Esculin Hydrolysis Reaction by *Escherichia coli*

ABRAHAM MISKIN¹ AND STEPHEN C. EDBERG^{2*}

Department of Microbiology, Kaplan Hospital, Rehovot, Israel,¹ and Division of Microbiology, Department of Pathology, Montefiore Hospital and Medical Center, Albert Einstein College of Medicine, Bronx, New York, 10467²

Received for publication 15 November 1977

The literature contains variable reports concerning the hydrolysis of esculin by members of the family *Enterobacteriaceae* and particularly *Escherichia coli*. We examined 113 strains of fresh clinical isolates of *E. coli* and assessed the ability of colonies in a population to hydrolyze esculin with and without preincubation in inducible substrates at 24, 48, and 72 h. The number of strains capable of fermenting salicin, a sugar with a β -glucoside linkage like esculin, was studied under the same conditions. A strip test that measured the presence of the constitutive glucosidase was also performed with and without preincubation in inducible substrates. No *E. coli* strain was able to produce constitutive enzyme; preincubation in esculin and salicin resulted in an induction of the β -glucosidase. The number of colonies able to hydrolyze esculin increased with time. Only those strains preincubated in esculin or salicin were able to produce a positive constitutive strip test. Because the β -glucosidase of *E. coli* is inducible, one should employ, when using growth media, a light inoculum obtained by touching the top of a colony with a bacteriological wire and read the reaction between 18 and 24 h, or perform a rapid strip or spot test.

Esculin is β -D-glucose-6,7-dihydroxycoumarin, a compound derived from the horse chestnut tree. It has vitamin P activity. The molecule can be hydrolyzed at the beta linkage to yield two end products—glucose and esculetin (6,7-dihydroxycoumarin). Hydrolysis can be determined either by measuring acid production from the fermentation of glucose or by the black color formed in the reaction of esculetin with ferric ions. Generally, iron is incorporated into a growth medium as ferric ammonium citrate.

The hydrolysis of esculin and its detection by using iron was first described for enteric bacilli by Meulen (7) in 1907. In the 1930's, Vaughn, Levine, and their associates (5, 9) used the hydrolysis of esculin as a taxonomic tool in their description of the family *Enterobacteriaceae*. Although included in many diagnostic charts, the reaction was not widely used for many years. In 1971 Wasilauskas (10) reported the use of bile-esculin agar, heretofore used for the identification of group D *Streptococcus*, for the characterization of *Enterobacteriaceae*. He reported that in 24 h of incubation, *Escherichia coli* did not hydrolyze esculin. Lindell and Quinn (6), using a very heavy inoculum, found 2% of *E. coli* positive in 4 h and 18% positive in 24 h. The Center for Disease Control (3, 4) found 30.9% positive at 48 h of incubation and 51% positive at 72 or more h of incubation. Edberg et al. (1, 2) studied the reaction by the species on both

growth- and non-growth-supporting media and found none positive at 4 h, 0.3% to 0.8% positive at 24 h, 35% positive at 48 h, 53% positive at 72 h, and 61% positive at 120 h (Table 1). No other species in the family showed this marked increase in hydrolysis with time. *E. coli* was the only species in the family that demonstrated distinct differences in growth and nongrowth tests. These observations suggested that *E. coli* β -glucosidase was inducible.

To evaluate these variant reports, a study was undertaken to determine the nature of the esculin hydrolysis reaction by *E. coli*. Salicin, a sugar sharing with esculin in the β -glucoside linkage, was used as a related substrate in parallel tests with esculin. Growth and nongrowth tests, performed after preincubation with both compounds, were undertaken to establish whether the enzyme was constitutive or inducible.

MATERIALS AND METHODS

Bacterial strains. The 113 *E. coli* strains studied were fresh clinical isolates from the General Bacteriology Section of the Division of Microbiology and Immunology of Montefiore Hospital and Medical Center, the Albert Einstein College of Medicine. The strains were identified by the scheme of Edwards and Ewing (2, 3) by conventionally prepared media made according to directions from the manufacturers.

Studies on induction of the enzyme. All 113 strains of *E. coli* were transferred to Trypticase soy

TABLE 1. *Reported esculin hydrolysis reactions by E. coli*

Source	No. of strains	Cumulative positive reactions (%) at progressive incubation times (h):					
		4	24	48	72	96	120
Center for Disease Control (3)		ND ^a	X ^b	31	51 ^c	Y ^d	Y
Wasilauskas (10)	69	0	0	ND	ND	ND	ND
Lindell and Quinn (6)	126	2	18	ND	ND	ND	ND
Edberg et al. (1)	510	0	0	ND	ND	ND	ND
Edberg et al. (2)	974	0	0.5	35	52	58	61

^a ND, Not done.^b X, Included in 48-h data.^c 72 h or greater.^d Y, Included in +72-h data.

agar (TSA) slants (Baltimore Biological Laboratories, Cockeysville, Md.) and incubated at 35°C for 24 h. From this medium, a suspension was made in 0.85% NaCl (normal physiological saline, NPS) to yield 10⁶ bacteria per ml. To quantitate the number of *E. coli* cells in a population capable of hydrolyzing esculin, for each of the 113 strains 0.001 ml was removed from the suspension with a calibrated bacteriological loop and evenly distributed over the surface of a bile-esculin agar plate (Difco Laboratories, Detroit, Mich.). It has been shown previously (2) that bile-esculin is as effective as Vaughn-Levine medium for the determination of esculin hydrolysis. The number of esculin-hydrolyzing colonies per plate was determined after 4, 24, 48, and 72 h to ascertain if the number of positive colonies increased with time or if the number of positive colonies were constitutively determined. In addition, a TSA salicin fermentation tube (Baltimore Biological Laboratories) was inoculated.

To determine the effect of incubation in the presence of inducing substrates, 10⁵ organisms from a 24-h culture on TSA were added to 0.9 ml of the following: (i) 0.5% proteose peptone no. 3-0.1% esculin in NPS, (ii) 0.5% proteose peptone no. 3-0.5% salicin in NPS, and (iii) 0.5% proteose peptone no. 3 in NPS.

Esculin was obtained from ICN Pharmaceuticals Inc. (Cleveland, Ohio), and salicin and proteose peptone no. 3 were obtained from Difco Laboratories.

After 24 h at 35°C, 0.001 ml was removed from each of three tubes and evenly distributed over the surface of a bile-esculin plate. As with the previous experiment, the number of colonies showing a positive reaction was scored after 24, 48, and 72 h of incubation at 35°C. A salicin fermentation tube was inoculated in parallel and read after 48 and 72 h.

Determination of inherent esculin-hydrolyzing capability in a non-growth-supporting medium. An experimental esculin hydrolysis strip (W7645-8, General Diagnostics, Warner-Lambert Co., Morris Plains, N.J.), shown to be notably more sensitive (1) than the rub-in strip described in the literature (8), was used to determine the presence of constitutive β -glucosidase in a nongrowth environment before and after incubation in substrates. A suspension equivalent to a 0.5 MacFarland standard was made for each strain from TSA; 0.3 ml of this suspension was pipetted into a test tube (13 by 100 mm), and the test strip was added according to directions supplied by the manufacturer. The test strip was also inoculated after prein-

cubation in both esculin and salicin broths, as described. All strip reactions were read after 4 h of incubation at 35°C.

Effect of bile salts. To determine if the presence of bile affected the hydrolysis of esculin, 25 random strains were inoculated as described in saline, esculin, and salicin preincubation broths containing oxgall (Difco Laboratories) at a 4% final concentration. These broths were treated as described except that subculturing was made on esculin agar without bile.

Effect of the end-product glucose. To determine the effect of glucose on the hydrolysis reaction, six strains known to hydrolyze esculin after 48 h were chosen. A 10⁶ bacterial suspension was made from TSA and inoculated into the following media: (i) 0.5% proteose peptone no. 3-NPS, (ii) 0.5% proteose peptone no. 3-0.1% esculin in NPS, and (iii) 0.5% proteose peptone no. 3-0.1% esculin in NPS-1% glucose in NPS.

The tubes were incubated at 35°C and tested for esculin hydrolysis by the addition of ferric ammonium citrate (final concentration, 0.05%) to a portion every 24 h for 72 h.

RESULTS

The percentages of positive *E. coli* strains tested from Trypticase soy agar, as determined on bile-esculin agar plates and salicin fermentation tubes, are presented in Table 2. After 24 h of incubation, 1 of 113 strains was positive on bile-esculin agar. After 48 h, an additional 31 strains hydrolyzed esculin. Of the 32 strains positive on bile-esculin agar at 48 h, 31 showed 5% or less esculin-positive colonies in the population as determined by quantitative counting. At 72 h, an additional 25 strains became positive. Of the 25 strains that became positive between 28 and 72 h, 17 demonstrated less than 5% esculin-hydrolyzing colonies in the population. On individual plates, additional colonies continued to hydrolyze esculin each 24 h, so that after 7 days of incubation 16 of these 17 strains showed over 95% positive colonies.

Of the 32 strains that hydrolyzed esculin after 48 h, 23 fermented salicin. After 72 h of incubation an additional seven strains fermented salicin. Only two strains hydrolyzed esculin but did

TABLE 2. *Effect of preincubation in inducing substrates on the ability of E. coli to hydrolyze esculin*

Incubation time (h)	Initial determination ^a in:				Determination after induction ^a in:					
	Bile-esculin agar		Salicin fermentation		Saline		Esculin		Salicin	
	Strains positive	% Positive	Strains positive	% Positive	Strains positive	% Positive	Strains positive	% Positive	Strains positive	% Positive
24	1	0.9	ND ^b	ND	1	0.9	30	27	34	30
48	32	28	41	36	33	29	62	55	69	61
72	57	51	61	54	56	50	64	57	69	61

^a Initial determination refers to reactivity before exposure to the substrate, and determination after induction refers to reactivity after preincubation in the substrate.

^b ND, Not done.

not ferment salicin; however, only 1% of the colonies of each of these strains showed a positive reaction on bile-esculin agar.

A total of 25 strains were esculin negative after 48 h of incubation and esculin positive at 72 h of incubation; of these, 10 fermented salicin in 48 h, and 16 fermented salicin after 72 h. Of the nine strains in this group that hydrolyzed esculin at 72 h, but did not ferment salicin in the same time period, six demonstrated less than 2% esculin-positive colonies in the population, and the other three demonstrated less than 5% positive colonies. Conversely, at 48 h nine strains fermented salicin but did not hydrolyze esculin; at 72 h this number was reduced to four strains.

Table 2 shows the effect of preincubation for 24 h in saline, esculin, and salicin on the percentage of esculin-hydrolyzing and salicin-fermenting colonies. The saline control tube yielded the same number of esculin-positive *E. coli* as found when the inoculum was taken from a TSA slant. Table 2 shows the marked difference preincubation in substrates had on the induction of β -glucosidase. Direct inoculation from TSA yielded 1 of 113 strains positive after 24 h; after preincubation in esculin and salicin the percentage of those positive increased to 27 and 30, respectively. Concomitant increases were seen after 48 and 72 h of incubation (Table 2). Although it appeared that salicin was a more efficient inducer of β -glucosidase than was esculin, the differences—by chi-square analysis—were not significant. As reconfirmed in these series of experiments, those strains that hydrolyzed esculin but did not ferment salicin demonstrated less than 5% positive esculin hydrolytic colonies in a population. Preincubation in salicin and esculin uniformly resulted in a larger percentage of cells in the population capable of hydrolyzing esculin at all incubation periods than that found with no preincubation in a substrate (Table 2).

None of 113 strains hydrolyzed esculin directly from a TSA slant as determined by using the

non-growth-supporting PathoTec strip. After preincubation in the presence of the inducing agents esculin and salicin for 24 h, the percentages of *E. coli* capable of producing a positive 4-h test were 44 and 52, respectively (Table 3).

The possible inhibitory effect of the end-product glucose was examined by using six strains that hydrolyzed esculin after 48 h. In the presence of 1% glucose, it took 96 h, or an additional 48 h, for these six organisms to produce detectable β -glucosidase. In the presence of both esculin and glucose, these strains required 72 h, or an additional 24 h, to produce the enzyme.

There was no difference observed between percent and/or time required for a positive reaction with 25 strains incubated with or without 4% oxgall.

To determine whether we were selecting strains within a population or whether the population as a whole was being affected, five strains able to hydrolyze esculin in 72 h and one strain able to hydrolyze esculin in 24 h were serially transferred three times on bile-esculin agar. After these transfers all strains were able to hydrolyze esculin in 24 h. The six strains were then subcultured six times on both 5% sheep blood agar and MacConkey agar, and all reverted to their natural states. After subculture

TABLE 3. *Effect of the preincubation in inducing substrates on the ability of E. coli to hydrolyze esculin in 4 h in a non-growth-supporting substrate-impregnated strip*

Pre-incubation medium	Effect at preincubation period of:			
	No preincubation		24 h	
	Strains positive	% Positive	Strains positive	% Positive
TSA/TSB ^a	0	0	0	0
Saline	0	0	0	0
Esculin	0	0	50	44
Salicin	0	0	59	52

^a TSB, Trypticase soy broth.

on bile-esculin agar three times, all strains were PathoTec strip positive; after subculture on non-esculin-containing agar, all strains became PathoTec strip negative.

DISCUSSION

Esculin hydrolysis is a useful tool for the differentiation of the family *Enterobacteriaceae*, but the literature has reported discrepant results (1-4, 6, 10) (Table 1). Each investigator employed somewhat different test conditions and periods of incubation. Most of the variation has centered within the species *E. coli*. This study attempted to define whether some cells in a bacterial population are able to constitutively hydrolyze esculin and that in esculin-containing medium they manifest themselves and become predominant, or whether the population as a whole cannot hydrolyze the glucoside and the enzyme must be induced. The experiments reported here support the latter view.

Of the 113 strains of fresh clinical *E. coli* studied here, only 1 was able to hydrolyze esculin within 24 h. Each additional 24-h incubation brought further numbers of positive colonies in a population. The examination of individual members in a population revealed that single colonies became positive as time progressed, indicating that induction was occurring. In addition only a very small percentage of cells were initially positive (less than 5%), with the number of positive colonies increasing during each 24 h of incubation.

If the enzyme were not inducible, but present constitutively in a small number of cells in the population, one would not expect preincubation in inducing substrates to exert an effect. Preincubation in both esculin and the structurally related glucoside salicin not only accelerated the reaction, but resulted in a marked increase in the number of colonies in a given population capable of hydrolyzing esculin. Conversely, after induction the ability to hydrolyze esculin was lost through subculture on non-glucoside-containing media, a characteristic expected only if the enzyme were inducible. This view is supported by the studies using the non-growth-supporting esculin hydrolysis test strips. The strip measures constitutive rather than inducible enzyme. None of 113 strains of *E. coli* was positive at 4 h, but after incubation in inducing substrates, the percentage of those positive increased almost to that seen after 72 h of incubation on bile-esculin medium. A positive constitutive strip reaction was also lost after subculture on non-esculin-containing medium. The strip, therefore, appears to be a more useful taxonomic tool in determining esculin hydrolysis by *Enterobacteriaceae* than does growth medium because all *E. coli* are negative by this test,

whereas all other *Enterobacteriaceae* that are positive on growth media are positive by this rapid technique.

When used with an appreciation of the nature of the β -glucosidase reaction, esculin hydrolysis is a very useful test in the taxonomy of *E. coli* and other *Enterobacteriaceae*. Care should be exerted to use a light inoculum when using growth-containing media, and one should read results within 18 to 24 h. Practically, this inoculum can be obtained by touching the top of a colony with a bacteriological wire and stabbing the medium. A heavy inoculum would present more cells for induction, and an incubation period longer than 24 h would result in more cells being induced with time. A heavy inoculum or an incubation period greater than 24 h would increase positivity. This is the case in the literature (2-4, 10). Lindell and Quinn (6), using a very heavy inoculum, found 2% positive at 4 h and 18% positive in 24 h; Edwards and Ewing's group at the Center for Disease Control (3, 4) found 30.9% positive at 48 h and 51% positive at 72 or more h; and Wasilauskas (10) and Edberg et al. (1, 2) found less than 0.5% positive in 24 h. Wasilauskas (10) noted that positivity appeared to increase with time. Alternatively, one could employ a rapid test strip or spot test (1) in which enzyme induction would not occur during the short incubation period.

LITERATURE CITED

1. Edberg, S. C., K. Gam., C. J. Bottenbley, and J. M. Singer. 1976. Rapid spot test for the determination of esculin hydrolysis. *J. Clin. Microbiol.* 4:180-184.
2. Edberg, S. C., S. Pittman, and J. M. Singer. 1977. Esculin hydrolysis by *Enterobacteriaceae*. *J. Clin. Microbiol.* 6:111-116.
3. Edwards, P. R., and W. H. Ewing. 1972. Identification of *Enterobacteriaceae*, 3rd ed. Burgess Publishing Co., Minneapolis, Minn.
4. Ewing, W. H. 1973. Differentiation of *Enterobacteriaceae* by biochemical reactions. Center for Disease Control, Atlanta, Ga.
5. Levine, M. R., S. Vaughn, S. Epstein, and D. Anderson. 1932. Some differential reactions in the colonaerogenes group of bacteria. *Proc. Soc. Exp. Biol. Med.* 29:1022-1024.
6. Lindell, S. S., and P. Quinn. 1975. Use of bile-esculin agar for rapid differentiation of *Enterobacteriaceae*. *J. Clin. Microbiol.* 1:440-443.
7. Meulen, H. 1907. (Title unknown.) K. Akad. Wet. Amsterdam. In F. C. Harrison and J. van der Leek. 1909. Aesculin bile salt media for water analysis. *Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 2* 22:547-551.
8. Smith, P. B., D. L. Rhoden, and K. M. Tomforhrde. 1975. Evaluation of the PathoTec Rapid I-D System for identification of *Enterobacteriaceae*. *J. Clin. Microbiol.* 1:359-362.
9. Vaughn, R. H., and M. Levine. 1942. Differentiation of the "intermediate" coli-like bacteria. *J. Bacteriol.* 44:487-505.
10. Wasilauskas, B. L. 1971. Preliminary observations on the rapid differentiation of the *Klebsiella-Enterobacter-Serratia* group on bile-esculin-agar. *Appl. Microbiol.* 21:162-163.