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Thin-Layer Chromatographic Technique for Rapid Detection of Bacterial Phospholipases

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Silica gel thin-layer chromatography was employed to detect lecithinase activity induced from bacterial resting cell preparations incubated at 37 C for 4 h in the presence of purified egg yolk lecithin. *Bacillus subtilis, Bacillus cereus, Serratia marcescens,* and *Pseudomonas aeruginosa* hydrolyzed lecithin with the formation of free fatty acids as the sole lipid-soluble product. In none of the *Escherichia coli* and *Citrobacter freundii* strains tested could lecithinase activity be detected. Four among eight strains of *Enterobacter aerogenes* and one among 12 strains of *Proteus* tested produced negligible amounts of free fatty acid.

Phospholipids are widespread through tissue. Numerous attempts have been made to clarify the circumstances related to the capacity of bacteria to form enzymes responsible for their degradation. The production of these enzymes has been investigated in an attempt to associate their formation with toxicity and virulence and to use it in the classification of bacteria (4, 5, 9-11).

The most widely used method for the detection of bacterial phospholipase was based on the appearance of an opacity in egg yolk media inoculated with the test organisms. The underlying mechanism for this phenomenon is the splitting of phosphatidyl choline (lecithin) into lipid-soluble (diglycerides, fatty acids) and water-soluble (choline, phosphorylcholine) products. The lipid-soluble products, connected with the egg yolk lipid, and the loss of the emulsifying properties of lecithin cause the opalescence in the media. Although most of the bacteria giving the egg yolk reaction produce lecithinase C, the opacity reaction is not per se evidence of a lecithinase activity. *Staphylococcus aureus,* known not to possess lecithinase activity, show an egg yolk reaction. Additionally the presence of egg yolk substrate may hinder a true lecithinase activity, especially in the case of strong lipolytic bacteria (16).

In the present work we tried to establish a reliable thin-layer chromatographic technique for the fast and easy detection of bacterial lecithinase, appropriate for use in the clinical laboratory.

MATERIALS AND METHODS

Bacterial strains. For the evaluation of the technique, 71 bacterial strains were tested; 65 of them were recent clinical isolates and six were obtained from the National Collection of Type Cultures (NCTC; London). The clinical isolates included 15 strains of *Escherichia coli,* 12 strains of *Citrobacter freundii,* 10 strains of *Serratia marcescens,* 12 strains of *Proteus* (including four strains of *P. mirabilis,* two strains of *P. vulgaris,* two strains of *P. morganii,* two strains of *P. rettgeri,* and two strains of *Providencia*), 14 strains of *Pseudomonas aeruginosa,* and eight strains of *Enterobacter aerogenes.* Enterobacterial isolates were classified according to the scheme proposed by Cowan and Steel (1) while *Pseudomonas aeruginosa* strains were identified by previously published criteria (2). The typed strains were as follows: four strains of *S. marcescens* (NCTC 1377, 10211, 2446, 2847), one strain of *Bacillus subtilis* (NCTC 7861), and one strain of *Bacillus cereus* (NCTC 2599).

Lecithinase detection. Egg yolk lecithin, prepared according to Pangorn (17), was used as substrate; its purity, assayed chromatographically, was approximately 98%. Lecithin was used in the form of a colloidal solution prepared by adding an ethereal solution of lecithin to warm (65 to 70 C) 0.1 M tris(hydroxymethyl)aminomethane buffer, pH 8.0. The final concentration of lecithin in this solution was 0.1% (wt/vol).

A tube (13 by 100 mm) containing 0.7 ml of the stated colloidal solution of lecithin and 0.3 ml of 0.1 M $MgCl_2$ was inoculated with two standard loopfuls (3 mm diameter) of organisms from an 18- to 24-h culture grown on a heart infusion agar plate at 37 C. The incubation was carried out in a shaking bath at 37 C for 4 h. Blank incubation mixtures without added bacteria or with heat-inactivated bacteria were prepared and tested together with the assayed strains.

Incubation was stopped by the addition of 1 ml of a chloroform-methanol mixture (1:4, vol/vol), whereas the lipids were extracted by successive addition of 3 ml of chloroform-methanol (4:1, vol/vol) (14). After vigorous shaking for 30 s and centrifugation at 2,000 × *g* for 2 min the chloroform phase was evaporated to dryness under a stream of nitrogen. The residue was dissolved in 50 μl of chloroform-methanol (2:1, vol/vol) and spotted in equal amounts to separate precoated Silica Gel G plates (Merck, Darmstadt), 10 by 10 cm and 0.25 mm thick, with a 50-μl syringe (Hamilton Co. Inc., Whittier, Calif.). The spots were dried with a stream of air. The plates, loaded with the extracted lipids from six samples, were placed in the chromatographic tank (8).

Two developing systems were used. For the separation of neutral lipids the developing system consisted of light petroleum, ethyl ether, and glacial acetic acid (80:20:1, vol/vol/vol). The partition of phospholipids (3) was achieved with a mixture composed of chloroform, methanol, glacial acetic acid, and water (100:56:20:10, vol/vol/vol/vol).

The identification of the spots on the chromatoplate was accomplished by comparison of their R_f values with those of standard lipid samples. Chloroform solutions of oleic acid (Merck Co., Darmstadt, Germany), 1-octadecoyl glycerol, and 1-octadecoyl-2-hexadecoyl glycerol (Fluka Co., Switzerland) were run as standards for fatty acids, monoglycerides, and diglycerides, respectively, whereas chloroform solutions of L-α-lecithin and L-α-lysolecithin (Sigma Chemical Co., Ltd.) were run as standards for lecithin and lysolecithin, respectively. The reagents, applied to the plate in a spray, were as follows. Dragendorff reagent (19) was used for the detection of choline-containing lipids, whereas spots of all lipid compounds were obtained with 50% sulfuric acid (vol/vol) saturated with $K_2Cr_2O_7$ and ethanolic phosphomolybdic acid (10%; vol/vol). Free choline was determined qualitatively with Florence reagent (4) in the upper phase formed after the addition of the extracting solution to the incubation mixture.

RESULTS

Complete hydrolysis of lecithin was achieved with resting cells of *B. cereus* (NCTC 2599) (Table 1), free fatty acid (FFA) being the sole lipid-soluble product.

In regard to the clinical isolates of *Pseudomonas aeruginosa* tested, 11 out of the 14 strains exhibited a rather strong lecithinase activity with hydrolysis of about two-thirds of the lecithin present (Table 2). The only lipid-soluble degradative products of lecithin were FFA. *Pseudomonas aeruginosa* produce, like *B. cereus*, lecithinase C; consequently the formation of FFA only as the lipid-soluble product must be interpreted as the result of an excessive lipase activity against the diglyceride formed.

B. subtilis (NCTC 7861) hydrolyzed one-third of the quantity of lecithin present (Table 1). The hydrolysis of lecithin resulted in the formation of FFA as the sole lipid-soluble product.

The lecithinase activity varied from strain to strain in regard to *Serratia marcescens*, the most active being strain NCTC 1377, which degraded approximately four-fifths of the lecithin present (Tables 1 and 2).

No lecithinase activity was noted among the *E. coli* and *C. freundii* strains tested. One half of the *Enterobacter aerogenes* strains and one strain of *P. mirabilis* produced only a slight hydrolysis of lecithin (Table 2) with the formation of FFA.

With all the strains tested no further change in the amount of lecithin was observed, even if the incubation was prolonged to 24 h. It is worthwhile to note that under the present experimental conditions no lysolecithin or choline could be detected in any of the lecithinase-producing strains that were used in this study.

Degradation of lecithin did not occur with control assays involving bacteria-free or boiled-cell incubation mixtures. In the case of enterobacterial isolates, the study of unincubated assay mixtures revealed that, although some compounds are extracted with the applied procedure, they move in the chromatograms in front

TABLE 1. *Hydrolysis of lecithin by bacterial resting cells of typed strains*

Organisms	Lecithin hydrolyzed (%)[a]
S. marcescens	
NCTC 1377	80
NCTC 10211	60
NCTC 2446	70
NCTC 2847	50
B. subtilis NCTC 7861	20
B. cereus NCTC 2599	100

[a] Results obtained with heat-inactivated bacteria were considered as 100%.

TABLE 2. *Hydrolysis of lecithin by bacterial resting cells of clinical isolates*

Organisms	No. of strains tested	Lecithin hydrolyzed	
		No. of positive strains	%[a]
E. coli	15	0	
C. freundii	12	0	
S. marcescens	10	10	30–70
P. mirabilis	4	1	40
P. vulgaris	2	0	
P. morganii	2	0	
P. rettgeri	2	0	
Providencia spp.	2	0	
Enterobacter aerogenes	8	4	20–30
Pseudomonas aeruginosa	14	14	30–80

[a] See footnote to Table 1.

of FFA and are faintly stained with the detecting reagents. In the case of *Serratia marcescens* the extracting procedure produces prodigiosin, which disappears after the treatment with the reagents used. In *Pseudomonas aeruginosa* strains, where some bacterial lipid substances were extracted, the lipids moved in the area of FFA in the chromatograms. However, their quantity was too small to be confused with any FFA formed from the degradation of lecithin.

DISCUSSION

Egg yolk medium has been widely used for the study of bacterial lecithinase activity (15). The positive reaction, expressed as an opacity of the medium, given by the lecithinase C-producing bacteria, may be hindered if active lipase is present (16).

Additionally, the egg yolk reaction is given by bacteria known not to have a lecithinase C activity as it occurs with *S. aureus* (18) and *Serratia marcescens* (6, 12). The transesterification of cholesterol with the fatty acids of lecithin, through the action of an acyltransferase, was proven to be the primary reaction in the breakdown of lecithin (16).

The above data indicate that it would be incorrect to use egg yolk as a substrate for the detection of lecithinase activity even if the reaction products are estimated by chromatography. Only purified lecithin should be used for the study of lecithinase activity.

The method employed in the present investigation, a combination of thin-layer chromatography with simultaneous qualitative estimation of choline after short incubation of resting cells in the presence of lecithin, permits a direct, rapid, and easy detection of lecithinase activity. A similar technique has been described for the study of lecithinase A activity of *S. aureus* (13). However, this method employs chromatographic detection of the hydrolysis products in the ether extracts and water phase of cultures incubated for 48 h. Apparently the short incubation period prevents disappearance of the hydrolysis products that may be used further in synthetic, or degradative, reactions, thus impeding the evaluation of the true lecithinase activity.

The complete breakdown of lecithin in the assay mixtures inoculated with *B. cereus*, and the existence of *Serratia marcescens* strains hydrolyzing lecithin nearly completely, clearly demonstrate that our procedure offers optimum conditions for the study of lecithinase activity. Despite a contrary opinion (7), the degree of dispersion of lecithin particles achieved by adding it in the form of an ethereal solution is satisfactory for the action of lecithinase.

The present technique has the advantage that it allows estimation of the amount of the lecithin broken down and an appreciation of the reaction products. Thus it contributes to a better evaluation of lecithinase activity. The nonenzymatic hydrolysis of lecithin resulting from a lack of contamination of the hydrolysis products with bacterial substances extracted during this procedure favor the present technique.

Bacterial action on lecithin, as monitored by our technique, varied from species to species. *E. coli*, *E. aerogenes*, and *Proteus* failed to hydrolyze lecithin. This finding is in agreement with previous reports (4), although lecithinase has been detected by other methods.

Pseudomonas aeruginosa and *B. cereus*, known to produce lecithinase C, split lecithin with the formation of FFA as the sole lipid-soluble hydrolytic product. Presumably the diglyceride formed is immediately hydrolyzed by excessive lipase activity. Previous study from this laboratory (8) showed that *Pseudomonas aeruginosa* assayed under identical conditions hydrolyzed triolein to FFA without formation of any intermediates (e.g., mono- or diglycerides).

Despite the fact that previous reports state that choline is produced as a result of the activity of *Serratia marcescens* on purified lecithin (6) or lecithin in egg yolk (12, 16), we were unable to confirm this by our technique. The production of FFA as the sole product of hydrolysis does not provide evidence as to the kind of lecithinase(s) involved.

Athough the nature of lecithinase is not determined by our technique, it is useful as a rapid and simple laboratory test for the study of bacterial lecithinase activity in diagnostic laboratories.

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