APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Jan. 1983, p. 340–342
0099-2240/83/010340-03$02.00/0

Vol. 45, No. 1

# Congo Red Absorption by *Rhizobium leguminosarum*

BARBARA E. KNEEN AND THOMAS A. LaRUE*

*Boyce Thompson Institute for Plant Research, Ithaca, New York, 14853*

Received 21 June 1982/Accepted 14 October 1982

Congo red absorption is generally considered a contraindication of *Rhizobium*. However, *R. leguminosarum* takes up the dye on yeast extract-mannitol agar. The uptake of congo red varies among strains of *R. leguminosarum*, as shown elsewhere with strains of *R. trifolii* and *R. meliloti*. Congo red absorption does not distinguish rhizobia from other bacteria, but may be useful as a strain marker.

Congo red (diphenyldiazo-bis-α-naphthylaminesulfonate) is frequently included in culture media for isolating *Rhizobium* spp. or for testing the purity of *Rhizobium* cultures. In general, rhizobia produce white colonies or absorb the dye weakly, whereas many other bacteria, including closely related *Agrobacterium* spp., take up the dye strongly (3, 12, 13). Dye absorption is affected, however, by the composition of the medium and conditions of incubation. If the medium is not buffered, acid-producing strains cause the dye to turn purple (8). On nitrogen-free or synthetic nitrate-containing medium supplemented with 0.0025% congo red, rhizobia reportedly produce white colonies which can be differentiated from colored colonies of other soil bacteria (2, 4, 6, 8, 10). On nitrogen-rich yeast extract-mannitol agar containing congo red (CR-YMA), rhizobia cannot be easily distinguished from other organisms. Strains of *R. meliloti* and *R. trifolii* cultured at 24 to 28°C on CR-YMA are colored white, pink, orange, or red (1, 8).

Congo red absorption has been used as a strain marker in nodulation and competition studies with mixed *R. trifolii* inocula (1). In this note, we report our observations of such variability in congo red absorption among strains of *R. leguminosarum* grown on CR-YMA.

Twenty-five strains of *R. leguminosarum*, seven strains of other *Rhizobium* spp., three strains of *Agrobacterium tumefaciens*, and two other bacterial species (Table 1) were streaked on plates of CR-YMA (mannitol, 10 g; $K_2HPO_4$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; NaCl, 0.1 g; yeast extract (Difco Laboratories, Detroit, Mich.), 0.4 g; $CaCO_3$, 3 g; agar, 15 g; and water to 1,000 ml, pH 6.8, and 10 ml of a 1:400 aqueous solution of congo red [13]). Tests were also performed on CR-YMA lacking $CaCO_3$. Duplicate plates for each strain were incubated at 28°C for 4 to 5 days and colony colors were observed on plates against a white background. Colonies were then scraped from the agar and observed on a white plastic surface. Their colors were matched with shades in the Royal Horticultural Society Colour Chart (12). The identity of each strain of *R. leguminosarum* (9) was tested by adding 5 ml of rhizobial suspension to sterile 5-day-old pea seedlings (*Pisum sativum* cv. Sparkle) grown in sterile Dispo bottles (American Scientific Products, Rochester, N.Y.) containing vermiculite and nitrogen-free nutrient solution. Nodulation was scored 21 days after inoculation. All *R. leguminosarum* strains used in this study produced pink nodules and dark green shoots on pea plants. Effectivity was confirmed on nine of the strains by acetylene reduction assays.

Variation in the intensity of dye absorption was observed among the bacterial strains plated on CR-YMA at 28°C. Table 1 lists the colors of colonies on agar. When bacteria were transferred from CR-YMA onto a white surface, the colors of rhizobia appeared darker than when viewed on the orange-red agar surface. If $CaCO_3$ was omitted from the medium, strains producing acid caused the dye in both the colony and the medium to turn purple. On CR-YMA containing $CaCO_3$, purpling did not occur, and color determination was easier.

Of the 25 *R. leguminosarum* strains tested, several produced dark red colonies, not unlike those of non-rhizobial species. Other colonies were orange, pink, or almost white. Similar variability in color among strains of *R. meliloti* and *R. trifolii* grown on CR-YMA at 24 to 28°C has been observed by others (1, 8). We found that dye-absorption characteristics can be used as a aid in *R. leguminosarum* strain identification.

To determine which portion of the bacterium absorbs congo red, eight strains (designated *c* in Table 1), were grown in congo red-yeast extract-mannitol broth without $CaCO_3$ on a 30°C incubator-shaker for 2 days. The bacterial suspensions (25 ml) were centrifuged at 12,000 × *g* for 20 min and the pellet was suspended in yeast extract-mannitol broth and centrifuged. The layer of capsular material above the cells was intense red

TABLE 1. Absorption of congo red by bacteria grown on CR-YMA

| Strain | Source[a] | Color of bacteria | | |
|---|---|---|---|---|
| | | On CR-YMA | Color no.[b] | |
| | | | On CR-YMA | On white |
| *R. leguminosarum* | | | | |
| ATCC 10004[cd] | 1 | Very dark pink-red | 46C | 46C |
| RI300[cd] | 2 | Pink-orange[e] | 39B | 52A |
| 128C53[cd] | 3 | Orange-pink[e] | 43D | 50B |
| CC302 | 4 | Pale orange-pink | 37A | 37B |
| NA500 | 4 | Dark pink | 52B | 52A |
| NA501-1[cd] | 4 | Light pink[e] | 50C | 52B |
| NA502-1 | 4 | Dark pink-orange | 50A | 46C |
| NA503 | 4 | Orange-red | 42A | 45B |
| NA504-1[cd] | 4 | Light pink[e] | 39A | 52A |
| NA504-2 | 4 | Bright pink[e] | 51A | 52A |
| NA525 | 4 | Dark pink[e] | 47B | 52A |
| NA533[cd] | 4 | Orange-pink[e] | 31D | 38B |
| SU391[d] | 4 | Dark pink-red[e] | 43A | 45B |
| TomC6[cd] | 5 | Dark orange-red | 43B | 42A |
| TomC7[cd] | 5 | White-very pale pink[e] | 39B | 52D |
| BB48a | 6 | Pale pink-orange | 41B | 50A |
| BB54b | 6 | Very pale pink[e] | 37B | 37C |
| L28a | 6 | Pale pink[e] | 39C | 38B |
| PS3a | 6 | Light pink[e] | 43B | 52A |
| PS3b | 6 | White-pink[e] | 43C | 50A |
| PS4a | 6 | Dark pink[e] | 45C | 45B |
| 510P | 7 | Light orange-pink[e] | 37B | 48C |
| 511P | 7 | Light pink[e] | 39B | 52B |
| F77 | 8 | Pink-orange[e] | 37A | 48C |
| FPec16 | 8 | Pink[e] | 51B | 52A |
| *R. trifolii* | | | | |
| 162BB1-A | 9 | Light pink-orange[e] | 37B | 41C |
| 162P17-A | 9 | Orange | 34B | 43C |
| *R. phaseoli* | | | | |
| 127K80-A | 9 | Light orange-pink[e] | 37A | 35B |
| *R. meliloti* | | | | |
| 3-3 | 10 | Light pink | 43C | 52A |
| *R. japonicum*[f] | | | | |
| 61A76 | 3 | Light orange-pink | 35B | 40D |
| *Rhizobium* spp.[f] | | | | |
| CB756 | 3 | Pale orange-pink | 33D | 41B |
| 32H1 | 3 | Pale orange-pink | 37A | 41B |
| *A. tumefaciens* | | | | |
| A6 | 11 | Red | 46C | 45B |

TABLE 1—*Continued*

or purple, whereas the cells were white to orange, pink, or both. Congo red stains amyloid and cellulose (7, 11) and interacts with β-D-glucans (15). The dye probably binds to polysaccharides in the rhizobial capsule.

Yeast extract-mannitol agar is used more often than nitrogen-deficient media because it supports better growth of rhizobia (4). Fred and Waksman (5) described the isolation of nitrogen-fixing bacteria on CR-YMA and implied that it could be used as a selective medium, but they did not describe colony colors. Marked absorption of congo red from CR-YMA at 26 to 28°C is generally considered a contraindication of *Rhizobium* (3, 13, 14). We found this to be untrue. If color on CR-YMA is used in enumeration, the number of rhizobia may be underestimated. Moreover, if only colonies not absorbing congo

TABLE 1—*Continued*

| Strain | Source[a] | Color of bacteria | | |
|---|---|---|---|---|
| | | On CR-YMA | Color no.[b] | |
| | | | On CR-YMA | On white |
| A277 | 11 | Light red | 47C | 45A |
| B6806 | 11 | Dark pink-red | 39B | 52A |
| *Micrococcus leuteus* | 12 | Dark orange-red | 43A | 44A |
| *Bacillus sphaericus* | 12 | Orange-red | 43B | 42A |

[a] Sources: 1, American Type Culture Collection, Beltsville, Md.; 2, J. Beringer, John Innes Institute, Norwich, England; 3, Nitragin Co., Milwaukee, Wis.; 4, Prairie Regional Lab, National Research Council, Saskatoon, Canada; 5, T. Lie, Wageningen, Netherlands, subcultured by M. Baase, Boyce Thompson Institute, Ithaca, N.Y.; 6, R. Islam, International Center for Agricultural Research for Dry Areas, Aleppo, Syria; 7, Volcani Institute, Bet-Dagan, Israel; 8, N. Ulgen, Soil and Fertilizer Research Institute, Yenimahalle, Ankara, Turkey; 9, A. Eaglesham, Boyce Thompson Institute; 10, D. Viands, Cornell University, Ithaca, N.Y.; 11, E. Nester, University of Washington, Seattle, Wash.; and 12, Department of Microbiology, Cornell University.

[b] Designates color in Royal Horticultural Society Colour Charts.

[c] Grown in congo red-yeast extract-mannitol broth and centrifuged.

[d] Shown to be effective in nitrogen fixation by acetylene reduction.

[e] Purpling or darkening of the medium and colony color occurred when $CaCO_3$ was omitted from CR-YMA medium.

[f] Slow-growing strains were scored at 10 days old.

red are selected when isolating root nodule bacteria, culture collections of rhizobia will include only those with limited ability to take up congo red. Thus, the general rule that rhizobia fail to absorb the dye will be perpetuated.

We gratefully acknowledge the technical assistance of M. Baase.

This research was supported by U.S. Department of Agriculture grant CRGO-79-00103.

## LITERATURE CITED

1. **Bromfield, E. S. P., and D. G. Jones.** 1980. A strain marker in *Rhizobium trifolii* based on the absorption of congo-red. Zentralb. Bacteriol. Parasitenkd. Infekionskr. Hyg. Zweite. Naturwiss. Abt. Allg. Landwirtsch. Tech. Mikrobiol. **135**:290–295.
2. **Bryan, C. S.** 1938. Identification of *Phytomonas, Azotobacter*, and *Rhizobium* or *Achromobacter* upon initial isolation. Soil Sci. **45**:185–187.
3. **Dye, M.** 1980. Functions and maintenance of a Rhizobium collection, p. 435. *In* N. S. Subba Rao (ed.), Recent advances in biological nitrogen fixation. Holmes and Meier Publishers, Inc., New York.
4. **Fred, E. B., I. L. Baldwin, and E. McCoy.** 1932. Root nodule bacteria and leguminous plants. University of Wisconsin Studies in Science, No. 5, Madison.
5. **Fred, E. B., and S. A. Waksman.** 1928. Laboratory manual of general microbiology. McGraw-Hill Book Co., New York.
6. **Giltner, W.** 1921. Laboratory manual in general microbiology, 2nd ed. John Wiley & Sons, Inc., New York.
7. **Gurr, E.** 1960. Encyclopedia of microscopic stains. The William & Wilkins Co., Baltimore.
8. **Hahn, N. J.** 1966. The congo-red reaction in bacteria and its usefulness in the identification of rhizobia. Can. J. Microbiol. **12**:725–733.
9. **Jordan, D. C., and O. N. Allen.** 1974. Family III. Rhizobiaceae CONN 1938, 321, p. 261. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
10. **Kellerman, K. F.** 1911. The relation of crown gall to legume inoculation. U.S. Department of Agriculture Bureau of Plant Industry circular 76.
11. **Lillie, R. D.** 1977. Biological stains. 9th ed. Biological Stain Commission. The Williams & Wilkins Co., Baltimore.
12. **Royal Horticultural Society.** 1966. Colour chart. The Royal Horticultural Society, London.
13. **Vincent, J. M.** 1970. A manual for the practical study of the root-nodule bacteria. International Biological Programme handbook no. 15. Blackwell Scientific Publications, Oxford.
14. **Vincent, J. M., P. S. Nutman, and F. A. Skinner.** 1979. The identification and classification of *Rhizobium*. Identification methods for microbiology, 2nd ed. Soc. Appl. Bacteriol. Tech. Ser. **14**:49–69.
15. **Wood, P. J.** 1980. Specificity in the interaction of direct dyes with polysaccharides. Carbohydr. Res. **85**:271–287.