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Note

Detection of inhibitors of *Erwinia carotovora* and *E. herbicola* on thin-layer chromatograms

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Published methods for detecting antibacterial compounds on TLC plates[1–4] have usually involved placing the chromatogram in contact with a seeded agar medium to allow diffusion of the compound, with subsequent incubation of the agar medium and observation of zones of inhibition of the bacterium. These zones may be intensified by several methods, particularly by the use of tetrazolium salts, of which *p*-iodonitrotetrazolium violet (INT) has been reported as being one of the most sensitive[5].

This note describes methods for detecting inhibitors of *Erwinia carotovora* and *E. herbicola* directly on TLC plates. Good results have been obtained with a method that is based on the ability of the organism to hydrolyze aesculin[6], and this method may be applicable to a wide range of bacteria as an alternative to the use of tetrazolium salts.

MATERIALS AND METHODS

Bacteria

Three strains of *E. carotovora* var. *atroseptica* (*E. atroseptica*) (G120, BL/CL 30/101, BL/CL 30/120), three strains of *E. carotovora* var. *carotovora* (*E. carotovora*) (BL/M 46/1, BL/CL 30/104, BL/CL 30/135), two strains of *E. herbicola* var. *herbicola* (708, G142) and *Bacillus cereus* M8 were from a collection maintained in this laboratory.

Extraction of garlic (Allium sativum)

Tissue (20 g) from fresh garlic bulbs was extracted with 200 ml of distilled water by treatment in an MSE microhomogeniser. The extract was centrifuged (30,000 *g*; 30 min), the supernatant liquid was filtered, evaporated to dryness under reduced pressure at 40°, and the residue was extracted with 2 ml of methanol (fraction 1) and then with 2 ml of water (fraction 2).

Culture media

Cultures were grown in Trypticase-soy-broth (TSB) (Baltimore Biological Labs., Baltimore, Md., U.S.A.) (20 ml of medium in 100-ml flasks) at 25° for 24–72 h on a shaker with a 1-in. rotary movement at a speed of 250 rpm.

Detection of antibacterial activity on agar plates

Plates of Trypticase-soy-agar (BBL) were surface-seeded with a culture of the appropriate bacterium in TSB. A measured volume of the solution to be tested was placed on a sterile filter-paper pad 6 mm in diameter (Antibiotic Assay Discs, Whatman), which was placed on the seeded plate. After incubation at 25° for 24 h, the diameter of the zones of inhibition were recorded. Control tests with solvent gave no zones of inhibition.

Thin-layer chromatography

Prepared TLC plates (silica gel 60 or silica gel 60, F254, Merck) were washed by ascending development with methanol before use. The chromatograms were developed with cyclohexane–ethyl acetate (1:1, v/v).

Detection of antibacterial activity on TLC plates

The antibacterial compounds used were coumarin (BDH, Poole, Great Britain), streptomycin sulphate (Sigma, St. Louis, Mo., U.S.A.) and constituents present in the extracts of garlic. After development, the plates were dried to remove solvent. Cultures of bacteria in TSB were centrifuged (3,000 *g* for 20 min), the supernatant liquid was discarded, and the sedimented bacteria were re-suspended in fresh TSB to give an absorption of 0.84 at 560 nm (equivalent to approx. 10^9 bacteria per ml). This suspension was sprayed on to the TLC plates using a fine spray (7CR, Quickfit); approximately 18 ml of suspension were used for a 20 $\times$ 20 cm plate. The plates were then dried in a stream of cold air, just sufficiently to remove any film of water and give a translucent appearance, before being incubated at 25° overnight in boxes lined with wet filter paper to maintain high humidity. After incubation, the plates were dried until opaque and immediately sprayed with one of the following solutions: (1) aesculin spray [aesculin (BDH), 0.2% w/v; ammonium ferric citrate, 0.1% w/v; yeast extract (Difco), 0.5% w/v, in distilled water]; (2) an aqueous solution of INT (Grade 1, Sigma), 2 mg$\cdot$ml^{-1} (see ref. 5) (3) an aqueous solution of 2,3,5-triphenyltetrazolium chloride (TTC) (BDH), 20 mg$\cdot$ml^{-1} (see ref. 5). The plates were then again incubated at 25° in the lined boxes to allow hydrolysis of aesculin or reduction of tetrazolium salts to occur.

In the conditions used, aesculin sprayed on to TLC plates appeared colourless in tungsten light, strongly fluorescent in UV radiation (366 or 254 nm) and was not significant affected by the iron salt. A similar quantity of aesculetin gave a yellow colour in tungsten light and was much less fluorescent than aesculin in UV radiation[7], the iron salt gave a brown colour with aesculetin in tungsten light and a decrease in fluorescence in UV radiation. On plates sprayed with the bacteria and subsequently with the aesculin spray, hydrolysis of aesculin resulted in development of a brown colour, the zones of inhibition of bacteria remaining colourless. Under UV radiation of 366 nm (or 254 nm for the plates without a fluorescent additive), zones of inhibition were fluorescent against a dark background.

On plates sprayed with the bacteria and subsequently with INT or TTC, zones of inhibition appeared colourless against a violet or a red background, respectively.

RESULTS AND DISCUSSION

Tests of antibacterial activity against *E. atroseptica* G 120 on agar plates showed that 2 mg of coumarin, 100 μg of streptomycin sulphate and 20 μl of the extracts of garlic produced zones of inhibition of 13, 23 and 17–18 mm in diameter, respectively.

When attempts were made to detect antibacterial activity of these compounds on TLC plates, none of the strains of *Erwinia* reduced TTC during incubation for 24 h; in contrast, *B. cereus* M8 reduced this compound in 2 h. All the strains of *Erwinia* hydrolyzed aesculin and reduced INT in 2–24 h, and both these sprays could be used to detect inhibition of bacterial growth. The tests with streptomycin sulphate, which was spotted on to plates, but not developed with a solvent mixture, showed that, with this water-soluble compound, careful spraying resulted in well-defined zones of inhibition, without distortion due to the aqueous sprays.

The times required for hydrolysis of the aesculin and for reduction of the INT differed according to the strain of *Erwinia* used. Generally, the reaction occurred within 2–7 h at 25°, but incubation overnight was sometimes necessary. Clear results could be obtained with both sprays, but with some strains of *Erwinia* slight inhibition by coumarin was revealed more clearly by the aesculin spray than by the INT. Examination under UV radiation intensified the zones of inhibition on plates sprayed with aesculin (see Fig. 1).

The sensitivity of detection of inhibitors on the TLC plates was assessed by comparing the areas of zones of inhibition with those obtained on agar plates. Tests

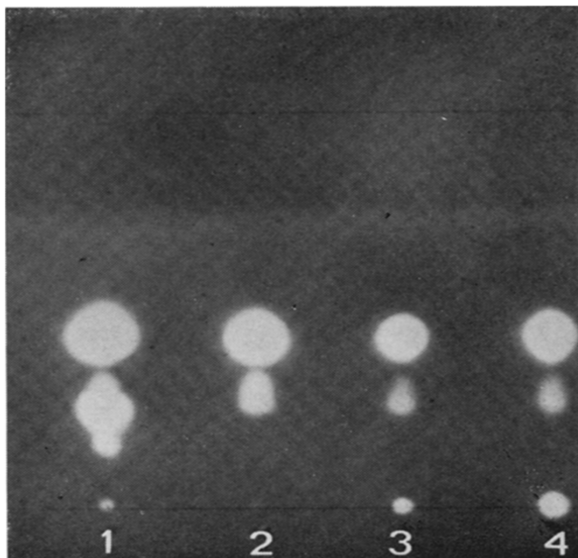

Fig. 1. Zones of inhibition of *E. carotovora* var. *atroseptica* G120 produced after chromatography of extracts of garlic on a silica gel 60, F254 plate in cyclohexane–ethyl acetate (1:1, v/v). The plate was then sprayed with bacteria and subsequently with aesculin spray. Photographed under UV radiation (366 nm). 1,2 = 10 μl and 5 μl (respectively) of fraction 1; 3,4 = 10 μl and 5 μl (respectively) of fraction 2 (see Materials and methods). The photograph was taken on High-Speed Ektachrome Type B film (Kodak), a black-and-white internegative being made on Plus X film (Kodak).

were made with coumarin (1–2 mg) against two strains each of *E. atroseptica* and *E. carotovora* and with garlic extracts (20 μl) against one strain of *E. atroseptica*. In each instance, larger areas of inhibition were formed on the TLC plates than in tests on the agar plates.

The results with extracts of garlic showed that these methods could be applied successfully to compounds in a crude extract from plant tissue. The aesculin spray has also been used to demonstrate inhibition of *E. atroseptica* by rishitin in extracts from potato tissue[8].

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