Journal of Industrial Microbiology, 6 (1990) 143–148
Elsevier

SIM 00269

Effect of selective media on loss of congo red binding in *Shigella flexneri*

James L. Smith, Brian J. Dell and Saumya Bhaduri

U.S. Department of Agriculture, ARS, Eastern Regional Center, Philadelphia, PA, U.S.A.

(Received 6 August 1989; revised 9 November 1989; accepted 16 November 1989)

Key words: Congo red binding; Plasmid; *Shigella flexneri*; Curing; Virulence

SUMMARY

The effect of growth of *Shigella flexneri* on various selective media on retention of congo red (CR) binding ability was determined to evaluate the effectiveness of isolation techniques regarding maintenance of the virulence plasmid. When *S. flexneri* was surface-plated onto selective agars and the resulting colonies replica plated onto CR plates, no white colonies indicative of loss of virulence were found despite repeated trials. However, when *S. flexneri* was grown in liquid media (agar was removed from agar-containing media by centrifugation), white colonies were found upon plating onto CR plates. Most common selective media for shigellae produced fewer than 5–10 white colonies/1000 red colonies. However, growth in broth prepared from violet red bile agar, desoxycholate citrate agar, and SS agar gave more than 100 white colonies/1000 red colonies. Loss of CR binding was demonstrated when *S. flexneri* was grown in broth containing tergitol 7, sodium dodecyl sulfate, bile salts #3, crystal violet, eosin y, or methylene blue. However, concentrations of selective agents that led to loss of CR binding were much higher than those used in selective media. Results indicate that under usual conditions of isolation of *S. flexneri* from food and clinical specimens, CR binding appears to be a relatively stable character with most selective media; however, use of violet red bile agar, desoxycholate citrate agar, and SS agar may lead to substantial loss of congo red binding indicating that the isolates may not be virulent.

INTRODUCTION

Virulence of *Shigella* species is multifactorial and dependent on genes present on both the chromosome and a large plasmid [4]. In *Shigella flexneri*, the large virulence plasmid (140 MDa) contains the genes which encode the ability of the cells to produce keratoconjunctivitis, to invade HeLa cells, and to bind congo red (CR) with concomitant production of red colonies on CR agar [4]. Thus, the congo red phenotype is associated with virulence and loss of the large virulence plasmid or a deletion in the plasmid that results in loss of congo red binding is correlated with loss of virulence [7].

Chambers et al. [1] indicated that the gene for congo red binding does not contain the information necessary to give invasive capacity of *S. flexneri*. However, Maurelli et al. [7], Sakai et al. [9], and Daskaleros and Payne [2,3], present evidence indicating that congo red binding is an essential component of the virulence complex in *S. flexneri*. Sasakawa et al. [10] show that subculturing a virulent strain of *S. flexneri* in liquid media resulted in simultaneous loss of invasiveness and congo red binding due to loss of the large virulent plasmid or to a deletion or a single-site IS insertion in that plasmid. On agar plates, congo red negative colonies were much larger than the red positive ones, indicating that the growth rates are quite different [10]. Thus, the avirulent non-congo red binding cells will overgrow the virulent wild-type cells because of their faster growth rate.

Plasmid curing has been shown to occur upon treating bacterial cells with a variety of agents including intercalating dyes, antibiotics, sodium dodecyl sulfate, and high growth temperatures [8,11]; but the effect of selective media upon plasmid curing has received only limited study. Hill and Carlisle [5] have shown that some strains of enterotoxigenic *Escherichia coli* not only lost plasmids but also lost the ability to produce heat-labile and heat-stable enterotoxins upon cultivation in lauryl tryptose broth (contains sodium dodecyl sulfate) at 37 °C followed by cultivation in EC medium (contains bile salts #3) at 44.5 °C. Using a similar system, Jungmann and de Souza Ferreira [6] demonstrated that when *E. coli* lost the F_0lac

Correspondence: J.L. Smith, Microbial Food Safety, U.S. Department of Agriculture, ARS, Eastern Regional Research Center, 600 East Mermaid Lane, Philadelphia, PA 19118, U.S.A.

plasmid, approximately 90% of the cells became lactose negative. We examined the effect of selective media, various chemicals and environmental conditions on CR binding in *S. flexneri* 5348 to assess the potential for loss of virulence associated characteristics during routine isolation.

MATERIALS AND METHODS

Microorganism. A CR positive (red) colony of *Shigella flexneri* 5348 (obtained from D.W. Niesel, University of Texas) was picked from a 24 h CR plate and inoculated into 100 ml tryptic soy broth (Difco; TSB) contained in a 1000 ml Erlenmeyer flask and grown 16–18 h at 37 °C under shaken conditions (200 rpm). CR plates were prepared from TSB plus 2% (w/v) agar containing 0.01% (w/v) congo red. Dye (100 mg) was dissolved in 10 ml distilled water, sterilized by autoclaving, and added aseptically to 990 ml autoclaved agar before pouring plates.

Effect of selective media on CR binding; (a) solid medium. Solid media were prepared according to manufacturer's directions. Agar (2% w/v) was added to liquid media and then prepared according to manufacturer's directions. The culture, grown in TSB (see above), was diluted so that each inoculated plate contained 100 to 200 colonies after 24 h incubation at 37 °C. Using the RepliPlate (FMC Bioproducts, Rockland, ME) colony transfer pad technique, the colonies from each solid medium were replicated onto CR plates. The replicated plates were incubated at 37 °C; at 24 to 48 h, colonies were examined for CR binding.

Effect of selective media on CR binding; (b) liquid medium. Liquid media were prepared according to manufacturer's directions. Agar containing media were dissolved in cold distilled water and then centrifuged to pellet the agar; the supernatant fluid was decanted into flasks (25 ml for each 250 ml Erlenmeyer flask) and the media prepared according to manufacturer's directions (either autoclaving or boiling without autoclaving). Flasks were inoculated with a diluted culture from TSB shaken at 37 °C for 16–18 h. The inoculum was adjusted so that each flask contained approximately 2×10^6 cells/ml. The inoculated flasks were incubated shaken (200 rpm) at 37 °C. At 24 h, appropriate dilutions from each flask were plated onto CR plates utilizing the spiral plater (Spiral Systems, Inc., Cincinnati, OH). Plates were incubated at 37 °C and observed for CR binding after 24 h.

Effect of chemicals and environmental conditions on CR binding. Various test compounds were dissolved in double strength TSB, the pH adjusted to 7.0–7.2 if necessary, distilled water added to bring the volume to 25 ml (in 250 ml flasks) and the mixtures sterilized by autoclaving. Alternatively, stock solutions of heat sensitive compounds were sterilized by filtration through a Nalgene 0.2 μm syringe filter (solutions were neutralized to pH 7.0 if necessary before filtering) before adding to sterile double-strength TSB; sterile distilled water was added to make the volume to 25 ml. In a study of the effect of pH on CR binding, double-strength TSB was adjusted to various pH values by use of 0.1 N NaOH or HCl, made to 25 ml with distilled water, dispensed into 250 ml flasks and sterilized by autoclaving. The effect of temperature on CR binding was studied by incubating TSB (25 ml in 250 ml flasks) at various temperatures. Each flask received approximately 2×10^6 cells/ml and was incubated shaken (200 rpm) at 37 °C for 24 h unless otherwise stated. At the end of incubation, dilutions of each culture were plated onto CR plates as described above.

Source of chemicals and media. Unless otherwise noted, all media and media components as well as brilliant green were obtained from Difco Laboratories. Inorganic salts, sodium lactate, sodium citrate, sodium acetate, and glucose were obtained from J.T. Baker Chemical Co. All other reagents were obtained from Sigma Chemical Co.

RESULTS AND DISCUSSION

Initial experiments consisted of growing *S. flexneri* 5348 in TSB for 16–18 h and then plating dilutions of the culture onto a solid form of the selective media (2% agar was added to liquid media) listed in Table 1, and incubating at 37 °C for 24–48 h. Plates containing 100–200 colonies were replicated by the use of the RepliPlate technique onto CR plates followed by incubation at 37 °C for 24–48 h. Colonies lacking the ability to bind CR were not found when *S. flexneri* was initially plated onto various selective agars and then replicated onto CR agar despite repeated attempts (data not shown).

However, when *S. flexneri* was grown in either liquid media or in agar media with the agar removed, incubated at 37 °C on a reciprocating shaker (200 rpm) for 24 h, and then plated onto CR plates, white colonies were found (Table 1). The various selective media have differential effects on the loss of CR binding by *S. flexneri.* TSB was always run in each experiment as a control; the data indicated growth of *S. flexneri* in TSB for 24 h resulted in approximately 5 white colonies for every 1000 red colo-

TABLE 1

Effect of growth of *Shigella flexneri* 5348 in selective liquid media on subsequent CR binding

Medium	n[a]	Log no. of red colonies at 24 h	Log no. of white colonies at 24 h	no. of white/1000 red
		Mean (S.D.)	Mean (S.D.)	
Tergitol 7 agar[a]	6	9.12 (0.134)	5.90 (0.449)	0.76
Nutrient broth	6	8.65 (0.308)	5.65 (0.305)	1.00
A-1 broth	6	9.28 (0.219)	6.30 (0.164)	1.05
Brilliant green bile broth 2% (BBL)	6	8.38 (0.333)	5.45 (0.0)	1.17
Levine EMB agar	6	8.59 (0.245)	5.73 (0.241)	1.38
Brilliant green bile broth 2% (Difco)	6	8.31 (0.372)	5.55 (0.155)	1.74
GN broth	6	9.35 (0.115)	6.62 (0.138)	1.86
EMB agar	6	8.65 (0.162)	5.94 (0.351)	1.95
Brain heart infusion broth	6	8.16 (0.457)	5.53 (0.192)	2.34
Lysine iron agar	6	9.21 (0.218)	5.66 (0.337)	2.82
XLD agar	6	8.25 (0.085)	5.77 (0.636)	3.31
Klinger iron agar (Difco)	6	8.70 (0.232)	6.34 (0.422)	4.37
MacConkey agar	6	8.95 (0.128)	6.15 (0.431)	5.01
Selenite cystine broth	6	7.53 (0.264)	5.27 (0.298)	5.50
Tryptic soy broth	19	9.81 (0.253)	7.57 (0.325)	5.75
Shigella broth + novobiocin (3 μg/ml)	6	9.65 (0.162)	7.57 (0.268)	8.32
Tryptose phosphate broth	6	9.00 (0.218)	6.94 (0.216)	8.71
XL agar base Taylor	6	8.64 (0.256)	6.58 (0.305)	8.71
Kliger iron agar (BBL)	6	8.40 (0.156)	6.35 (0.675)	8.91
Desoxycholate agar	6	8.47 (0.076)	6.43 (0.236)	9.12
Endo agar (Oxoid)	6	8.27 (0.063)	6.45 (0.391)	15.13
Lauryl tryptose broth	6	8.03 (0.285)	6.46 (0.099)	26.90
Desoxycholate lactose agar	6	8.03 (0.229)	6.54 (0.312)	33.11
Violet red bile agar	6	7.42 (0.342)	6.70 (0.284)	109.57
Desoxycholate citrate agar	6	8.29 (0.071)	7.61 (0.341)	208.92
SS agar	6	7.77 (0.258)	7.79 (0.340)	1047.21

[a] Agar was removed from agar containing media by centrifugation of liquid suspension.

nies (Table 1). Growth of the organism in most media tested showed little loss of CR binding, with approximately 15 or fewer white colonies appearing for every 1000 red colonies (Table 1). Lauryl tryptose both gave approximately 27 white colonies/1000 red colonies and desoxycholate lactose agar gave approximately 33 white colonies/1000 red colonies. Three media (with agar removed by centrifugation) gave increased numbers of white colonies: growth in violet red bile gave approximately 191 white colonies for every 1000 red colonies; desoxycholate citrate gave approximately 209 white/1000 red colonies; and SS produced 1047 white colonies/1000 red colonies (Table 1).

A large number of compounds were added to TSB and tested for their effect on CR binding (Table 2, Fig. 1). Those compounds which were found to have little or no effect (i.e., loss of CR binding was approximately that found with TSB alone) at levels which permitted growth are shown in Table 2. These compounds included salts, anionic detergents, non-ionic detergents, sugars, disodium EDTA, and the dyes, basic fuschin and brilliant green.

Data with compounds that led to loss of CR binding when present during growth of *S. flexneri* are shown in Fig. 1. The anionic detergents, tergitol 7 and sodium dodecyl sulfate, were particularly effective in inducing loss of CR binding. The highest concentration of tergitol 7 tested gave more than 200 000 white colonies/1000 red colonies.

TABLE 2

Compounds that did not result in loss of CR binding in *S. flexneri* 5348

Compound tested	Levels tested (%)[a]
Sodium chloride[b]	1.0–3.0
Sodium nitrite	0.01–0.03
Sodium lactate[b]	1.0–10.0
Sodium citrate[b]	1.0–3.0
Sodium acetate[b]	0.1–1.0
Sodium thiosulfate	0.5–5.0
Sodium sulfite	0.1–1.0
Sodium taurocholate	1.0–5.0
Sodium glycodeoxycholate	1.0–7.5
Sodium cholate	1.0–5.0
Sodium taurodeoxycholate	1.0–5.0
Triton X-100	1.0–10.0
Tween 80	1.0–10.0
Polyoxylene ether W-1	1.0–10.0
Tween 20	1.0–10.0
Tergitol NP7	1.0–10.0
Lactose[b]	1.0–10.0
Fructose	1.0–4.0
Glucose[b]	1.0–5.0
Disodium ethylene dinitrilotetraacetic acid	0.001–0.01
Basic fuchsin	0.001–0.004
Brilliant green	0.000001–0.000016

[a] Levels tested permitted growth.

[b] These compounds were added to TSB before autoclaving. The other compounds including those used in Fig. 1 were sterilized by filtration and then added to sterile TSB.

However, sodium dodecyl sulfate was not quite as effective as tergitol 7, yielding approximately 3000 white colonies/1000 red colonies at the highest concentration. The dyes, eosin Y, methylene blue chloride, and crystal violet, were also active in inducing loss of CR binding in *S. flexneri* but were not as effective as the anionic detergents (Fig. 1). Bile salts #3 was moderately active whereas sodium deoxycholate (an anionic detergent) had only weak activity in inducing loss of CR binding (Fig. 1).

Several compounds which caused a loss of CR binding (Fig. 1) are also present as components of some tested media in Table 1. Tergitol 7 is present in Tergitol 7 agar at a level of 0.01% which is much lower than that necessary to induce loss of CR binding (Fig. 1). Sodium dodecyl sulfate is found in lauryl tryptose broth at 0.01%; at that level, the detergent is not very effective in inducing loss of CR binding (Fig. 1). MacConkey agar and violet red bile agar both contain crystal violet and bile salts #3. Bile salts are present in both media at 0.15%, while MacConkey agar contains 0.0001% crystal violet and violet red bile contains 0.0002% crystal violet. The levels of bile salts and crystal violet present in MacConkey agar do not explain why there is little loss of CR binding with that medium, but neither do the levels found in violet red bile agar explain why there is a much greater loss of CR binding than might be expected (Table 1 and Fig. 1).

Eosin Y (0.04%) and methylene blue (0.006%) are present in EMB and Levine EMB agars. The level of methylene blue present in these media would suggest that more loss of CR binding should be expected (Table 1, Fig. 1). Sodium deoxycholate is present in desoxycholate lactose agar at 0.05%, in desoxycholate agar at 0.1%, and in desoxycholate citrate agar at 0.5%. Desoxycholate lactose or desoxycholate citrate media showed greater loss of CR binding than expected on the basis of the level of sodium desoxycholate present (Table 1, Fig. 1). The loss of CR binding when *S. flexneri* is grown in selective media is complex and does not appear to be related solely to single components found in these media. Rather it appears related to the complex mixture of compounds present.

When the pH of TSB was varied from 5.0 to 8.0, there was no increase in loss of CR binding as compared to the control TSB (data not shown). Long term incubation (up to 120 h) at 24, 37 and 42 °C also did not lead to an increase in loss by CR binding (data not shown). Growth was not always observed at 43 or 44 °C; when it occurred, it was delayed by several days. CR binding of colonies from delayed growth at 43 and 44 °C was quite variable with some cultures showing little or no loss of CR binding, whereas other cultures showed considerable loss of binding. Jungmann and de Souza Ferreira [6] found that the incubation of cultures of *E. coli* containing the F_0lac plasmid at temperatures of 44.5 °C or above was the most important factor in loss of the F_0lac plasmid. The type of media used also was important but played a secondary role to temperature.

The data obtained in this study indicate that with few exceptions, the individual components of commercially available selective media at the levels present in those media have little or no effect on loss of CR binding in *S. flexneri* 5348. Only when concentrations of certain of these components were increased to much higher levels (Fig. 1) did they lead to loss of CR binding. At pH values and temperatures that permitted growth, no major loss of CR binding occurred. Since loss of CR binding probably

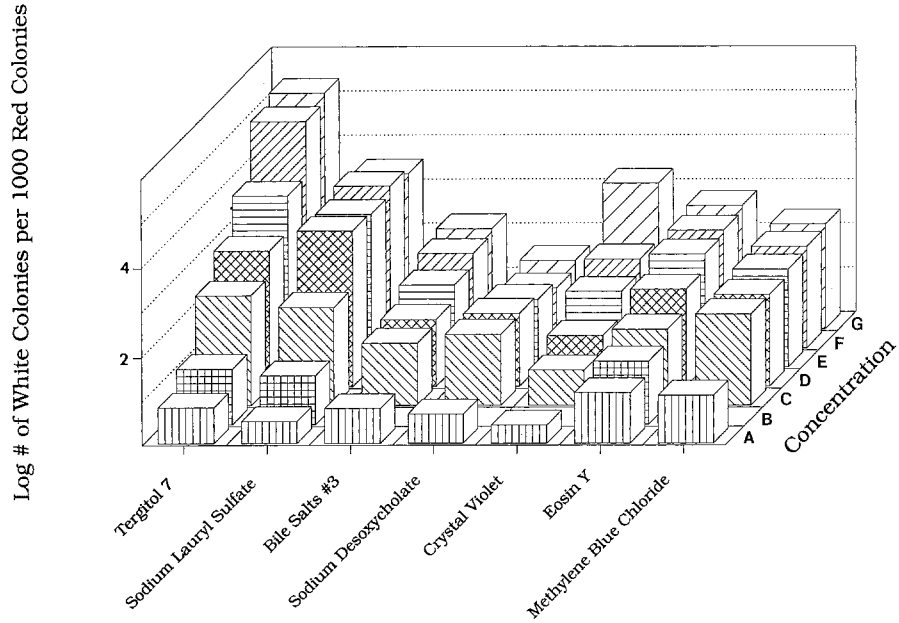

Fig. 1. Effect of various compounds on the loss of CR binding by *Shigella flexneri* 5348. Each bar represents the mean of 3–5 separate experiments. Concentration in percent, tergitol: A = 0.0%, B = 0.05, C = 0.1, D = 0.2, E = 0.3, F = O.4, G = 0.5; sodium dodecyl sulfate: A = 0.0%, B = 0.01, C = 0.02, D = 0.04, E = 0.06, F = 0.06, G = 0.1; bile salts #3: A = 0.0%, C = 0.32, D = 0.64, E = 0.96, F = 1.28, G = 1.60; sodium deoxycholate; A = 0.0%, C = 0.2, D = 0.4, E = 0.6, F = 0.8, G = 1.0; crystal violet: A = 0.0%, C = 0.0000625, D = 0.000125, D = 0.0001875, F = 0.00025, G = 0.000375; eosin Y: A = 0.0%, B = 0.1, C = 0.2, D = 0.4, E = 0.6, F = 0.8, G = 1.0; methylene blue: A = 0.0%, C = 0.002, D = 0.004, E = 0.006, F = 0.008.

indicates loss of the virulence plasmid, under usual isolation conditions for shigellae most selective media should permit the isolation of virulent shigellae (i.e., CR positive) if they are present in food or clinical samples. However, the use of violet red bile, desoxycholate citrate, and SS agars may be contraindicated because of substantial loss of congo red binding with the interpretation that virulence plasmid loss or deletion may have occurred.

REFERENCES

1 Chambers, C.E., S.L. Stockman and D.W. Niesel. 1985. Thermoregulated expression of a cloned congo red binding activity gene of *Shigella flexneri.* FEMS Microbiol. Lett. 28: 281–286.

2 Daskaleros, P.A. and S.M. Payne. 1985. Cloning the gene for congo red binding in *Shigella flexneri.* Infect. Immun. 48: 165–168.

3 Daskaleros, P.A. and S.M. Payne. 1987. Congo red binding phenotype is associated with hemin binding and increased infectivity of *Shigella flexneri* in the HeLa cell model. Infect. Immun. 55: 1393–1398.

4 Hale, T.L. and S.B. Formal. 1986. Genetics of virulence in *Shigella.* Microb. Pathogen. 1: 511–518.

5 Hill, W.E. and C.L. Carlisle. 1981. Loss of plasmids during enrichment for *Escherichia coli.* Appl. Environ. Microbiol. 41: 1046–1048.

6 Jungmann, D.M. and L.C. de Souza Ferreira 1987. Plasmid curing of *Escherichia coli* and *Salmonella typhimurium* by treatment with sodium docecyl sulfate and high temperature incubation. Rev. Microbiol., Sao Paulo 18 (2): 178–183.

7 Maurelli, A.T., B. Blackmon and R. Curtiss III. 1984. Loss of pigmentation in *Shigella flexneri* 2a is correlated with loss of virulence and virulence-associated plasmid. Infect. Immun. 43: 397–401.

8 Michel-Briand, Y., J.M. Laporte, G. Couetdic and P.J. Sansonetti. 1986. Elimentation of a virulence plasmid from *Shigella sonnei* and *Escherichia coli* by antibiotics. Ann. Inst. Pasteur Microbiol. 137B: 291–295.

9 Sakai, T., C. Sasakawa, S. Makino, K. Kamata and M. Yoshikawa. 1986. Molecular cloning of a genetic determinant for congo red binding ability which is essential for the virulence of *Shigella flexneri.* Infect. Immun. 51: 476–482.

10 Sasakawa, C., K. Kamata, T. Sakai, S.Y. Murayama, S. Makino and M. Yoshikawa. 1986. Molecular alteration of the 140-megadalton plasmid associated with loss of virulence and congo red binding activity in *Shigella flexneri.* Infect. Immun. 51: 470–475.

11 Trevors, J.T. 1986. Plasmid curing in bacteria. FEMS Microbiol. Rev. 32: 149–157.