

# Isolation of Salmonellae from Food Samples

## III. Dulcitol Lactose Iron Agar, a New Differential Tube Medium for Confirmation of Microorganisms of the Genus *Salmonella*

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The many differential media designed for the clinical microbiologist have reflected his interest in the delineation of a wide variety of genera within the enteric group of organisms. The traditional differential tube media, Russell double sugar agar, Krumweide triple sugar agar, Kligler iron agar, and triple sugar iron agar (Hajna, 1945), have been invaluable aids in the prompt establishment of the biochemical patterns which are of generic significance in the identification of *Enterobacteriaceae*. These media were created especially for versatility in order to secure the widest applicability. In the laboratory of the food analyst, the greater emphasis placed specifically on the detection and enumeration of members of the genus *Salmonella* imposes a requirement for a medium designed to facilitate that technique. In previous papers, the limitations of the enrichment broths (Silliker and Taylor, 1958) and the capabilities of differential plating media (Taylor *et al.*, 1958) have been demonstrated and discussed. The need for a simplified test for confirmation of presumptively positive colonies of *Salmonella* on brilliant green agar plates became increasingly apparent as these works progressed. The tubed differential media used were of limited value because of the multiplicity of enteric organisms capable of producing reactions identical to the salmonellae. This, in turn, necessitated the use of many additional biochemical tests, such as several carbohydrate fermentations, indole and urease tests. An investigation to reduce the time and labor involved in biochemical confirmation of suspect salmonellae culminated in the differential medium herein described.

### MATERIALS AND METHODS

Dulcitol lactose iron agar (DLI) is a medium designed to differentiate between the genus *Salmonella* and other enteric genera. In its most effective form, it is a double-poured medium having slant and butt of different composition. It is designed to disclose the fermentation of lactose and dulcitol and the production of hydrogen sulfide. The directions given will make 2 L of the completed medium.

### *Solution A (slant):*

Phenol red agar base (Difco <sup>1</sup> /BBL <sup>2</sup> )	30 g
Lactose, CP	10 g
Ferrous ammonium sulfate	0.2 g
Sodium thiosulfate	0.2 g
Water, distilled	1 L

### *Solution B (butt):*

Phenol red agar base	30 g
Water, distilled	1 L

Solutions A and B are brought to a rolling boil in separate containers, autoclaved for 20 min at 15 lb pressure, and cooled to 55 C. A Seitz-filtered 10 per cent solution of dulcitol, CP, is added to solution B in the amount of 20 ml per L (0.2 per cent). If there is a noticeable color change after the addition of the carbohydrate, restore the pH to about 7.4 with a few drops of 1 N NaOH. Upon occasion when a relatively crude dulcitol was used in lieu of CP grade, there was some color change, albeit without gas production, after incubation with nondulcitol fermenting organisms. This is assumed to result from the fermentation of trace carbohydrate impurities which, when coupled with the range of the phenol red indicator, is sufficient to produce a slight acid reaction. The same dulcitol in bromcresol purple sugar broth (BCP) is used to verify the fermentation and has always been reliable. Crude dulcitol is now used in BCP broth and CP grade in the DLI slants.

Solution B is dispensed aseptically in 15 by 125 mm screw-capped tubes in 2 to 3 ml amounts and allowed to solidify. Solution A is added in like amounts and slanted to form a tube half slant and half butt. Sterile separatory funnels are excellent for dispensing the solutions.

The medium is inoculated by streaking and stabbing, incubated for 18 to 24 hr at 37 C, and the reactions read.

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RESULTS AND DISCUSSION

When Kligler's or triple sugar iron agar (TSI) slants are used for the presumptive detection of salmonellae, *Proteus*, Arizona, and Ballerup-Bethesda group organisms produce reactions identical to those of the salmonellae, that is, alkaline slant, H<sub>2</sub>S blackening, and acid and gas in the butt. A search was initiated for a carbohydrate fermented exclusively by salmonellae which would supplement the primary differentiation afforded by lactose. No such carbohydrate was found, but dulcitol was observed to approach this ideal most closely. The obvious advantages secured by the inclu-

sion of dulcitol in the medium are shown in table 1. Since neither the *Proteus* nor Arizona groups ferment dulcitol, these are easily distinguished from salmonellae which ferment it readily in 24 hr with but few exceptions. Many organisms of the Ballerup-Bethesda group are able to ferment dulcitol, however, and these constitute the only false positives which occur in the use of the DLI medium.

Separation of the salmonellae from the Ballerup-Bethesda groups is effected by use of the ninhydrin test. This is the only other biochemical reaction necessary to complete the confirmation of salmonellae short of antigen analysis. The ninhydrin test (Carlquist, 1956) is used with one change in technique: no centrifugation is used, 5 to 10 min of standing effecting suitable separation of the chloroform layer. Salmonellae are ninhydrin positive; Ballerup-Bethesda group organisms are not (table 1).

When H<sub>2</sub>S producing organisms are incubated on TSI or Kligler's iron agar slants, the fermentation of the glucose in the butt is often obscured by excessive blackening. This is of relatively small importance because all such organisms are glucose fermenters and the assumption is that a positive butt has been obscured. The significance of dulcitol fermentation as a differential reaction makes it imperative that the result be observed. The omission of the sulfide indicator would lose a valuable diagnostic characteristic. On the other hand, early trials with the medium produced equiv-

TABLE 1  
Fermentation reactions of enteric groups

Group*	Lactose	Dulcitol	H <sub>2</sub> S	Ninhydrin
Salmonella	-†	+	(+)	+
Arizona	X	-	+	+
Ballerup-Bethesda	X	(+)	+	-
Escherichia	+	V	(-)	+
Klebsiella	+	V	-	+
Providence	-	-	-	-
Proteus	-	-	(+)	V

\* Nomenclature and most fermentation reactions shown, from Kauffmann, 1951.

† Key to reactions: - = negative; + = positive in one day; (+) = most strains positive, a few negative; (-) most strains negative, a few positive; X = delayed positives or negative; V = variable with serotype or strains.

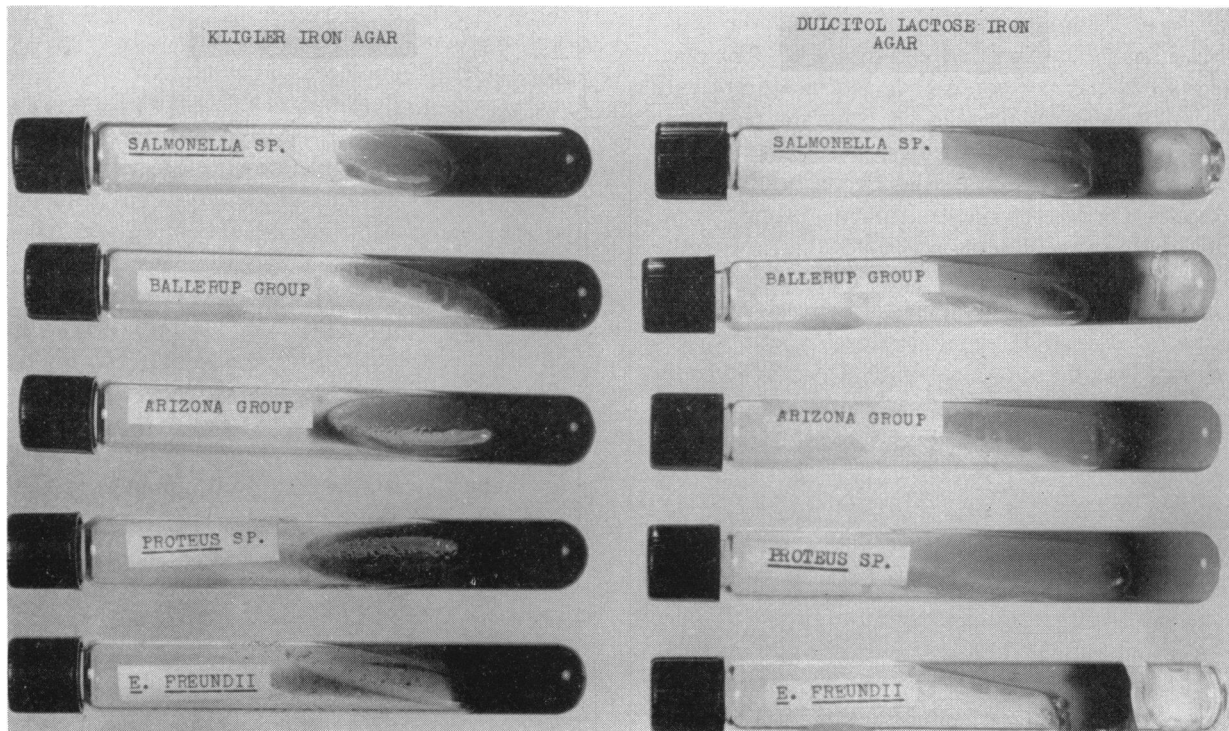


Figure 1. A comparison of the biochemical reactions of closely related enteric organisms on Kligler's iron agar and dulcitol lactose iron agar slants.

ocal results because many organisms, especially protei, blackened all but the uppermost portion of the slant. The amount of the indicator was reduced with no appreciable improvement in the medium. Different compositions for butt and slant portions of the medium were used in order to limit the blackening reaction. Since separate pouring would be used, there was no need to mix the carbohydrates, so amounts of dulcitol and lactose to be used were determined by trial. The final formula evolved is one in which the dulcitol is confined to the butt in such a concentration that fermentation reactions are unequivocal. The medium is stable and it is not normally kept refrigerated but allowed to stand at room temperature. Under these conditions, there has been no noticeable migration on the indicator or carbohydrates and exceedingly crisp and clear-cut reactions characterize the medium. For purposes of comparison, duplicate cultures of organisms were used to inoculate Kligler's iron agar and DLI. The total blackening of the butt of the Kligler's may be observed in contrast to the distinctive separation of fermentation reactions shown by the DLI. All of the

organisms except *Escherichia freundii* were identical in appearance on Kligler's; on DLI, *E. freundii* differed from *Salmonella* and *Ballerup* by having a yellow slant, the latter differed from *Proteus* and *Arizona* by having yellow fractured (acid and gas) butts (figure 1). A schematized presentation of the correlation of typical reactions to commonly encountered organisms from enrichment broths is shown in figure 2. The typical coliform and *E. freundii* reactions should, of course, be screened out of contention by their appearance on brilliant green agar plates or other such differential plating media but, unfortunately, they will occasionally show as lactose-negative or doubtful colonies when first isolated because of initially delayed fermentation reactions.

In use, the medium has proved itself both valid and reliable. In the course of more than 150 isolations of naturally occurring salmonellae, more than 95 per cent of these organisms have produced the characteristic appearance of red slant, yellow fractured butt, and black-banded middle. The only exceptions have been one dulcitol negative *S. worthington*, and a few H<sub>2</sub>S-negative strains of various serotypes.

The need for a single medium, DLI, to replace two media such as Kligler's and dulcitol broth is best understood from the viewpoint of the requirements of *Salmonella* enumeration by the most probable number (MPN) method, discussed in a previous paper of this series (Silliker and Taylor, 1958). By this method, in a 9 tube MPN determination, for example, 3 dilutions of sample would be used to inoculate triplicate enrichment broths and thence streaked to brilliant green agar plates. From these 9 plates a statistically valid number of *Salmonella*, suspect colonies, usually from 3 to 8 per plate, must be picked for identification as salmonellae in order to determine the population density. This could amount to 72 cultures per sample, and many samples require more than a 9 tube determination. If one were to use the two media mentioned in place of DLI, it would obviously entail twice the storage space for the tubed media, two inoculations per culture, twice the incubator space, two readings of reactions, and twice the amount of glassware to disinfect, handle and wash. Under these circumstances the reduction from two media to one has been amply justified in our experience.

SUMMARY

A new differential tube medium, dulcitol lactose iron agar, which facilitates the biochemical separation of genus *Salmonella* from other commonly encountered enteric organisms, is presented.

The marked superiority of dulcitol lactose iron agar medium over Kligler iron agar and triple sugar iron agar slants is demonstrated to result from use of a carbohydrate less frequently fermented by nonsalmonellae than glucose, and from the method of construction in

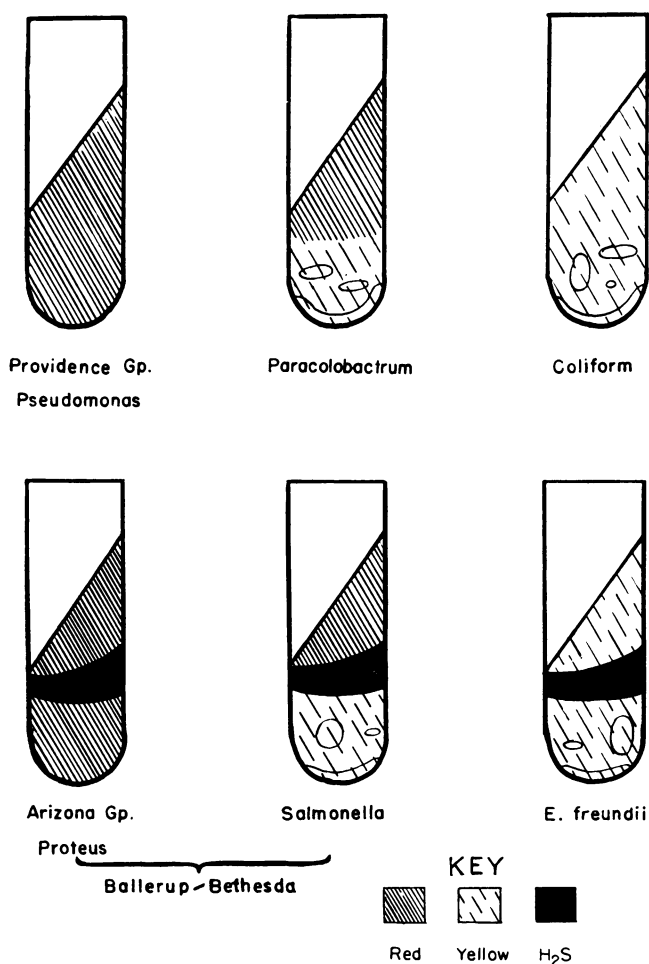


Figure 2. The characteristic reactions of enteric organisms on dulcitol lactose iron agar slants.

which the butt reaction is never obscured by excessive H<sub>2</sub>S formation.

The only nonsalmonellae which produce the characteristic salmonella reactions on dulcitol lactose iron agar are Ballerup-Bethesda paracolons which may be differentiated by the ninhydrin test.

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## The Inactivation of Spores of *Bacillus globigii* and *Bacillus anthracis* by Free Available Chlorine<sup>1</sup>

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The germicidal action of chlorine and chlorine-yielding compounds has been studied by a number of investigators. However, two important variables were not considered: the effect of pH changes and the distinction between free available, combined available, and combined chlorine.

Studies conducted by Tilley and Chapin (1930) on anthrax spores indicated that nitrogen trichloride, chlorine in neutral solutions, and chlorine plus N/50 HCl were effective in 15 min with available chlorine concentrations of 10 mg per L or more. In a more recent study (Fair *et al.*, 1947), titratable free chlorine residuals of 1 and 3 mg per L produced a 99.95 per cent kill of *Bacillus anthracis* spores at pH 5.0 and 25 C in 60 and 30 min, respectively. Based on the amount of hypochlorous acid present, Moore (1951) stated that 3 mg per L produced 100 per cent kill of *B. anthracis* spores in 30 min at 25 C.

This investigation was undertaken to determine the effects of free available chlorine, hereinafter referred to as FAC, upon the spores of *Bacillus globigii* (*Bacillus subtilis* morphotype *globigii*) and *Bacillus anthracis* under the following conditions: (1) hydrogen-ion concentrations of 6.2, 7.2, 8.6, and 10.5; (2) exposure temperatures of 4 and 22 C; and (3) exposure periods of 2 and 48 hr. The results obtained should provide practical information on water treatment plant operation in times of disaster.

<sup>1</sup> This work was performed under contract with the Department of the Navy, Bureau of Yards and Docks.

#### MATERIALS AND METHODS

*Test cultures.* *Bacillus anthracis* no. 811 and *Bacillus globigii* no. 102 were obtained from The Ohio State University.

*Development of media.* In initial studies with spore-forming microorganisms, media which would induce rapid germination during incubation at 35 C were tested. Nutrient agar plus 0.5 per cent yeast extract and 0.3 per cent sodium citrate seemed to be most suitable for *B. globigii* spores. Tryptose agar plus 0.2 per cent liver extract proved to be adequate for *B. anthracis* spores.

Studies were also made to determine the medium which would attain maximum sporulation of the test organisms under specified conditions. A mineral medium containing 0.1 per cent Casamino acids, 0.25 per cent glucose, 0.5 per cent yeast extract, 0.01 per cent manganese sulfate, 0.0001 per cent ferrous sulfate, and 3 per cent agar, adjusted to pH 6.8 with sodium hydroxide, provided the greatest number of mature viable spores.

*Vegetative cell autolysis.* The presence of vegetative cells in spore suspensions might lead to endogeneous respiration, resulting in the germination of spores during FAC evaluations. Germination of the spore suspensions under the conditions of the investigation could result in spurious observations. Autolysis obtained by a combination of heat shock plus incubation at 45 C, for 24 hr, proved to be the most efficient method for destroying vegetative cells.