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Differentation Between Virulent and Avirulent *Yersinia enterocolitica* Isolates by Using Congo Red Agar

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Cultivation of clinical isolates of *Yersinia enterocolitica* of diverse geographical origin on a medium containing 5 μg of Congo red per ml disclosed two colony types. These were designated CR^+ and CR^- according to their ability to bind Congo red. CR^+ strains bore plasmids of between 40 and 50 megadaltons and were positive in several tests of *Y. enterocolitica* virulence, including autoagglutination, reduced growth on magnesium oxalate agar, resistance to the bactericidal effect of serum, and lethality for iron-overloaded mice. CR^- strains were plasmidless and were negative in all these assays. The Congo red reaction provides a simple and efficient means of screening *Y. enterocolitica* for virulence and is the best available method for identifying individual plasmid-bearing colonies.

Yersinia enterocolitica has been implicated in a broad array of clinical conditions, including acute gastroenteritis, mesenteric adenitis, septicemia, arthritis, and erythema nodosum (3). Although the determinants responsible for the pathogenicity of *Y. enterocolitica* in these disorders have not been fully identified, it has been established that virulence is associated with the presence of plasmids with masses of 40 to 82 megadaltons (Md) (6, 11, 18, 26).

Properties determined by these plasmids include autoagglutination (12), calcium dependence (5, 6), production of V and W antigens (5), detachment of tissue culture monolayers (18), pathogenicity for mice (21), alteration in outer membrane proteins (2, 18), and serum resistance (15). Most of these characteristics are temperature dependent.

The ability of *Yersinia pestis* to absorb hemin and Congo red from agar media is correlated with virulence (8, 24). This correlation has also been noted for other bacteria (17). In this paper we report that the capacity to take up Congo red is also associated with virulence of *Y. enterocolitica* and that this property is plasmid mediated. The Congo red reaction, therefore, provides a simple indicator of virulence in *Y. enterocolitica*.

MATERIALS AND METHODS

Bacteria. Seven strains of *Y. enterocolitica* isolated from patients in Belgium, Canada, South Africa, Sweden, and the United States were examined (Table 1). Bacteria were screened for the presence of virulence-associated plasmids on magnesium oxalate (MOX) agar (6, 7), and the colonial variants thus obtained were frozen at −20°C as thick suspensions in 34% glycerol–1% peptone.

Congo red agar. Stock cultures initially were plated onto the Congo red agar described by Payne and Finkelstein (17). Although some differential pigmentation was evident, color differences were not great and became less pronounced with prolonged incubation. Several different base media were investigated. That which gave the most pronounced differential pigmentation was Congo red acid-morpholinepropanesulfonic acid pigmentation (CRAMP) agar. CRAMP agar contained 0.2% (wt/vol) galactose, 0.2% Casamino Acids, and 5 μg of Congo red per ml in a basal salts solution (14) composed of 50 mM NaCl, 40 mM morpholinepropanesulfonic acid, 10 mM NH_4Cl, 2.5 mM $Na_2S_2O_3$, 1.4 mM K_2HPO_4, 0.4 mM $MgSO_4$, and 10 mM Tricine (Sigma Chemical Co., St. Louis, Mo.), pH 5.3 (unadjusted), and solidified with 1.4% agarose. On this medium, *Y. enterocolitica* displayed two colony types: intensely colored (CR^+) colonies which bound Congo red and colonies which remained nonpigmented (CR^-) (Fig. 1).

Congo red binding assay. The ability of bacteria to bind Congo red was tested by a modification of the method of Payne and Finkelstein (17). Strains were grown in a 1% tryptone–0.25% yeast extract (TYE) broth with shaking at 25 or 37°C for 30 h. Cells were centrifuged, washed with phosphate-buffered saline, pH 7.2, suspended to a concentration of 10^9 cells per ml in phosphate-buffered saline containing 30 μg of Congo red per ml, and incubated at 4, 25, or 37°C with shaking for 12 h. At hourly intervals, samples were removed and centrifuged. The absorbance of the supernatant at 488 nm was measured, and the concentration of Congo red remaining was determined by comparison with a standard curve. Uptake of greater than 15 μg per 10^9 cells was scored as a positive result.

Calcium dependency. Virulent strains of *Y. enterocolitica* require calcium for growth at 37°C (6). Calcium dependency was tested with MOX agar (7) which

TABLE 1. *Y. enterocolitica* strains examined

Strain	Sero-group	Country of origin	Rapid Congo red uptake[a]	Mouse lethality (LD_{50})[a,b]	Calcium dependence[a]	Serum resistance[a]	Auto-agglutination[a]	Sereny response[a]	Plasmid size (Md)
WA									
CR^{+}[c]	O8	United States	+	50	+	+	+	+	40
CR^{-}[c]			−	$>1.0 \times 10^5$	−	−	−	−	ND[d]
30.42.67									
CR^{+}	O3	Sweden	+	2.0×10^5	+	+	+	−	47
CR^{-}			−	$>5.0 \times 10^8$	−	−	−	−	ND
135									
CR^{+}	O3	South Africa	+	NT[e]	+	+	+	−	47
CR^{-}			−	NT	−	−	−	−	ND
518									
CR^{+}	O9	Belgium	+	6.4×10^6	+	+	+	−	50
CR^{-}			−	$>1.4 \times 10^8$	−	−	−	−	ND
526									
CR^{+}	O3	Belgium	+	NT	+	+	+	−	50
CR^{-}			−	NT	−	−	−	−	ND
4209									
CR^{+}	O5	Canada	+	7.4×10^3	+	+	+	−	50
CR^{-}			−	$>2.5 \times 10^8$	−	−	−	−	ND
6003									
CR^{+}	O3	Canada	+	NT	+	+	+	−	44
CR^{-}			−	NT	−	−	−	−	ND

[a] See text for explanation.
[b] LD_{50}, 50% lethal dose.
[c] CR^{+} and CR^{-} indicate capacity of bacteria to bind pigment or not to bind pigment, respectively, on CRAMP agar.
[d] ND, No plasmids detected.
[e] NT, Not tested.

contained 0.02 M sodium oxalate and 0.02 M $MgCl_2$ in Columbia agar base (Oxoid Ltd., Basingstoke, Hampshire, England). After overnight incubation at 37°C, plasmid-bearing *Y. enterocolitica* gave rise to pinpoint colonies, whereas plasmidless strains yielded colonies 0.5 to 1 mm in diameter.

Autoagglutination. Strains were tested for autoagglutination in tissue culture medium as previously described (12). It was found, however, that autoagglutination could readily be demonstrated in phosphate-buffered saline by using cells grown for 24 h at 37°C in TYE broth, washing them in phosphate-buffered saline, and suspending them to a concentration of 10^9 cells per ml. Suspensions were incubated at 25 and 37°C with shaking. Autoagglutination was apparent after 1 h at 37°C. No autoagglutination occurred at 25°C.

Serum sensitivity. Resistance of strains to the bactericidal effects of normal human serum was tested by the method of Pai and DeStephano (15), modified first by selecting CR^{+} and CR^{-} variants on CRAMP agar and second by growing the cells with shaking in TYE broth rather than on MOX agar. Under these conditions, serum-sensitive strains (CR^{-}) gave no survivors after 1 h, whereas serum-resistant (CR^{+}) bacteria survived and began to multiply after 2 h.

Mouse lethality. With the exception of serogroup O8, *Y. enterocolitica* strains are avirulent for mice. However, iron-overloaded mice have been used to demonstrate virulence of serogroups O3 and O9 (13, 20, 23). Accordingly, strains investigated for mouse lethality were suspended in iron dextran before use. Cells grown for 48 h on nutrient agar at 25°C were harvested in saline, washed, and suspended to a concentration of 10^8 cells per ml. Decimal dilutions were prepared in 0.85% saline and mixed with equal volumes of 20% (vol/vol) iron dextran (Imferon; Fisons Pty. Ltd., Sydney, Australia) in saline. Determinations of the 50% lethal dose (19) were undertaken in groups of five BALB/c adult female mice, where each mouse received an intraperitoneal injection of 1 ml of a particular bacterial suspension. Controls included mice inoculated with the highest concentration of cells without iron dextran and mice which received 10^8 heat-killed bacteria with iron.

Guinea pig keratoconjunctivitis (Sereny) test. Strains were grown on nutrient agar at 25 or 37°C for 48 h. A thick paste of CR^{+} bacteria was inoculated into the conjunctival sac of one eye of an adult male guinea pig (22). The other eye was inoculated with the CR^{-} derivative of the same strain. Serogroups other than O8 were also inoculated with 10% iron dextran. Guin-

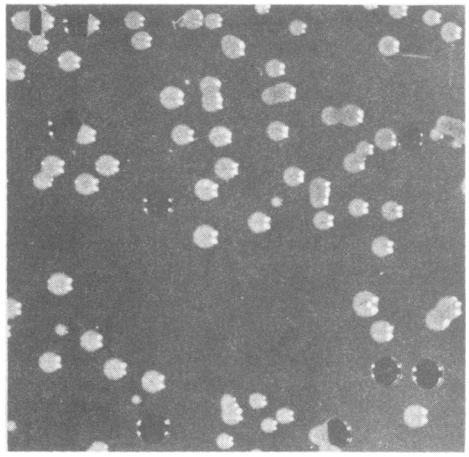

FIG. 1. CR^+ and CR^- colonies of *Y. enterocolitica* strain 30.42.67 on CRAMP agar after 48 h at 25°C. Dark (CR^+) colonies were approximately 1 mm in diameter.

ea pigs were observed for the development of purulent keratoconjunctivitis for 7 days.

Isolation and characterization of plasmids. Plasmid DNA was isolated from overnight cultures in TYE broth at 25°C by the method of Birnboim and Doly (1) or Portnoy et al. (18). Molecular weights of *Y. enterocolitica* plasmids were determined by comparing their mobility with those of standard plasmids from *Escherichia coli*. Standard plasmids used were R6K (26 Md), R46 (33 Md), RP4 (36 Md), TP114 (41 Md), and TP113 (57 Md) (10).

RESULTS

Pigmentation on Congo red media. In initial experiments with CRAMP agar adjusted to pH 7.0, all *Y. enterocolitica* strains examined displayed two colony types after 72 h at 25°C. CR^+ variants were an intense red, whereas CF^- colonies ranged from colorless to pale pink. Subsequently, it was found that when the pH of the medium was left unadjusted (pH 5.3), differential pigmentation was more pronounced and unequivocal: CR^+ colonies became dark violet after 48 h at 25°C, whereas CR^- colonies remained colorless and translucent (Fig. 1). CRAMP agar modified by omitting galactose or by substituting galactose with glucose, glycerol, lactose, sucrose, or mannitol was also investigated. In each case, either acid production was so excessive that differential pigmentation was obscured or growth was unacceptably scanty. Similar findings have been reported for *Y. pestis* (4).

The stability of the Congo red reaction of *Y. enterocolitica* was investigated by subculturing CR^+ strains on CRAMP agar. In these experiments, CR^+ colonies always yielded a mixture of CR^+ and CR^- colonies, with the proportion of CR^+ colonies ranging between 70 and 97%. In contrast, CR^- colonies remained nonpigmented.

The behavior of CR^+ and CR^- colonies was further examined in a rapid Congo red binding assay. CR^+ cells grown at 25°C bound Congo red after 6 h, but only when the assay was performed at 37°C. The same bacteria grown at 37°C, however, demonstrated rapid uptake of dye (greater than 15 $\mu g/10^9$ cells in 1 h) at 4, 25, and 37°C. CR^- cells, on the other hand, bound very little dye, even after 12 h, irrespective of growth and assay temperatures.

Correlation between pigmentation and virulence. CR^+ and CR^- pairs derived from each strain were examined for various virulence-associated characteristics. In every case, pigmentation was correlated with virulence, as shown by lethality for iron-overloaded mice, resistance to the bactericidal effects of human serum, autoagglutination at 37°C, and reduced growth on MOX agar at 37°C (Table 1). Although the Sereny test has been used as a test for virulence of *Y. enterocolitica* (6, 26), we found that only CR^+ derivatives of the serogroup O8 strain WA evoked clear-cut keratoconjunctivitis.

To confirm the correlation between ability to bind Congo red and other virulence characteristics, we determined the Congo red reaction of *Y. enterocolitica* strains which expressed these characteristics. Surviving colonies from serum killing experiments always contained an overwhelming majority of CR^+ cells, as did autoagglutinating strains. Pinpoint colonies on MOX agar also contained CR^+ cells (usually about 4 to 20%), whereas large colonies were almost exclusively CR^-.

Correlation between pigmentation and plasmid carriage. As shown in Table 1 and Fig. 2, all CR^+ strains harbored plasmids between 40 and 50 Md. Plasmids were not detected in CR^- derivatives. Furthermore, in all strains examined, loss of plasmid resulted in loss of Congo red binding, as well as other virulence properties.

DISCUSSION

Experimental procedures to determine *Y. enterocolitica* pathogenicity are costly, unreliable, or both. Although the virulence of certain serogroups can be demonstrated effectively in laboratory animals (4, 16, 18, 21, 23), such tests are generally not suitable for routine diagnostic use. In vitro methods such as serum resistance (15) and autoagglutination (12) are laborious, and the widely used test for calcium dependence has the disadvantage that virulent strains grow poorly, if at all, on MOX agar (6, 15). Our results indicate that pigmentation on CRAMP agar differentiates simply and rapidly between virulent and avirulent strains of *Y. enterocolitica*.

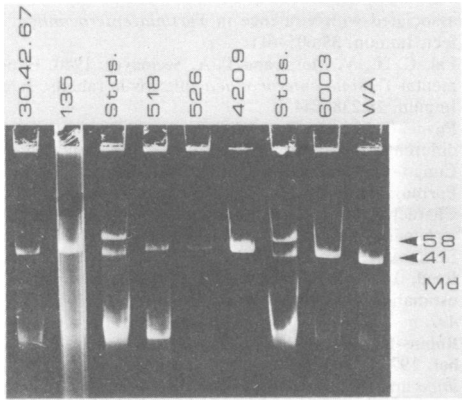

FIG. 2. Agarose gel electrophoresis of plasmid DNA from seven CR^+ strains of *Y. enterocolitica*. The lower band in each track is linear DNA (predominantly chromosomal fragments). Standards (Stds.) were TP114 (41 Md) and TP113 (57 Md). Molecular weight assignments in Table 1 were confirmed against additional standards covering a broader range of molecular weights (data not shown).

The optimum growth temperature of *Y. enterocolitica* is around 25°C, and despite the elevated body temperature of host animals, strains are more virulent when grown at 25°C (4). Congo red uptake on solid media was observed at 25 and 37°C, although other virulence-associated properties, such as autoagglutination, serum resistance, and calcium dependence, were evident at 37°C only. The finding that uptake of Congo red in the rapid binding assay was more pronounced when cells were grown at 37°C is of interest. The reasons for the apparent discrepancy between growth and Congo red uptake from solid and liquid media are not clear, but it is important to note that the binding assay involves cells suspended in buffer rather than growing in culture medium.

Ability to bind Congo red appeared to be encoded by the virulence-associated plasmid of *Y. enterocolitica*: all CR^+ (and no CR^-) strains reported here, and many others examined subsequently, harbored a plasmid and expressed other virulence attributes that have been reported to be plasmid determined. Although virulence was plasmid mediated in all strains examined, the plasmids involved differed in molecular weight. Thus, in epidemiological studies, it is not sufficient to search for plasmids of a particular molecular weight as indicators of *Y. enterocolitica* virulence, especially since virulence-associated plasmids in *Y. enterocolitica* span a broad range of molecular weights (6, 11, 18, 26).

The correlation between ability to bind Congo red and virulence was most clearly demonstrated by the finding that only CR^+ strains, regardless of serogroup, were lethal for mice. Indeed, Congo red reactivity was the criterion used for selecting strains to be tested. It has been difficult to date to demonstrate virulence of *Y. enterocolitica* in animals. Only serogroup O8 is lethal for mice or induces a positive Sereny reaction. Our results demonstrated, however, that iron-overloaded mice may be used to examine virulence of serogroups O3, O5, O8, and O9.

Our data indicate that two conditions must be met for mouse virulence of these serogroups. These conditions are: readily available iron and the presence of a plasmid which encodes binding of Congo red. Virulence of *Y. pestis* has also been shown to be critically dependent on iron (9), and in that organism, binding of Congo red corresponds to the ability to take up hemin (24). The CR^+ *Y. enterocolitica* strains studied here also become pigmented on the hemin agar described by Jackson and Burrows (8) (data not shown). Since Congo red uptake parallels hemin uptake, it is possible that the ability to bind Congo red reflects a plasmid-determined system for assimilating iron, which is similar to that specified by ColV plasmids in *E. coli* (25).

The relationship between Congo red binding, iron metabolism, and the virulence-associated plasmid of *Y. enterocolitica* is currently under investigation. Regardless of the nature of this relationship, it is evident that pigmentation on solid growth media containing Congo red (or hemin) provides a simple and rapid indication of probable virulence in *Y. enterocolitica*. Furthermore, unlike MOX agar, which inhibits the growth of, and therefore tends to select against, plasmid-bearing *Y. enterocolitica*, CRAMP agar permits the identification of bacterial colonies containing a large proportion of plasmid-bearing cells.

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