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Convenient Agarose Medium for Simultaneous Determination of the Low-Calcium Response and Congo Red Binding by Virulent Strains of *Yersinia enterocolitica*

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A simple, efficient method for identification and differentiation of *Yersinia enterocolitica* containing virulent plasmid-bearing clones is described. The method is based on temperature of incubation and the low calcium response of the organism in an agarose Congo red medium.

Yersinia enterocolitica is now recognized as a food-borne pathogen of significance to humans. Not all strains of *Y. enterocolitica* can cause disease. Of the many serobiotypes of *Y. enterocolitica*, only a few of these have been implicated in disease (5–7, 12). The virulent strains of *Y. enterocolitica* contain a 40- to 45-MDa plasmid which is involved in virulence (5–7, 12). A number of plasmid-mediated phenotypic characteristics have been described and used to distinguish between virulent, plasmid-bearing (P^+) and avirulent, plasmidless (P^-) strains of *Y. enterocolitica* (1–3, 5–7, 12). Recently, we developed a simplified assay based on crystal violet binding (3) and calcium dependency, also known as low calcium response (LCR) (5, 9), as indicators of virulence in *Y. enterocolitica*. A disadvantage of the former technique is that the extra step of flooding plates with crystal violet solution kills the cells. Furthermore, because of the instability of the virulence plasmid at 37°C, it is difficult to isolate P^+ strains after its detection, since plasmid loss results in the loss of virulence and the concomitant disappearance of the associated phenotypic characteristics. The problem is quite obvious in clinical laboratories in which incubation at 37°C is a standard procedure. This leads to the confusion of whether one is dealing with virulent or nonvirulent strains. The mouse virulence assay (7, 12–14) can be used for the isolation of P^+ strains of *Y. enterocolitica*; however, the procedure is complex and impractical for routine use, particularly in field laboratories. The present study describes a convenient medium that is useful for the simultaneous determination of Congo red (CR) uptake and LCR and that permits the rapid identification and subsequent isolation of P^+ strains of *Y. enterocolitica*. In addition, the growth conditions required for the expression of CR binding are described.

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Five different P^+ and their isogenic P^- strains, GER (serotype O:3), EWMS (serotype O:13), PT18-1 (serotypes O:5, O:27), O:TAC (serotype O:TACOMA), and WA (serotype O:8), representing five serotypes of *Y. enterocolitica*, were used in this study. Detailed descriptions of the strains and sources are given elsewhere (4). Agarose (Sigma Chemical Co., St. Louis, Mo.) and agar (Difco Laboratories, Detroit, Mich.) were used as gelling agents for the preparation of calcium-deficient brain heart infusion (BHI), agarose (BHO), and calcium-adequate BHI agar (BHA) (5). Taking advantage of the noninhibitory nature of CR (Sigma), the dye was added at the desired concentration prior to autoclaving of BHO and BHA. These two media were designated CR-BHO and CR-BHA, respectively. Various levels of calcium were also added to CR-BHO to determine its effect on CR binding. The addition of CR did not alter the concentration of calcium in either CR-BHO (238 μM) or CR-BHA (1,500 μM), which remained calcium deficient and calcium adequate, respectively (5). P^+ and P^- cells were grown separately in BHI broth for 18 h at 25°C with shaking. The cells were diluted to 10^3 cells per ml and surface plated on CR-BHO and CR-BHA (5). The plates were then incubated at 37 and 25°C. CR binding as indicated by distinct pinpoint red colonies was observed after the plates were incubated at 37°C for 24 h. The LCR colony morphology was also determined as described previously (5).

The presence of plasmid DNA in bacteria was determined by the method of Bhaduri (2). Tests for crystal violet binding, LCR, autoagglutination, hydrophobicity by latex particle agglutination, and mouse virulence were performed as previously described (3, 5, 10).

We observed that when P^+ and P^- strains were cultivated at 37°C on CR-BHO, two colony types were readily discernible after 24 h. The P^+ strain formed red pinpoint colonies having a colony size of 0.36 mm in diameter (Fig. 1A). The P^- colonies did not bind CR but formed much larger white or light-orange colonies (1.37 mm in diameter) (Fig. 1B). The size and colony morphologies of P^+ and P^- strains in CR-BHO were similar to those found in a typical calcium-deficient medium as reported previously (5, 15). On calcium-adequate CR-BHA, colonies of both P^+ and P^- remained white or light orange and their respective colony sizes were similar, as reported previously (5) (Fig. 1C and D). When cells were grown at 25°C under similar conditions neither P^+ nor P^- strains bound CR. P^+ cells formed white or light-orange small colonies of 0.8 mm in diameter, whereas the P^- cells formed large white or orange colonies of 1.9 mm in diameter. On the basis of color contrast between the colony and the medium, optimum concentration of CR in the binding assay was found to be 75 μg/ml. The actual dye content of the CR powder was taken into account in deter-

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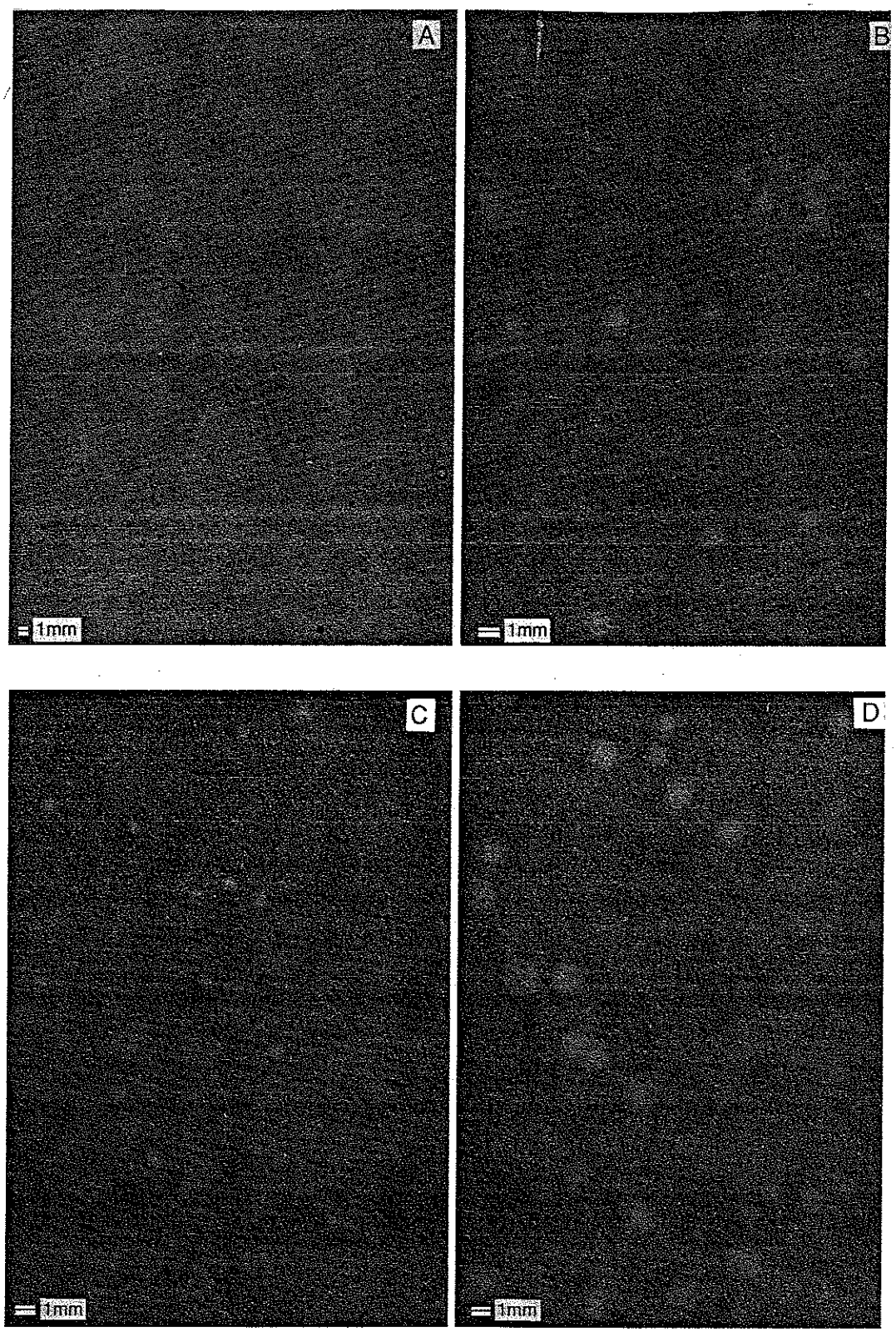

FIG. 1. CR binding of colonies of *Y. enterocolitica* GER (serotype O:3) with cells grown on CR-BHO and CR-BHA for 24 h at 37°C. (A) Virulent P^+ cells showing red (dark) pinpoint colonies (0.36 mm in original diameter). (B) Avirulent P^- cells showing large white or light orange colonies (1.37 mm in original diameter). (C) Small white or light orange colonies (0.97 mm in original diameter) of virulent P^+ cells. (D) Large white or light orange colonies (1.9 mm in original diameter) of avirulent P^- cells. The concentration of CR used in the binding assay was 75 μg/ml.

TABLE 1. Efficiency of CR binding in mixed cultures of virulent and avirulent strains[a]

Sample	Estimated no. of colonies in the mixture		No. (%) of colonies which bound to CR[b]
	Avirulent	Virulent	
A	67	4	4 (100)
B	63	10	10 (100)
C	49	30	30 (100)
D	35	50	50 (100)
E	21	70	70 (100)
F	7	90	90 (100)

[a] Virulent cells of *Y. enterocolitica* GER (serotype O:3) were mixed in various ratios with cells from plasmidless GER strain and surfaced plated on CR-BHO. The mixed colonies were incubated at 37°C for 24 h. The number of virulent colonies was determined by the appearance of red pinpoint colonies.
[b] Average percentage of efficiency was 100%.

mining the concentration. At reduced concentrations, the color intensity of the P^+ colonies was low. At the elevated concentrations, the background color intensity was high. The CR binding technique could identify quantitatively individual plasmid-bearing colonies from a mixed culture of P^+ and P^- strains (Table 1). As the calcium concentration of CR-BHO was increased from 238 to 1,276 μM by the addition of exogenous calcium chloride, the P^+ cells did not abolish the CR absorption until it was about 1,276 μM (Table 2). In contrast, colony size gradually increased, abolishing the unique colonial morphology as reported previously (5). This technique has been successfully applied to different serotypes of *Y. enterocolitica*. CR binding was clearly associated with the virulence plasmid. Thus the agarose-based medium was able to detect expression of both LCR and CR binding.

Comparison of CR absorption to crystal violet binding and hydrophobicity for strain GER (serotype O:3) in calcium-deficient (BHO, CR-BHO) and calcium-adequate (BHA, CR-BHA) media at both 37 and 25°C showed CR binding occurred at 37°C but not at 25°C in calcium-deficient medium, indicating non-LCR characteristics. In contrast, crystal violet binding and hydrophobicity by latex particle agglutination were expressed in both calcium-deficient and calcium-adequate media only at 37°C, indicating that these are non-LCR virulence properties. Identical data were obtained for the other four strains representing four serotypes used in this study.

Studies on CR binding by virulent strains of *Y. enterocolitica* have brought about considerable confusion. Initially, CR binding was reported to be associated with the virulence plasmid but was not temperature or calcium dependent (13). However, subsequent studies reported CR binding by virulent *Y. enterocolitica* to be associated with the chromosome (3, 14). A reevaluation in the present study, using BHI with either agarose or agar as gelling agent, indicates that CR binding is indeed associated with the virulence plasmid and requires an environment containing low levels of calcium at 37°C for its expression. The recent study by Riley and Toma (15) describing the association of CR binding with LCR by using CR-magnesium oxalate agar among various strains of *Y. enterocolitica* is consistent with our results. However, the use of oxalate to complex calcium in CR-magnesium oxalate agar (5, 15) raised the possibility that other essential minerals may be sequestered (8) thereby affecting bacterial behavior (5). In fact, the colony size of the P^+ cells on magnesium oxalate agar was smaller than that on BHO after the same period of incubation, suggesting that oxalate either may be toxic or may bind other essential minerals (5, 8). The binding of CR appears to be unrelated to crystal violet binding and hydrophobicity. Two independent phenomena may be involved since the expression of the latter two virulence properties are non-LCR. Moreover, the combined use of CR-BHO, crystal violet binding, and hydrophobicity by latex particle agglutination provides a method for accurately differentiating between pathogenic and nonpathogenic *Y. enterocolitica*.

TABLE 2. Effect of calcium concentration on CR binding by plasmid-bearing virulent strains of *Y. enterocolitica*

Medium and addition of exogenous calcium (μM)	Final calcium concentration (μM)	CR binding	Diam of colony (mm)
CR-BHO			
None	238	+	0.36
100	338	+	0.42
150	388	+	0.50
238	476	+	0.78
600	838	+	0.87
800	1.038	+	0.94
1,038	1.276	−	1.20
CR-BHA (no added calcium)	1,500	−	1.13

Studies were done to determine the usefulness of CR-agarose medium for direct isolation of plasmid-bearing virulent strains of *Y. enterocolitica* after its detection based on CR binding. The P^+ cells were diluted and surface plated on CR-BHO as described above and incubated at 37°C. Colonies were observed at 12, 24, 48, and 96 h. At 12 h, red pinpoint colonies were seen. At 24 and 48 h and even more so at 96 h, these colonies retained the red center, but a white opaque circumference appeared around the red center (Fig. 2A and 2B). (The white border around the red center of the colony appears darker in the figure because of the red background of the agar plate, red-pigmented center, and reflected light source during the photography of the colonies.) The time required for the appearance of the white border around the red center of the colonies depends on the serotype used. Thus CR-BHO facilitated in differentiating P^+ cells from P^- cells for subsequent isolation of P^+ cells. At 12 h, red pinpoint colonies were picked so that those colonies would have less chance of losing the plasmid. Hence, the red pinpoint colonies at 12 h were picked up aseptically by a sterile inoculating needle, and transferred to 5 ml of BHI broth. The inoculated broth was grown at 25°C for 18 h. A portion of the original culture was diluted to 10^3 cells per ml and surface plated on CR-BHO and BHA. The plates were incubated at 37°C for 24 h. The remaining culture was stored at 4°C for further confirmation of the presence of the virulence plasmid and as a source of P^+ strains for future experiments. The presence of the virulence plasmid in the recovered cells was indicated by the binding of CR in the CR-BHO plate and by flooding the BHA plate with crystal violet. To further confirm that the red pinpoint portion of P^+ colonies maintained the presence of the plasmid whereas the white border of the colonies was devoid of the virulence plasmid, the following tests were performed. At 24 h, cells were removed with a sterile inoculating needle from both the red center and the white border of the colonies. The presence of the virulence plasmid at the red center and absence in the white border from the same colony was confirmed as described above. The cells from the red center were con-

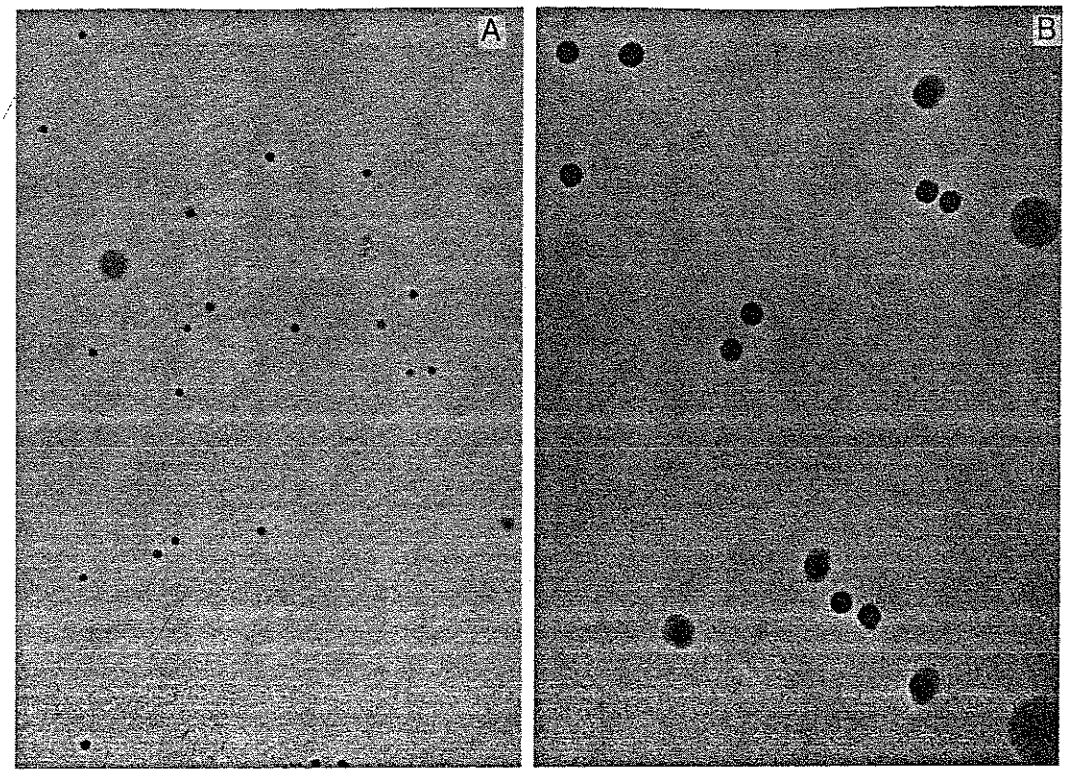

FIG. 2. CR binding of colonies of *Y. enterocolitica* GER (serotype O:3) with virulent P^+ cells grown on CR-BHO for 24 and 48 h at 37°C. (A) White border around the red center of the colony after 24 h of incubation. (B) White wider border around the red center of the colony after 48 h of incubation.

firmed to be P^+, whereas the cells from the white border were found to be P^-. The virulence of the stored recovered cells in BHI was further confirmed by the presence of plasmid and a number of virulence-associated properties, including crystal violet binding, CR uptake, autoagglutination, hydrophobicity, and mouse virulence test results. The presence of the virulence plasmid in the red pinpoint colonies surrounded by the white border from the original CR-BHO was also confirmed by hybridization with a synthetic 24-base oligonucleotide DNA probe (SP12) (11) prior to recovery (16). The recovery technique was successfully applied to all five serotypes of *Y. enterocolitica*. The recovery of the plasmid-bearing cells varied from 5 to 95%, indicating strain variation in the stability of the plasmid.

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