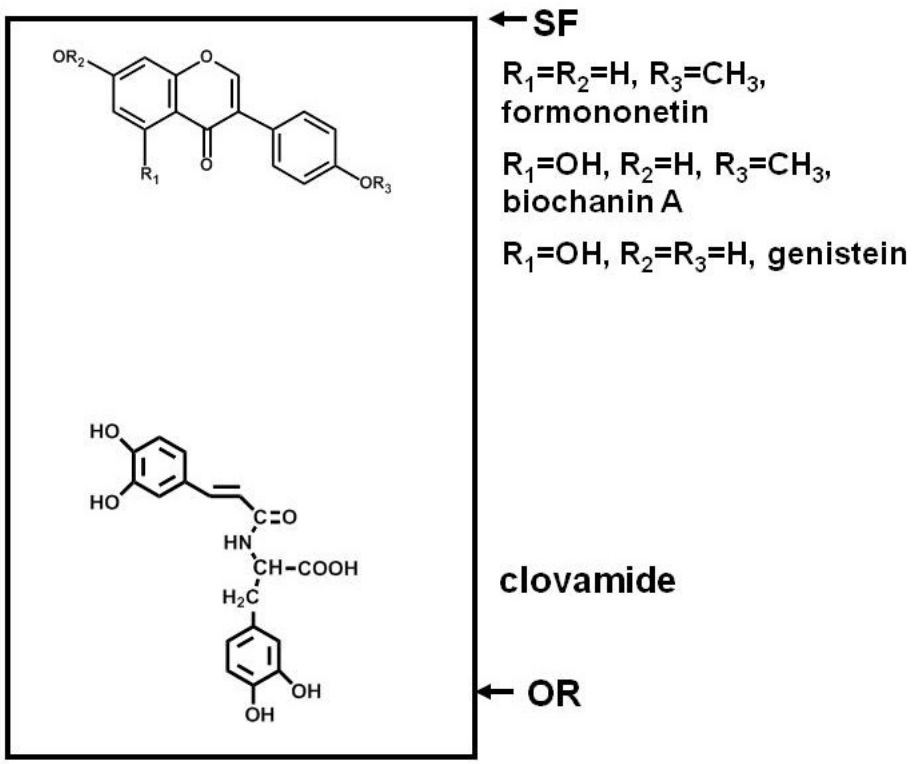

Figure 1. Diagram illustrating distributions of compounds of different polarities after separation on a silica thin-layer chromatographic (TLC) plate. Phenolic compounds of red clover (*Trifolium pratense* L.) are used as an example. Polar compounds, such as clovamide, have a strong affinity for a polar adsorbent like silica and remain near the origin (OR), while less polar compounds, such as the three isoflavones near the solvent front (SF), partition more readily into the solvents (which are less polar than silica unless water, acids, or bases are included) and migrate farther up the plate.

After separation of an extract on a TLC plate, test microorganisms can be exposed to all compounds on the plate, thus speeding the identification of the active components of an extract[2]. If a fungal or bacterial culture is exposed to the chromatogram, microbial growth will occur everywhere except over areas with growth-inhibitory compounds. Zones of inhibition then can be visualized by observing the contrast between mycelial growth and the growth-free areas if fungi have been applied[3] or by spraying with compounds that change color when reduced or hydrolyzed by living cells[4]. Although the use of paper or thin-layer chromatograms for antimicrobial assays was first applied to antibiotics[5] and fungicides[3,6], plant extracts are now frequently screened for antimicrobial compounds with this method, often referred to as bioautography. The protocols described herein apply to bioautography of thin-layer chromatograms. TLC is widely used because it is relatively rapid and can be performed on different adsorbents (*e.g.* silica, starch, alumina), as well as providing good resolution and sensitivity[1].

Plant extracts can be prepared for TLC in many ways. Common methods include extracting plant material in alcohol-water mixtures such as 80% ethanol[7,8], possibly with the addition of acid or base[9]. Following an extraction in such solvents, which contain some water and are possibly acidic or basic, extracts must be concentrated so that they can be applied to TLC plates in a minimal volume. The concentration of alcohol-water extracts can be achieved by partitioning with water-immiscible organic solvents[8] or with a mixture of such solvents, such as ethyl acetate-ethyl ether (1:1, v/v)[10,11]. Different plant metabolites are extracted into different organic solvents, depending on their polarities. To ensure that plant organic acids or bases are extracted into organic solvents at this stage, the pH of an alcohol-water extract can be raised or lowered with a water-soluble acid or base to convert dissociated analytes into their nondissociated forms, which are then soluble in neutral organic solvents[9]. The organic phase can then be evaporated under reduced pressure or under nitrogen and adjusted to the desired volume for TLC. The pH of the extract is unlikely to be lethal to bioassay microorganisms due to the partitioning of analytes into neutral solvents, small final volume, and evaporation of the extract on the TLC plate prior to separation.

Both fungi and bacteria are employed as test microorganisms in bioautography of plant extracts[2]. Spores of some fungi, such as *Cladosporium cucumerinum*, germinate on TLC plates (apart from areas with inhibitory compounds) if sprayed onto plates in a nutrient solution and incubated in a moist environment for several days[3]. The dark mycelium of *C. cucumerinum* on noninhibitory zones provides a sharp contrast to zones free of mycelial growth. Although bacteria have been applied to thin-layer chromatography (TLC) plates in the same manner[4,12], bacteria are also poured over TLC plate surfaces in agar overlays[13,14]. Yeast, such as *Candida albicans*, can be applied in agar overlays as well[14]. Alternatively, TLC plates can be placed face down onto agar inoculated with bacteria[10,15] or yeast[8], a method known as contact bioautography[2].

We describe a method for contact bioautography to screen for antimicrobial phenolic compounds from red clover (*Trifolium pratense* cv. Kenland). The test microorganism is *Clostridium sticklandii*, a ruminal hyper ammonia-producing bacterium (HAB) and obligate anaerobe. Although the separations used do not resolve all components of the extract, they facilitate identification of zones of antimicrobial activity, thus narrowing the pool of possible antimicrobial compounds. The protocol utilizes standard procedures for TLC[1]. The protocol also

describes some of the techniques required for culturing obligate anaerobes for such an assay, a usage of contact bioautography[15] and a visualization method with a tetrazolium salt, which stains living cells[2,4].

Protocol

1. Preparation of Plant Extract

1. See Kagan and Flythe[10] for extraction of phenolic compounds from *Trifolium pratense* cv. Kenland.
2. To extract other compounds in other plants, check the phytochemical analysis literature for plant- or metabolite-specific extraction methods (many are described), or look for protocols such as those of Khurram *et al.*[7,8] which isolate many compounds with a wide range of polarities.

2. Preparation of Thin-layer Plates

1. Clean TLC plates by developing in one or more polar, neutral solvents, in order to move adsorbed contaminants away from the zone of development.
    1. In a fume hood, prepare enough cleaning solvent (*e.g.* 15-100 ml of ethyl acetate-methanol 2:1, v/v) to cover the bottom of the TLC developing chamber, as well as the lower edge of a TLC plate when set inside the chamber.
    2. Use commercially available glass TLC developing chambers (different sizes available, with lids) or foil-covered Pyrex beakers or preserving jars.
    3. Use scissors to cut aluminum- or plastic-backed (flexible) silica gel plates, which come in various sizes (20 cm x 20 cm and smaller), to fit the available developing chamber. (Caution: silica can cause lung damage if inhaled. Work in a fume hood, and handle TLC plates with gloves to avoid getting skin oils onto the silica.)
    4. Insert the plates into the chamber, with the tops leaning against the chamber walls. Plates should not touch each other. Cover the chamber and let the solvent move up the plate by capillary action.
    5. When solvent has reached the top of the plates, remove plates from chamber and arrange in a standing position within the fume hood until solvent has evaporated.
    6. Check to see if impurities have migrated near the top of the TLC plate by looking for a yellow band under visible light, or a fluorescent band under ultraviolet (UV) light (see the “impurity front” or IF in **Figure 2B**). If the majority of the plate still has a yellowish tinge, repeat the cleaning process.
    7. After removing TLC plates from the chamber, discard the solvent. Allow residual solvent to evaporate completely before using the chamber for Protocol 2.
    8. To remove residual moisture that can affect migration of compounds on silica[16], prop the plates upright in a drying oven at 100 °C (10-15 min for a 20 cm x 20 cm plate, and 5 min for 7 cm x 10 cm plates).
    9. If a 100 °C drying oven is not available, heat plates for a longer period of time at lower temperatures (*i.e.* 40 min at 60 °C).
    10. After the plates are dry, let them cool to ambient temperature before loading.

3. Preparation of Developing Chambers for Extract Separation

1. Use scissors to cut a piece of filter paper slightly below chamber height, and about half the chamber perimeter in width. This paper acts as a wick to draw solvent up the chamber wall and saturate the chamber with solvent vapors, thus improving reproducibility of separations[1].
2. In a fume hood, mix solvents (ethyl acetate-methanol 4:1, v/v, for this study). Pour solvent mixture into the chamber and cover. Wait until the entire wick is wet with solvent, indicating chamber saturation, to put plates into chamber.

4. Loading and Development of TLC Plates

1. Lightly mark the origin with pencil. If the TLC plate adsorbent is soft and easily damaged, make marks at edges. Compounds should be above the surface of the developing solvent when plates are inserted into the TLC chamber.
2. Dissolve extracts in enough organic solvent (in this case, methanol) to have a concentrated solution instead of a turbid suspension.
3. Load samples and standards as narrow bands with a microliter syringe or capillary micropipettes, leaving a 1 cm border on the sides of the plate. Allow the bands to dry (fanning the plate or loading it in a fume hood helps).
4. If a greater concentration of sample is needed on a plate, "overspot" by loading samples again on the dried bands.
5. With forceps or tongs, set plate(s) inside the saturated TLC chamber. Plates should not touch the wick because it may provide solvent to the plates at points of contact, thus altering the path of compound migration. Cover chamber and let plates develop.

5. Preparation of Plates for Bioassay

1. Remove plate(s) from TLC tank before the solvent front reaches the top of the plate, and mark the height of the solvent front with a pencil. Let plate dry in fume hood.
    1. Develop any remaining TLC plates in the same TLC chamber, which is generally usable for an entire day if kept closed. Remake solvent mixture for TLC chamber if the amount of solvent in the chamber decreases notably.

2. After plates are dry, visualize bands under visible or UV light, and delineate bands with a pencil. A viewing chamber with a portable UV lamp is convenient, especially if the lamp can detect compounds at both 254 nm (short-wave UV) and 365 nm (long-wave UV).

Note: At this point or after the next step, plates can be wrapped in plastic wrap, covered with foil, and stored at -20 °C. Maximum storage time depends on compound stability. Because silica is not neutral, some compounds may degrade while on the TLC plate.

3. Photograph or draw plates.
    1. Use a gel photodocumentation system if one equipped with overhead UV and/or visible lights is available.
    2. If no commercial photodocumentation system is available, photograph plates inside of a box lined with dark paper or cloth, with a camera set on a tripod and a portable UV light clamped to a ring stand[17]. When photographing plates that have no fluorescent indicator, use a blue light filter over the camera lens to improve band appearance[17].

4. To aid in characterizing bands, calculate retention factor (R_f) values by measuring distances traveled by compound and solvent front, and dividing the former by the latter (**Figure 2A**).

6. Bacterial Culture and Assay

1. Prepare and inoculate media under anaerobic conditions. The culture used in this case was *Clostridium sticklandii* strain SR, which was obtained from the culture collection of James B. Russell, Cornell University, Ithaca, NY.
    1. See Flythe and Kagan[13] and the materials list for a description of HAB media.

2. Grow the culture to exponential or stationary phase. Use sterile anaerobic techniques when working with anaerobic microorganisms.
3. Inoculate the culture (1% v/v) into molten agar after the temperature of the agar (0.75% w/v) has decreased to less than 60 °C. Mix gently and immediately bring into an anaerobic chamber (95% CO_2-5% H_2 atmosphere for HAB media) to pour.
4. Pour into 15 mm x 100 mm plastic Petri dishes, and allow to solidify.
5. With scissors, cut the TLC plate into zones containing band(s) of interest. Lay bands face down onto agar plates, marking bands on the plate backing to keep track of original orientation on TLC plate. Lay an unused TLC strip onto an agar plate as a control.
6. Incubate agar plates, agar side down, in the anaerobic chamber (24 hr, 39 °C).

7. Visualization of Bioassayed Agar Plates

1. With forceps, remove bands from agar plates while they are in the anaerobic chamber.
2. Add 1% (w/v) tetrazolium red, prepared in water, dropwise onto the surfaces of the agar plates. Allow the color to develop for at least 20 min, or until the control plate turns completely red, before removing from the anaerobic chamber. The anaerobic bacteria will begin to lose viability immediately after removal from the anaerobic chamber, but the color is stable for more than 24 hr.
3. Photograph under visible light.

Representative Results

Representative silica TLC separations of red clover (*Trifolium pratense* cv. Kenland) extracts, containing phenolic compounds, are shown in **Figure 2**. Separation of red clover extract in ethyl acetate-hexane (9:1, v/v), over 8.5 cm, resulted in five bands, one incompletely resolved from the origin (**Figure 2A**). However, **Figure 2B** demonstrates that about twice as many bands were revealed when a different sample of red clover extract (from the same cultivar, but grown in a separate plot at the same farm) was separated over a greater distance (15 cm instead of 8.5 cm) and with two successive developments in different solvent systems. The chromatogram of **Figure 2B** was developed first in ethyl acetate-hexane (9:1, v/v), and then allowed to dry and separated in ethyl acetate-methanol (78:22, v/v). These results demonstrate that both plate size and choice of solvent system (or series of solvent systems) can affect the number of compounds separated on a chromatogram. In this particular case, elution and high-performance liquid chromatography (HPLC) of bands from other TLC separations of the same extracts confirmed that the compounds remaining at or near the origin consisted primarily of phenolic compounds (phenolic acids or isoflavones) conjugated to polar moieties such as amino acid derivatives, sugars, or malic acid. In contrast, isoflavones not conjugated to polar moieties migrated farther up the TLC plates[10].

The results of a bioassay of the TLC plate shown in **Figure 2A** with *Clostridium sticklandii*, a ruminal hyper ammonia-producing bacterium (HAB), are shown in **Figure 3**. Adding a 1% (w/v) aqueous solution of tetrazolium red to the agar plates after a 24 hr incubation resulted in a bright red color when living cells were stained. Incubation of band 1 (biochanin A) and part of band 2 (formononetin) on bacteria-seeded agar resulted in a well-defined zone of inhibition (**Figure 3A**). However, incubation of the remainder of band 2, along with bands 3 and 4, did not inhibit bacterial growth (**Figure 3B**). These results demonstrated that biochanin A was inhibitory to *C. sticklandii* growth, but formononetin was not.

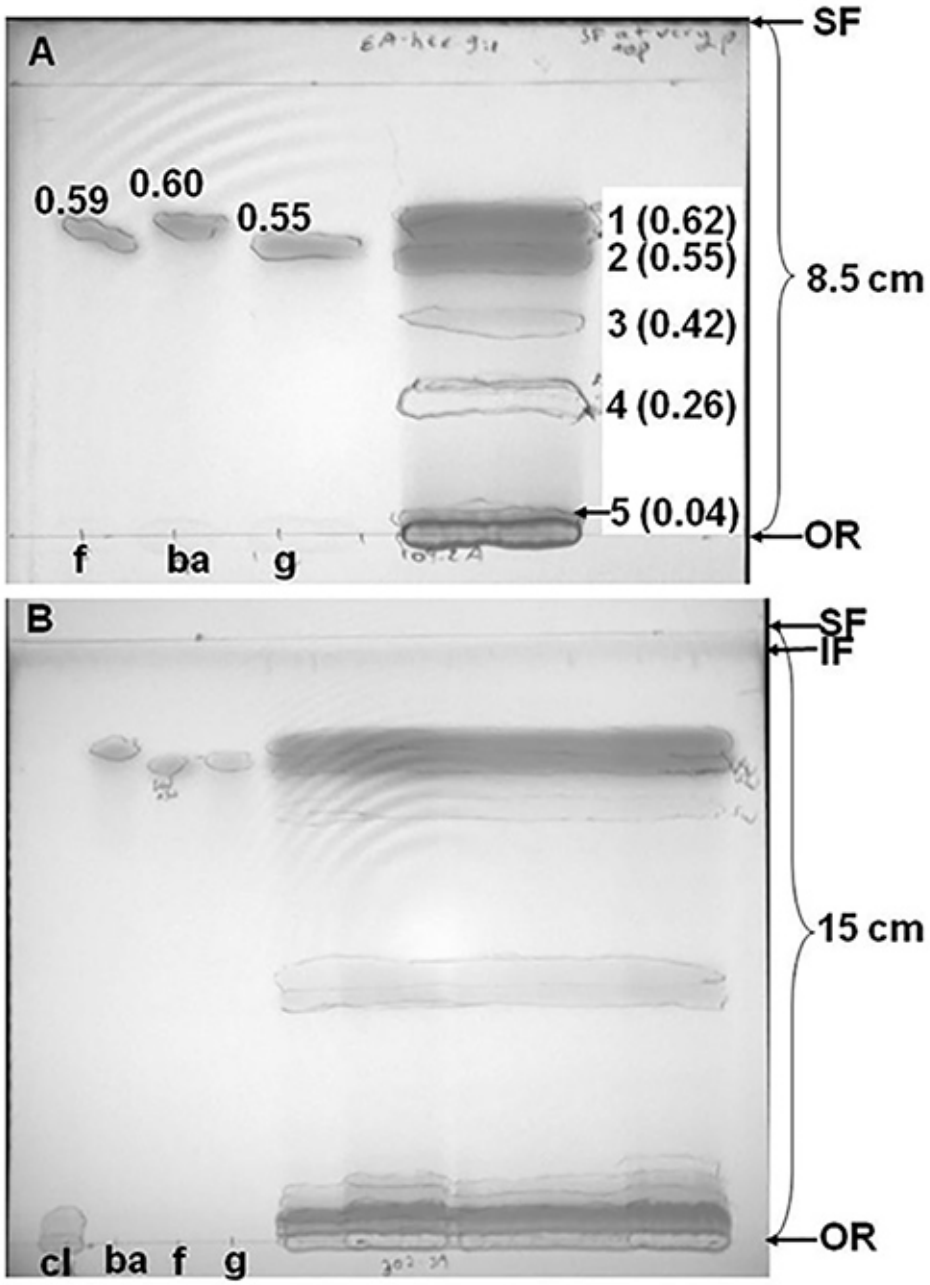

Figure 2. Silica thin-layer chromatographic (TLC) separations of extract of *Trifolium pratense* cv. Kenland, developed in (A) ethyl acetate-hexanes (9:1, v/v), or (B) that same solvent followed by development in ethyl acetate-methanol (78:22, v/v). The distance migrated by the solvent between the origin (OR) and solvent front (SF) is indicated near the brackets between those two boundaries. In **Figure 2B**, the "impurity front" (IF, location of the impurities moved up the plate by prewashing in polar solvents) is seen as a dark band near the top of the plate. Extracts, prepared from 180-250 mg freeze-dried red clover leaves and stems, were dissolved in methanol, and 10% of extract (**A**) or 32% (**B**) was loaded onto silica plates. Standards loaded were formononetin (f, 7.1 nmol), biochanin A (ba, 7.4 nmol), genistein (g, 9.8 nmol), and clovamide (Cl, **Figure 2B** only, 8.7 nmol). Bands were circled while viewed under a hand-held UV lamp at 254 nm (short-wave UV) and 365 nm (long-wave UV). A digital camera was used to photograph the plate under short-wave UV. The numbers to the right of the clover extract bands in **Figure 2A** refer to the zones cut out for bioassays. Retention factors (R_f, distance traveled by band/distance traveled by SF) are in parentheses after band numbers in **Figure 2A**.

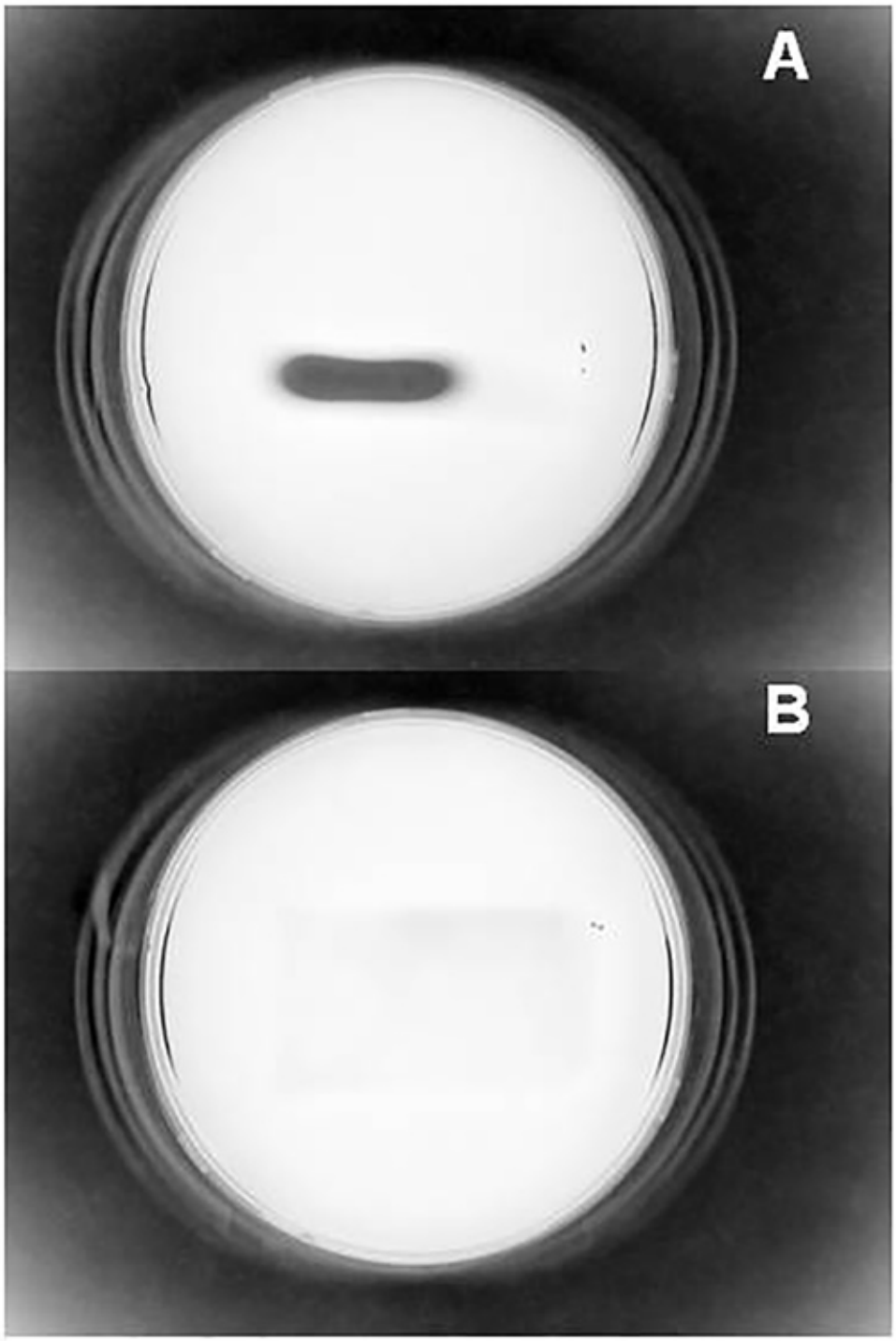

Figure 3. Results of TLC plate bioassays of (A) band 1 and the incompletely resolved upper part of band 2 from the TLC plate of Figure 2A, and (B) bands 2-4 from the same TLC plate. Bands were laid face down onto HAB medium inoculated with *Clostridium sticklandii* and incubated anaerobically 24 hr at 39 °C. At the end of this incubation period, the bands were removed, and the agar plates were stained with an aqueous solution of 1% (w/v) tetrazolium red and photographed under visible light.

Discussion

This protocol describes a simple method for separating an extract into subsets of compounds and assaying those subsets by contact bioautography. The method is quite similar to one used by Chomnawang *et al.*[15] to screen for plant metabolites inhibitory to gonorrhea-causing bacteria. The type of bioautography employed to screen for antimicrobial plant compounds depends on many factors, including the test microorganism, the laboratory setup, and the preferences of the person(s) performing the bioassay. Contact bioautography, the method used in

this study, has been criticized for depending on the ability of a compound to diffuse into inoculated media[12]. Zones of inhibition over compounds at low concentrations, or with little capability to diffuse into media, may not be detectable if growth occurs in the agar below the diffusate. Also, a zone of inhibition created by one band may spread beyond the band's initial boundaries, thus possibly masking inhibitory zones created by neighboring compounds[14]. However, contact bioautography is an easy method to use, and the ability to stack Petri plates minimizes the amount of space needed. In addition, laying TLC bands onto agar eliminates the risk of band dissolution and spreading that can occur when a TLC plate is overlaid with inoculated agar or sprayed with the test microorganism.

Obtaining information from any of the above types of bioautography depends on the chromatographic conditions, test microorganism concentration, and culture/incubation conditions. Chromatographic resolution of antimicrobial bands is not indispensable, but sensitivity may be greater for a well-resolved band[6]. Bands can be separated by development on longer plates or in multiple solvent systems in one dimension (**Figure 2B**), or in two dimensions by turning a TLC plate 90 degrees before development in a second solvent[16,19]. Polar compounds may be difficult to separate on silica without using solvent mixtures that contain acids or bases. These may be lethal to the test microorganism[12], although the use of small amounts of acids has been reported[14,15]. Alternative solvent systems may include water or methanol as components[20,21]. Polar compounds can also be resolved on different types of TLC plates, such as plates coated with C_{18} adsorbent or microcrystalline cellulose. However, growth[12] or sensitivity to the visualization agent[14] may be negatively impacted on some of these adsorbents.

Many different combinations of test microorganism and media can be used for bioautography. However, several factors must be considered when selecting microorganism and media. The media should be tested to determine that there are no components that reduce the tetrazolium salt and cause a nonbiological color change. The agar should also be light enough in color to provide contrast between the stained cells and unstained zones of inhibition. A microorganism that can only grow on the surface of agar can be removed along with the TLC band or washed off during staining. Additionally, the selection of microorganism/medium combination that minimizes gas production will reduce bubbles in the agar. Rumen HAB grow in a carbonate-buffered, carbohydrate-free medium with a mixture of amino acids or peptides as the growth substrate. The agar plates are light gold and transparent. When *C. sticklandii* is grown in the medium, most of the products are soluble (volatile fatty acids, ammonia). Very little gas is produced, which results in a smooth agar plate with no bubbles.

This bioassay has a couple of potential applications. The size of the zone of inhibition can be used as a rough estimate of the amount of the inhibitory compound, since the radius of the inhibitory zone is proportional to the logarithm of the amount of the compound causing the inhibition[17,22]. Perhaps the most common use of TLC plate bioassays is to narrow the range of possible antimicrobial compounds in a plant extract. After inhibitory zones are identified by a bioassay, the corresponding regions on a duplicate TLC plate can be eluted with a solvent, such as methanol or ethyl acetate, and analyzed by HPLC with ultraviolet spectroscopy detection to begin characterizing bioactive compounds[10,14].

Disclosures

Mention of trade names or commercial products in the article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the USDA. The authors declare no competing financial interest.

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