LOCALIZATION OF CAROTENOID PIGMENT IN THE CYTOPLASMIC MEMBRANE OF *XANTHOMONAS JUGLANDIS*

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Received for publication 25 June 1963

ABSTRACT

STEPHENS, WILLIAM L. (University of California, Davis), AND MORTIMER P. STARR. Localization of carotenoid pigment in the cytoplasmic membrane of *Xanthomonas juglandis*. J. Bacteriol. **86**:1070–1074. 1963.—The kinetics of release of carotenoid pigment from cells of *Xanthomonas juglandis*, disrupted by sonic or ballistic treatment, showed the pigment to be localized in the cell envelope. Cells subjected to ballistic disintegration released carotenoid at a rate identical to that of a membrane component (reduced nicotinamide adenine dinucleotide oxidase) and at a slightly higher rate than a wall component (hexosamine). Thus, the membrane portion of the cell envelope is the most probable locus of the unique carotenoid pigment of *X. juglandis*.

In the past few years, numerous cytological studies (reviewed by Marr, 1960*a*, *b*) have implicated the bacterial cytoplasmic membrane as the structure which contains respiratory enzymes and carotenoid pigments. The localization has recently been pinpointed with greater precision by Pangborn, Marr, and Robrish (1962), who presented electron micrographs of thin sections of *Azotobacter agilis* disrupted by sonic treatment, osmotic shock, or ballistic disintegration, which revealed a network of internal membranes attached to the cell envelope. Ballistic disintegration of envelopes, previously emptied of cytoplasm by osmotic shock, resulted in the loss of the internal membranes and a concomitant release of reduced nicotinamide adenine dinucleotide (NADH) oxidase. Thus, these intracytoplasmic membranes appear to be the most probable locus of the respiratory enzymes of the cell.

In the present study, we used the method designed by Marr and Cota-Robles (1957) for the purpose of determining whether a cell component originates in the cytoplasm or arises by comminution of a larger structure during disruption of the cell. The rationale of their method follows from the fact that sonic treatment ruptures the cell by a single physical process, which releases all the free soluble and particulate contents of the cytoplasm; subsequently, there is further disintegration of the envelope. Samples are removed at various times during sonic oscillation, centrifuged, and the kinetics of disappearance of a cell component from the sedimented pellet are thereby determined. Substances lost from the pellet at a rate equal to the decrease in viable count are assumed to be totally released from the cell at the time of rupture and, therefore, contained in the cytoplasm. Components lost from the pellet at rates lower than the decrease in viability are assumed to be released only after comminution of the cell envelope.

Mathews and Sistrom (1959) applied the principle of Marr and Cota-Robles to show that the carotenoid pigments and the enzymes of the succinic oxidase system are located in the cell envelope of *Sarcina lutea*. Removing the cell wall with lysozyme, they were able to demonstrate that the carotenoid pigments, as well as the respiratory enzymes, are associated with the cell membrane. Gilby, Few, and McQuillen (1958) had shown earlier that carotenoids are a component of the membrane in *Micrococcus lysodeikticus*. The chromatophores, with which carotenoids are associated in photosynthetic bacteria (Schachman, Pardee, and Stanier, 1952), have been interpreted by Marr (1960*a*, *b*) as fragments of extensions of the cell membrane—a view which, on very much weaker experimental grounds, might be extended to the protein particles with which the carotenoids of nonphotosynthetic phytopathogenic bacteria are associated (Saperstein and Starr, 1955).

The recent demonstration (Starr and Stephens, 1963) that a distinctive and possibly unique

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carotenoid pigment exists in all yellow-pigmented species of *Xanthomonas* led to an interest in the localization of this unusual pigment in these bacterial cells. The present report summarizes our observations with a typical phytopathogenic xanthomonad, *X. juglandis*, and thus extends the rather scanty literature (*see* Starr, 1959) on the cytology of phytopathogenic bacteria.

Materials and Methods

Cultivation of the bacteria. Erlenmeyer flasks (250 ml) containing 100 ml of double-strength nutrient broth (16 g of Difco dehydrated nutrient broth per liter) were inoculated with *X. juglandis* strain ICPB-XJ103 (obtained from the International Collection of Phytopathogenic Bacteria, Department of Bacteriology, University of California, Davis) grown on yeast-glucose-$CaCO_3$ agar slants. These flasks were incubated at 28 C on a rotary shaking machine operating with an amplitude of 1.5 in. at 160 rev/min. After 24 hr, the 100-ml samples were checked for purity, and were added aseptically to 2-liter Erlenmeyer flasks containing 500 ml of double-strength nutrient broth. The 2-liter flasks were incubated under the same conditions mentioned above. After a second purity check, the cells were harvested in a Servall angle centrifuge (type SP/X) at 3440 $\times$ *g*. After harvest, the slime was removed, insofar as possible, by suspending the cells in distilled water in a Waring Blendor and centrifuging them at 2000 $\times$ *g* for 45 min at 4 C, with three repetitions of this treatment.

Disruption of cells by sonic oscillation. Samples (40 ml) of a suspension of cells [25 mg (dry weight)/ml] in 0.05 M phosphate buffer (pH 7.4) were treated for varying lengths of time in a Raytheon 10-kc sonic oscillator, operating at full electrical power (approximately 80 acoustical watts). The cup of the transducer was flushed with hydrogen, and the temperature of the samples was maintained at less than 5 C during the treatment. At the end of each time period, two 3-ml samples were removed, and the remaining 34 ml were centrifuged at 5000 $\times$ *g* for 10 min at 4 C. The 3-ml samples were used for turbidity and viable-count determinations. The pellets from the centrifugations were analyzed quantitatively for pigment.

Disruption of cells by ballistic disintegration. Samples (5 ml) of a suspension of cells [25 mg (dry weight)/ml] in 0.05 M phosphate buffer (pH 7.4) and 2 ml of glass beads (0.2 mm in diameter; Minnesota Mining and Manufacturing Co., St. Paul, Minn.) were added to the cup of a Mickle disintegrator. The cups were shaken with a 9-mm peak-to-peak displacement. The cups were chilled every 2 min in an ice bath, so that the temperature of the contents never exceeded 20 C during the treatment.

Viable counts. Total cell counts on the material disrupted by sonic oscillation were made with a model B Coulter counter. Since the decrease in total cell count during sonic treatment must result from cell rupture with the associated death of the cell (Marr and Cota-Robles, 1957), this change may also be interpreted as a decrease in viable count.

Turbidity determination. A Beckman model DU spectrophotometer was used for all measurements of turbidity. Optical density was measured at 650 mμ, at which wavelength the carotenoid pigments of *X. juglandis* do not absorb appreciably (Starr and Stephens, 1963).

Pigment determination. The carotenoid pigments were extracted and determined quantitatively by the method of Mathews and Sistrom (1959). To each milliliter of pellet suspension, 2.5 ml of 10% trichloroacetic acid were added. After centrifugation, the pellet was extracted with 5 ml of hot absolute methanol. Our previous experience with *X. juglandis* showed that this procedure removes substantially all of the carotenoid, unharmed. Furthermore, since the carotenoid of strain XJ103, like all xanthomonads, has a central absorption maximum at 441 mμ in methanol, the optical density of the crude methanol extract at that wavelength could be used as a measure of the total pigment content.

Assay for NADH oxidase. NADH oxidase was assayed by measuring the oxidation of the reduced pyridine nucleotide at 340 mμ in the Beckman model DU spectrophotometer. A quartz cell with a 1-cm light path contained 0.6 μmoles of NADH, 0.1 ml of a suitable dilution of the preparation containing NADH oxidase, and 150 μmoles of phosphate buffer (pH 7.4), made up to a final volume of 3.0 ml.

Hexosamine determination. Samples (1 ml) of the Mickle-treated material were removed periodically and centrifuged, and the sediment was hydrolyzed in 4 N HCl in a sealed tube at 100 C for 15 hr. The HCl was removed by vacuum distillation, and the hexosamine was determined by the Elson-Morgan reaction (Boas, 1953).

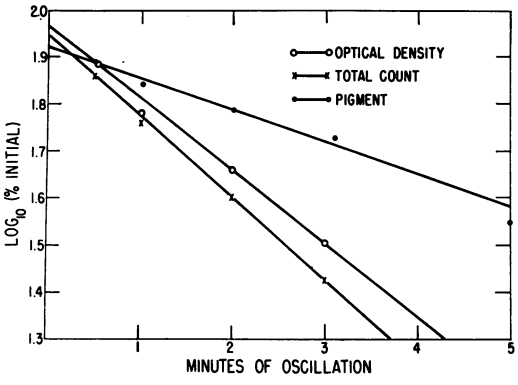

FIG. 1. *Turbidity, total cell count, and rate of release of carotenoid pigment of Xanthomonas juglandis as functions of the time of sonic treatment. Total cell counts were made with a model B Coulter counter.*

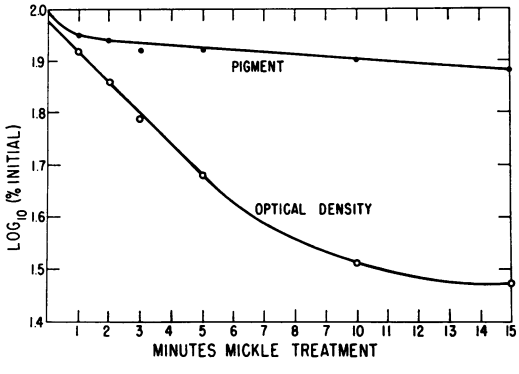

FIG. 2. *Turbidity and rate of release of carotenoid pigment of Xanthomonas juglandis as functions of the time of Mickle treatment.*

RESULTS AND DISCUSSION

Kinetics of release of cell components during sonic oscillation. Using the aforementioned kinetic approach of Marr and Cota-Robles (1957), we followed the disruption by sonic oscillation of cells of *X. juglandis* XJ103. A decrease in turbidity occurred at a lesser rate than the decrease in viable count, and the loss of pigment from the sedimentable material occurred at a much lower rate than the decrease in turbidity (Fig. 1).

Kinetics of release of cell components during ballistic disintegration. Whole cells of *X. juglandis* XJ103 were subjected to ballistic disintegration in a Mickle disintegrator. The rate of disappearance of pigment from the pellet was found to be much less than the rate of decrease in turbidity (Fig. 2). This is in good agreement with the results obtained by sonic oscillation. Pigment was lost from the pellet at a rate identical with the loss of the respiratory enzyme NADH oxidase (Fig. 3). Pigment was lost from the cells at a slightly higher rate than was hexosamine (Fig. 4).

The large difference observed between the rates of decrease in viable count and of loss of

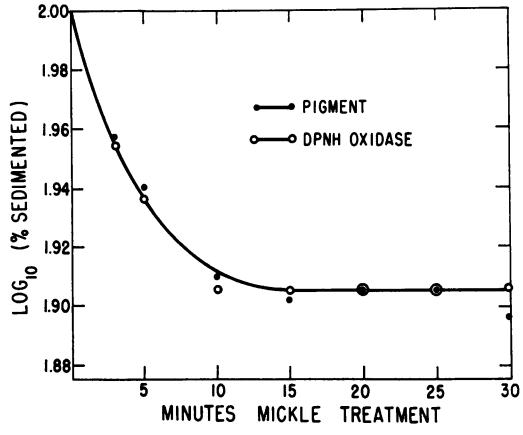

FIG. 3. *Release of NADH oxidase and carotenoid pigment during Mickle treatment of Xanthomonas juglandis cells. The values plotted are the logarithms of the per cent of the initial concentration of each component in the sediment after centrifugation at 5000 × g for 10 min.*

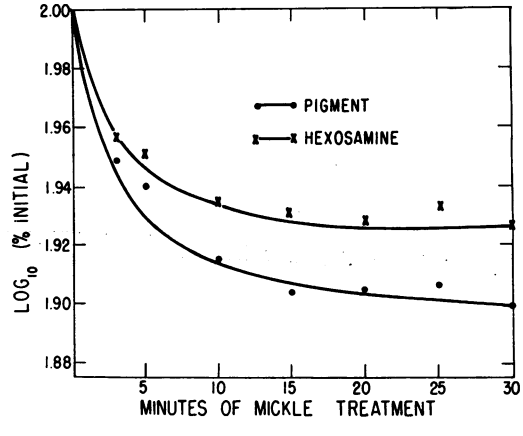

FIG. 4. *Release of hexosamine and carotenoid pigment during Mickle treatment of Xanthomonas juglandis cells. The values plotted are the logarithms of the per cent of the initial concentration of each component in the sediment after centrifugation at 5000 × g for 10 min.*

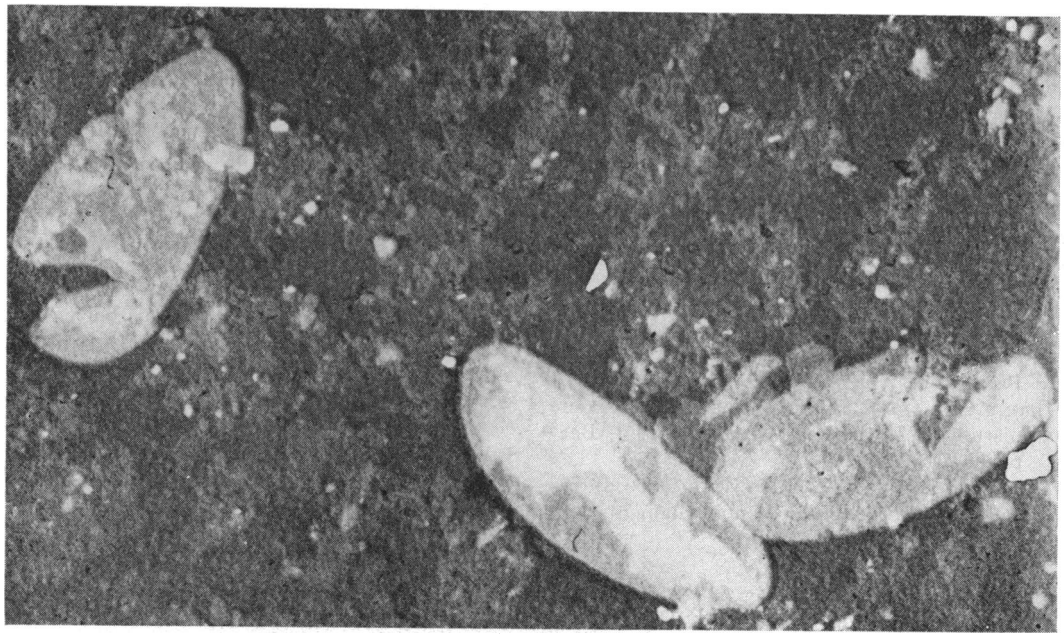

FIG. 5. *Electron micrograph of Xanthomonas juglandis after 30 min of Mickle treatment. Shadowed with uranium at a 4:1 angle. ×30,000. Photo: J. Pangborn.*

carotenoid from *X. juglandis* subjected to sonic oscillation suggests that the cell envelope is the locus of these pigments. The difference in rates of decrease in turbidity and of loss of pigment make it likely that the cell envelope of *X. juglandis* is a relatively resistant structure. During sonic oscillation, *A. vinelandii* (Marr and Cota-Robles, 1957) and *S. lutea* (Mathews and Sistrom, 1959) lost envelope components at the same rate as they decreased in turbidity, thus indicating that loss of an envelope component and decrease in turbidity are both functions of the same process, namely, comminution of the cell envelope. *X. juglandis*, subjected to sonic oscillation, lost pigment at a much lower rate than it decreased in turbidity; the latter process closely paralleled the decrease in viable count. The decrease in turbidity in this organism may result more from breakage of the cell envelope, with the resultant loss of cytoplasm, than from the subsequent comminution which releases the pigment.

Ballistically disintegrated cells of *X. juglandis* also displayed this same large difference between rates of pigment release and turbidity decrease. Phase microscopy of cells disrupted in this manner revealed a few intact cells at 5 min, and only ghosts at 10 min. The electron micrograph (Fig. 5) shows very little comminution of the ghosted cells after 30 min of Mickle treatment. These observations, and the plot of optical density seen in Fig. 2, support the view that decrease in turbidity results more from initial envelope breakage and loss of cytoplasm than from further comminution.

Our results with *X. juglandis*, thus, are consistent with other localization studies, which have shown that respiratory enzymes and carotenoid pigments are located in the membrane portion of the bacterial cell envelope. The identical rates of release of the NADH oxidase and the carotenoid, and the dissimilar rates of release of the hexosamine and the pigment, point to the cell membrane as the most probable locus for the carotenoid pigments of *X. juglandis*. The fact that release of wall components occurs at a rate only slightly lower than the rate of release of membrane components is understandable in light of the relative resistance to comminution displayed by xanthomonad cells.

ACKNOWLEDGMENT

This study was supported in part by research grant AI-01067 from the National Institutes of Health, U.S. Public Health Service.

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