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Congo Red-Agar Plating Medium for Detecting Pigmentation in *Pasteurella pestis*

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Ability to detect pigmented and nonpigmented *Pasteurella pestis* is essential in plague research, and is currently dependent on use of the synthetic hemin-agar of Jackson and Burrows. We have devised a new differential medium for this purpose, containing Congo red dye and common, commercially available laboratory media. The ease and simplicity of preparation make the Congo red-agar a practical routine laboratory tool in plague research. These findings, possibly indicating a common binding site for hematin and Congo red, should be useful in efforts to determine the chemical nature of a bacterial component associated with high virulence in *P. pestis*.

Ability to produce pigmented colonies on a defined medium containing hemin is a bacterial character correlated with the virulence of *Pasteurella pestis* (1, 5). Study of this bacterial character has yielded much information on its relationship in vivo to virulence and immunogenicity, and in vitro to other bacterial activities (2, 4, 10; R. R. Brubaker and M. E. Rosenhaft, Bacteriol. Proc., p. 132, 1969). Also, important interactions between this virulence determinant (P^+) and other determinants of virulence have been described, e.g., influences in vivo of the fraction I determinant and P^+ on antigenicity of the V and W virulence antigens (3) and influences in vitro of P^+ on pesticin I sensitivity of nonpestigenic (Pg^-) organisms (R. R. Brubaker, Bacteriol. Proc., p. 59, 1967). It is evident, therefore, that research on mechanisms of pathogenesis of plague is heavily dependent on practical laboratory methods for routine monitoring of virulence characters of working cultures. There is considerable interest, also, in the possible identity of the hematin-binding site.

Jackson and Burrows had reported that P^+ strains absorbed basic dyes as well as hemin from liquid medium more efficiently than did P^- strains (6). Under the conditions of preparation of plating media, it is assumed that the hemin is converted to hematin. Our interest in Congo red arose from discussions with E. J. Ordal regarding the possible identity of hematin-binding sites on *P. pestis*. Dr. Ordal had observed that colonies of the fish pathogen *Chondrococcus columnaris* stain with simple acid dyes such as Congo red (cited in reference 7). Since the hematin and Congo red molecules exhibit certain similarities, we decided to see whether Congo red would stain *P. pestis* colonies.

Recognition of P^+ has depended upon the use of the hemin-containing synthetic agar of Jackson and Burrows (6). Pigmented and nonpigmented colonies produced on the standard hemin-agar by a mixture of P^+ and P^- strain Kim-10 are illustrated in Fig. 1A. This medium provides a highly effective method of measuring the levels of P^+ and P^- organisms in a bacterial population. However, because it is difficult and time-consuming to prepare, its routine use in most laboratories has not been commensurate with its great value to the investigator. The purpose of this report is to describe the development of an easily prepared medium that differentiates between P^+ and P^- organisms on the basis of differences in absorption of Congo red dye.

MATERIALS AND METHODS

All chemicals constituting synthetic media or additives were reagent grade or equivalent. Carbohydrates and dyes were sterilized separately and added aseptically to the sterile media unless otherwise indicated. Hemin-agar was prepared according to Jackson and Burrows (6) in 10-liter lots, poured into disposable plastic petri dishes, and stored at 5 C in plastic bags until used. Bacterial strains were grown on slopes of Blood Agar Base (BBL) containing 0.1 M glucose and 0.025 M $CaCl_2$ at 26 C for 24 to 48 hr. Suspensions of cells were made in Difco Heart Infusion, and 0.1-ml volumes containing 50 to 100 organisms were spread evenly over the surface of dry test media with sterile glass rods. Inoculated test media, unless otherwise stated, were incubated at 26 C and observed daily for 7 days. Kim-10, a highly pigmented strain,

and a nonpigmented mutant selected from hemin-agar, were utilized throughout the study as the standard fog comparing the effectiveness of various media.

RESULTS AND DISCUSSION

Initial observations indicated that *P. pestis* would absorb Congo red from hemin-agar base. Growth on this agar was very scant, and the addition of a reducing agent failed to increase colony formation. It appeared that the dye was toxic under these circumstances; therefore, a complex medium composed of 1% acid-hydrolyzed casein (Colab Laboratories, Inc., Chicago Heights, Ill.) plus 2% agar (Difco) with 2% concentrated buffer, *p*H 7.0 (Fisher Scientific Co., Pittsburgh, Pa.), and 0.01% aqueous Congo red was tested. Although abundant growth occurred, dye absorption was not intense. It was possible, however, to distinguish between P+ and P− colonies. Addition of the metal salts and the carbohydrate galactose, which are used in hemin-agar, markedly enhanced the amount of dye absorption. Testing of various combinations of these compounds revealed that only galactose was necessary to promote adequate growth and optimal pigmentation within 72 to 96 hr. Xylose and glucose were unsatisfactory because of excessive acid production. Even though this medium provided satis-

TABLE 1. *Recovery and pigmentation ratios of P+ and P− Kim-10*

Medium	Total count	Percentage total	Percentage P+
Congo red-agar..	104	89	63
Hemin-agar......	112	91	66
Blood agar base..	123	100	—

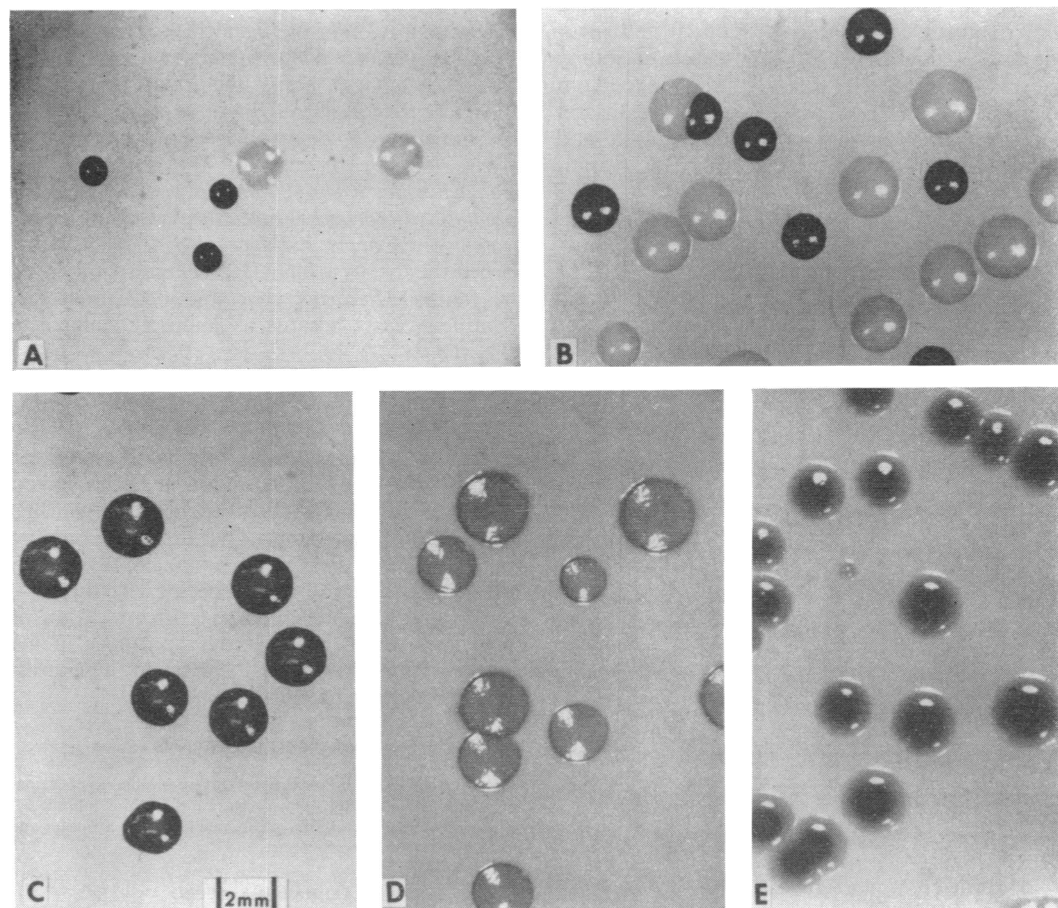

FIG. 1. *(A) P+/P− Kim-10, hemin-agar; (B) P+/P− Kim-10, Congo red-agar; (C) P+ Alexander, Congo red-agar; (D) P− EV76, Congo red-agar; (E) Pasteurella pseudotuberculosis, Congo red-agar.*

factory results, it required separate sterilization of all components, thereby complicating the preparation.

In attempts to eliminate this difficulty, a commercial dehydrated medium, Difco Blood Agar Base, containing added 0.2% galactose and 0.01% Congo red, was tested. Growth was abundant; however, dye absorption was poor and differentiation was inadequate. Inhibitors of hematin absorption are present in tissue extracts in unknown amounts. The source of nutrient in Blood Agar Base is an extract of beef heart which may contain inhibitors of hematin absorption such as uracil (1). An experiment was carried out to determine whether reduction of inhibitor by decreasing the amount of nutrients would allow sufficient growth of the organisms and provide optimal dye absorption.

Media composed of decreasing amounts of Heart Infusion plus 2% Difco agar, 0.01% Congo red, and 0.2% galactose were inoculated with mixtures of P^+ and P^- Kim-10 and incubated for 4 days at 26 C. Observation of colony size and color intensity indicated that the medium containing 1% HIB was optimal for growth and dye absorption. A comparison of recovery and pigmentation ratios of the test strains on hemin-agar and Congo red-agar is shown in Table 1. It can be seen that the results are similar for both media; however, the total counts were 89 to 91% of that obtained on standard plating medium. Reduction of Congo red concentration to 0.005% increased recovery to a level equaling the control; however, the intensity of pigmentation was considerably diminished. Therefore, when P^+ to P^- ratios are of primary interest, 0.01% dye should be used, and, if the medium is to be used for determining cell numbers as well as P, the level of Congo red should be reduced to 0.005%.

TABLE 2. *Comparison of pigmentation on hemin-agar and Congo red-agar*

Strain	Pigmentation	
	Hemin-agar	Congo red-agar
Fully virulent (P^+, VW^+)		
Kim-10	+	+
Alexander	+	+
M23	+	+
Yokohama	+	+
Siam	+	+
Washington	+	+
139-L	+	+
MP6	+	+
Attenuated (P^-, VW^+)		
NP Kim-10	0	0
NP Alexander	0	0
NP M23	0	0
EV76	0	0
M7	0	0
Avirulent (P^+, VW^-)		
Tjiwidej	+	+
TS	+	+
A12	+	+
Avirulent (P^-, VW^-)		
Harbin	0	0
Java	0	0
TRU	0	0

Although excellent differentiation between P^+ and P^- Kim-10 (as shown in Fig. 1B) was demonstrated, we compared other strains of *P. pestis*, well characterized in the literature, on Congo red-agar and hemin-agar. As can be seen in Table 2, Congo red-agar gave results equal to those obtained on hemin-agar. Strains Alexander (P^+), EV76 (P^-), and the closely related *P. pseudotuberculosis* strain PBl/+ showed typical reactions on Congo red-agar (Fig. 1C, D, E). *P. pseudotuberculosis* absorbed a small amount of dye, but, unlike *P. pestis*, the colony was rather transparent and smooth in appearance; thus, it was possible to distinguish between the two species. Finally, members of the genera *Salmonella, Shigella, Klebsiella, Pseudomonas, Proteus, Bacillus, Micrococcus*, and *Staphylococcus* were grown on Congo red-agar. Most species absorbed the dye to a slight degree, but members of the family *Micrococcaceae* and occasional unidentified yeasts became as intensely stained as *P. pestis*.

Although the basis for absorption of hematin and Congo red is still unknown, we were gratified to find that the differential absorption by P^+ and P^- colonies was essentially the same for the two compounds. The finding that Congo red as well as hematin is absorbed by P^+ organisms indicates that the chemical nature of the binding sites may be basic residues of mucopolysaccharides (7), proteins such as specific enzymes (8), or albumin-like substances (9). Identification of these sites would be of great importance in the determination of the role P^+ plays in the expression of full virulence in *P. pestis*.

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