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Detection and Differentiation of Iron-Responsive Avirulent Mutants on Congo Red Agar

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Agar medium containing Congo red dye differentiates virulent and avirulent colonies of *Shigella, Vibrio cholerae, Escherichia coli,* and *Neisseria meningitidis.* Like virulent plague bacilli, wild-type cells of these species absorb the dye and produce red colonies. Mutants or colonial variants have been isolated that fail to absorb the dye and produce colorless colonies. These mutants exhibit reduced virulence in the chicken embryo model, but their virulence is enhanced by supplementation with iron. Of those species tested, only *Neisseria gonorrhoeae* isolates failed to grow in the presence of this dye. Inhibition of growth by Congo red may thus provide a simple means for differentiating gonococci from other *Neisseria.*

An agar medium containing the dye Congo red was used by Surgalla and Beesley to differentiate between virulent colonies of *Yersinia pestis* possessing the P^+ determinant and avirulent colonies lacking this characteristic (17). Virulent P^+ colonies, which absorb hemin from a synthetic medium and thus appear pigmented, also absorb Congo red (9, 17). The P^- strains absorb neither hemin nor Congo red. Although normally less virulent, P^- strains are as lethal for mice as P^+ strains when iron is added to the inoculum (10). This increased virulence in the presence of iron is accompanied by an enhancement of growth in vivo to a level approximating that of P^+ strains. Iron has been shown to influence virulence of a variety of other microorganisms including *Escherichia coli* (1, 12), *Pseudomonas aeruginosa* (2), and *Mycobacterium tuberculosis* (11).

During the course of our studies on the role of iron in virulence of gonococci and other gram-negative pathogens (15), several mutants and colonial variants were obtained that were relatively avirulent in an experimental model but responded to the addition of iron with an increase in virulence. It was of interest to determine whether these mutants, like the P^- plague bacilli, could be differentiated from their virulent parent strains on the basis of Congo red absorption. This paper summarizes our observations on the use of media containing Congo red to detect and differentiate "iron-responsive" avirulent mutants.

MATERIALS AND METHODS

Bacterial strains. Strains used, their sources, and their appearances on Congo red media are listed in Table 1.

Media. *Neisseria* strains were grown on GC medium base (Difco) plus 1% defined supplement and 0.01% Congo red dye (Harleco or J. T. Baker). Trypticase soy agar (BBL) plus 0.01% Congo red was used for all other strains. Other dyes tested included trypan blue, janus green, metanil yellow, amido naphthol red, and fast green. Dyes were added either before sterilization of the medium or 1% aqueous solutions were autoclaved and added aseptically to the sterile molten agar.

Dilutions of bacterial suspensions were plated by a modification of the drop technique of Miles and Misra (14). Plates were incubated at 37°C (with 10% CO_2 for *Neisseria* strains) and examined with a stereoscope and reflected oblique lighting after 24 and 48 h of incubation. Colonies that absorbed Congo red dye (CR^+ colonies) became noticeably red in comparison with CR^- colonies, which remained translucent or white.

Chicken embryo methodology. Eleven-day-old chicken embryos were used as decribed previously (3 to 5, 15). Embryos were inoculated allantoically or on the chorioallantoic membrane (CAM) with 0.1 ml of serial dilutions of bacterial suspensions. Iron in the form of Imferon (Lakeside Laboratories) was added at a concentration of 500 μg/0.1 ml where indicated. Six to 10 embryos were used per dilution, and mean lethal dose values were calculated according to Reed and Muench (16).

Selection of mutants. CR^- mutants of *Shigella* and *E. coli* were naturally occurring colonial variants. The *Vibrio cholerae* and *N. meningitidis* mutants were induced by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (NTG) (Sigma). The procedure for NTG mutagenesis of *V. cholerae* was as described previously (6) and was modified for use with *N. meningitidis* by growing the cells in heart infusion broth (Difco) and reducing the NTG concentration to 25 μg/ml. Mutagenized cultures were plated on Congo red media, and

TABLE 1. *Bacterial strains used and their reactions on Congo red agar*

Strain	Source	Absorption of Congo red[a]
S. flexneri 2457 T	S. B. Formal	+
S. flexneri 2457 O	S. B. Formal; colonial variant of 2457 T	−
S. dysenteriae WR377	R. Wedgwood	+
S. dysenteriae WR377 CR^-	Colonial variant of WR377	−
S. sonnei MU370	SEATO Medical Research Lab., Bangkok, Thailand	+
S. sonnei MU370 CR^-	Colonial variant of MU370	−
V. cholerae 569B	N. K. Dutta	+
V. cholerae 569B CR^-	NTG mutagenesis of 569B	−
E. coli U169 (K-1^+)	G. McCracken, Jr.	+
E. coli U169 (K-1^+) CR^-	Colonial variant of U169	−
S. typhimurium LT2	P. Gemski	+/−[b]
S. typhimurium 9SR2	P. Gemski	+/−
N. meningitidis A-17	H. Schneider	+
N. meningitidis A-17 CR^-	NTG mutagenesis of A-17	−
N. meningitidis B-11	H. Schneider	+
N. meningitidis B-11 CR^-	NTG mutagenesis of B-11	−
N. gonorrhoeae F62: T1,T2,T3,T4	D. S. Kellogg, Jr.	No growth
N. gonorrhoeae 2686: T1,T4	T. M. Buchanan	No growth
N. gonorrhoeae D1-D7	Local isolates, seven strains	No growth
N. subflava	K. H. Johnston	+/−
N. sicca	K. H. Johnston	+/−

[a] By colonies on agar medium containing 0.01% Congo red.
[b] +/−, Slight uptake of dye.

colonies that failed to absorb the dye were selected after 48 h of incubation.

Congo red binding assay. Strains were grown on agar without Congo red. After overnight growth, the cells were harvested with phosphate-buffered saline (PBS), pH 7.2, centrifuged, and washed with PBS. The cells were resuspended to a concentration of 10^9 per ml (by absorbance at 600 nm) in PBS containing 0.002% Congo red. The mixture was incubated at 37°C with shaking for 1.5 h. After incubation, the cells were removed by centrifugation, and the amount of Congo red remaining in the supernatant was determined by measuring the absorbance at 500 nm and comparing with a standard curve.

RESULTS

The addition of Congo red to Trypticase soy agar or GC medium base facilitated the isolation of avirulent mutants of certain gram-negative pathogens. Wild-type *Shigella, E. coli, V. cholerae,* and *N. meningitidis* absorbed the dye from agar media, producing red colonies after 24 to 48 h of incubation. Colonies unable to absorb the dye and remaining relatively white (designated CR^-) were found to occur naturally or could be isolated after NTG mutagenesis (Table 1). A mixture of CR^+ and CR^- colonies of *Shigella flexneri* on Congo red is shown in Fig. 1. Mixtures of the two colony types of the other species had a similar appearance on Congo red agar, although CR^- mutants of these strains absorbed slightly more of the dye than the *Shigella* mutants upon prolonged incubation. The CR^+ isolates of the various *Shigella* strains all produced distinctly red colonies by 24 h of incubation, whereas the *E. coli, V. cholerae,* and *N. meningitidis* strains required at least 48 h of incubation to absorb sufficient dye to easily distinguish CR^+ and CR^- colonies. The *Salmonella typhimurium* strains tested absorbed the dye only slightly, and it was not possible to select CR^+ and CR^- colonies.

Other dyes were evaluated for their ability to discriminate between CR^+ and CR^- colony types. Trypan blue, a bis-azo dye structurally similar to Congo red, was absorbed by CR^+ but not by CR^- colonies. However, cells plated on media containing both Congo red and trypan blue appeared to absorb the Congo red preferentially. Metanil yellow also appeared to be absorbed in greater amounts by CR^+ than by CR^- colonies, but there was very little contrast between the two colony types on agar containing this dye. Janus green was absorbed slightly by both colony types, whereas fast green and amido naphthol red were absorbed by neither.

With the four genera tested, colony types that failed to absorb Congo red were 100- to 5,000-fold less virulent for chicken embryos than colony types that absorbed the dye (Table 2). As Jackson and Burrows observed earlier with *Y. pestis* (10), the addition of iron to the inoculum partially, if not totally, restored the virulence of these CR^- strains (Table 2).

The enhancement of virulence by iron was

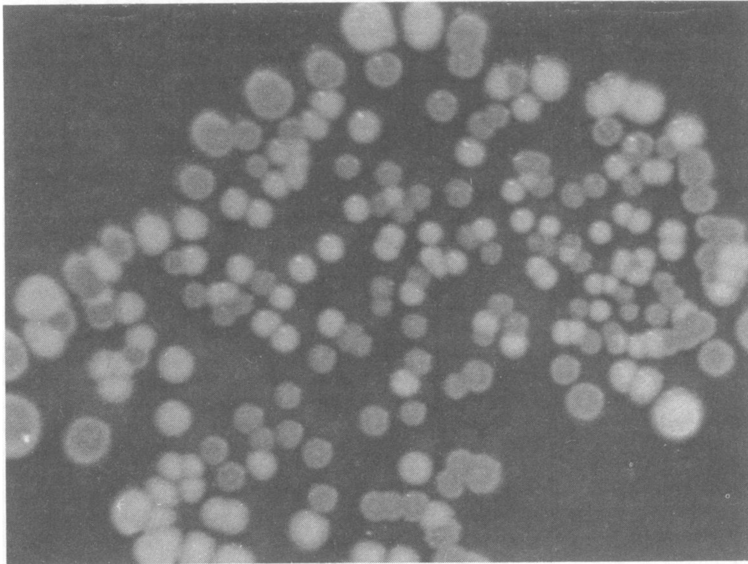

FIG. 1. *CR^+ and CR^- colonies of S. flexneri on Congo red agar.*

not due to in vivo selection of CR^+ colony types since, with the exception of the *N. meningitidis* mutant, CR^+ colonies were never recovered from infected embryos. The CR^- meningococcus occasionally yielded CR^+ colonies both in vivo and in vitro. Reversion was not observed with the other CR^- variants tested.

The iron supplement enhanced virulence of CR^- mutants when it was given simultaneously with the inoculum by the same route or when it was administered intravenously approximately 1 h before allantoic or chorioallantoic challenge. Other permutations were not tested. Dilution plating of blood samples taken at 16 to 24 h after inoculation revealed that supplementation with iron led to an increase in the numbers of bacteria in the blood of embryos inoculated with the relatively avirulent colony types of *V. cholerae* and *S. flexneri*. For example, 16 h after allantoic inoculation with 10^4 colony-forming units of avirulent *Shigella,* no bacteria were detected in the blood of the embryos (i.e., $<10^2$ per ml). In those embryos that had received iron, the level of bacteremia averaged 4.6×10^4 colony-forming units. Iron had no observable effect on growth or lethality of the highly virulent CR^+ colony types.

Unlike the other bacteria tested, the nine strains of *N. gonorrhoeae* tested failed to grow on agar containing Congo red. Filter paper disks (concentration disks, Difco) saturated with 0.5% Congo red produced zones of inhibition on plates inoculated with the gonococci but had no effect on meningococci, which grow and produce red colonies in the presence of this dye. Growth in the presence of Congo red may thus provide a simple means of distinguishing meningococci from gonococci. Two other *Neisseria* species were assayed for their growth and coloration in the presence of Congo red. Both *Neisseria subflava* and *Neisseria sicca* grew on Congo red agar, although *N. sicca* grew more slowly than on agar lacking the dye. These strains absorbed less of the dye than *N. meningitidis* and produced pink colonies.

The basis for Congo red inhibition of gonococ-

TABLE 2. *Enhancement of virulence of CR^- mutants by iron*

Strain	Route of inoculation	LD_{50}[a] at 24 h	
		Control	With added iron[b]
S. flexneri 2457 T	Allantoic	8.7×10^1	8.2×10^1
S. flexneri 2457 O (CR^-)	Allantoic	4.5×10^5	4.1×10^2
V. cholerae 569B	Allantoic	6.3×10^1	6.5×10^1
V. cholerae 569B CR^-	Allantoic	8.0×10^3	3.8×10^1
E. coli U169	CAM	2.0×10^1	2.0×10^1
E. coli U169 CR^-	CAM	3.1×10^4	1.2×10^2
N. meningitidis B-11	CAM	2.8×10^3	8.7×10^2
N. meningitidis B-11 CR^-	CAM	3.0×10^5	2.3×10^3

[a] LD_{50}, Mean lethal dose.
[b] Imferon, 500 μg of iron.

cal growth is not known. However, the binding of Congo red to gonococcal cells can be measured in a liquid assay similar to that used by Bates and Surgalla for hemin binding (personal communication). Both virulent (T1) and avirulent (T4) gonococci were found to absorb relatively large amounts of Congo red (Table 3). Wild-type *N. meningitidis, V. cholerae,* and *S. flexneri* also bound the dye, and, in agreement with the results obtained with agar medium, little or no Congo red was absorbed by CR⁻ mutants. The *Shigella* strain bound less of the dye than the other virulent bacteria in the 1.5-h incubation period used in this assay, despite the fact that it appeared to absorb the dye more rapidly than the other strains from agar media.

TABLE 3. *Congo red binding (in liquid) by agar-grown cells*

Strain	Congo red bound (μg/10^9 cells)[a]
N. gonorrhoeae F62 T1	14.4
N. gonorrhoeae F62 T4	15.0
N. meningitidis B-11	14.0
N. meningitidis B-11 CR⁻	4.3
S. flexneri 2457 T CR⁺	7.1
S. flexneri 2457 O CR⁻	0
V. cholerae 569B	8.4
V. cholerae 569B CR⁻	2.1

[a] Cells were exposed to 0.002% Congo red in PBS for 1.5 h at 37°C. After removing the cells by centrifugation, Congo red remaining in the supernatant was determined by absorbance at 500 nm, and bound Congo red was calculated by comparison with a control solution containing no cells.

DISCUSSION

Heart infusion agar containing Congo red was used by Surgalla and Beesley to differentiate virulent and avirulent colonies of *Y. pestis* (17). Most other genera they tested, such as *Salmonella* and *Shigella,* absorbed the dye only slightly from the medium they described. In the present study with media other than heart infusion agar, *Shigella, E. coli, V. cholerae,* and *N. meningitidis* were found to absorb the dye. Like the plague bacilli, these strains produce variants, either naturally or after exposure to a mutagenic agent, which absorb little or no Congo red and can easily be distinguished from the wild type. Mutants unable to bind the dye exhibit reduced growth and virulence in the relatively iron-poor environment of the chicken embryo. The addition of exogenous iron, however, enables these mutants to grow in vivo and establish infections. Avirulent gonococci also respond to the addition of iron in vivo (15). However, these mutants appear to be of a different type in that they absorb Congo red as well as do virulent colony types.

Congo red may prove useful for the selection of avirulent mutants suitable for live vaccines. The attenuated plague vaccine strain, EV76, is of this type and appears to differ from the virulent wild type only in its inability to absorb hemin and Congo red (9, 17). Similarly, colonial variants of *S. flexneri* which have previously been shown to be relatively avirulent for humans and laboratory animals and which have been shown to offer protection against subsequent challenge with virulent *Shigella* (7, 13) were found, in the present study, to be CR⁻.

Although effective capsular polysaccharide vaccines have been developed for serogroups A and C of *N. meningitidis,* there is presently no vaccine for serogroup B meningococci. A CR⁻ mutant of group B *N. meningitidis* was isolated during the present study and was found to be less virulent than the wild type in the sensitive chicken embryo model. The virulence of this and the other CR⁻ mutants is at least partially restored by iron. This suggests that these mutants are defective in their ability to acquire iron in vivo; that they can no longer compete effectively within the host for this essential element; and that they may therefore be unable to cause septicemic or disseminating infections in hosts with normal iron levels. If these types of mutants retain their ability to colonize mucosal surfaces, they may be able to stimulate local immune responses that will provide an effective barrier to subsequent invasion by virulent forms. With *N. meningitidis,* the carrier state is known to be an immunizing process (8). An important consideration, however, would be the genetic stability of the avirulent strain, and an appropriate candidate remains to be established. Nevertheless, we believe this approach to vaccine development merits further consideration.

In addition to its value as a means for selecting avirulent mutants, Congo red appears to provide a simple and rapid means of differentiating gonococci and meningococci. All strains of gonococci tested in the present study failed to grow on Congo red agar and exhibited zones of inhibition around Congo red disks placed on heavily streaked areas of the plates, whereas no zones of inhibition were detected with strains of *N. meningitidis,* the two nonpathogenic *Neisseria* tested, or any other of the genera used in this study. The use of Congo red disks might therefore be considered as an aid in the rapid diagnosis of gonorrhea.

The mechanism by which Congo red inhibits growth of gonococci but not meningococci is not

clear. Wild-type cells of both species absorb similar amounts of dye in liquid medium, and both *N. meningitidis* and *N. gonorrhoeae* absorb approximately twice as much of the dye as *Shigella* and *V. cholerae* absorb from liquid. It is possible that the sites of dye absorption and/or its subsequent fate differ in the two pathogenic *Neisseria* species. In general, however, the results obtained in liquid medium parallel those obtained in agar. CR^- mutants of *Shigella* appear to have lost completely the ability to absorb Congo red, whereas other CR^- mutants retain some binding capacity. These differences may represent variations in the amount or number of cell surface components that can bind Congo red or closely related dyes. The nature of such cell surface components has not been elucidated, but it is not unlikely that at least one of the structures that binds the dye is involved in iron transport.

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ADDENDUM

After submission of this manuscript, in a more extensive survey, some strains of *N. gonorrhoeae* were found to be capable of growth on Congo red agar, producing red colonies. These strains were penicillin-resistant, non-penicillinase producers (15a).

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