Density Changes during Encystment of *Azotobacter vinelandii*

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INTRODUCTION

Wyss, Neumann & Socolofsky (1961), Lin & Sadoff (1968) and Hitchins & Sadoff (1970) have described morphological changes which occur during the development of the desiccation-resistant resting cell, or cyst, of *Azotobacter vinelandii*. These changes are experimentally induced by the presence of 0.2 % *n*-butanol or 0.2 % sodium β -hydroxybutyrate (BHB) and have been observed to begin shortly after the termination of vegetative growth and to be complete within 5 days. Lin & Sadoff (1969*a*) also presented values for the density of *A. vinelandii* vegetative cells, cysts, and parts of cysts. This paper describes buoyant density changes during encystment and relates these changes to events in cyst morphogenesis.

METHODS

Azotobacter vinelandii (ATCC 12387), grown on Burk's nitrogen-free medium as modified by Kellogg (1957), was induced to encyst nearly synchronously by the method of Lin & Sadoff (1968). After growth at 33 °C on a rotary shaker for 18 h (mid-log phase) in broth supplemented with 1 % (w/v) glucose the *A. vinelandii* vegetative cells were washed free of glucose, transferred to 0.2 % BHB-containing broth, and incubated again. The 20 ml linear density gradients used in this study were formed of 50 to 35 % (v/v) Renografin 60, (E. R. Squibb Inc., New York, N.Y., U.S.A.) an aqueous solution of 52 % methylglucamine 3,5-diacetamido-2,4,6-triiodobenzoate and 8 % sodium 3,5-diacetamido-2,4,6-triiodobenzoate. Before use, the Renografin was supplemented with 0.005 M each of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ which protect *A. vinelandii* cysts from the disruptive effect (Goldschmidt & Wyss, 1968) of the 0.001 M-disodium ethylenedinitrilotetraacetate found in Renografin. The density of Renografin solutions and the linearity of gradients were established by weighing samples in a 3 ml pycnometer (Kimax Inc., Toledo, Ohio, U.S.A.). Density gradients were centrifuged at 25 °C for 1 h at 40000 g. *Azotobacter* cells were labelled with [^{14}C]sucrose by growing them in Burk's broth supplemented with 0.5 % unlabelled sucrose and 2.0 $\mu\text{Ci/ml}$ (0.57 $\mu\text{g/ml}$) uniformly labelled [^{14}C]sucrose (Amersham/Searle, Arlington Heights, Illinois, U.S.A.).

RESULTS AND DISCUSSION

After the densities of *Azotobacter vinelandii* cells (1.106 g/ml) and cysts (1.152 gm/ml) in Renografin had been established, [^{14}C]sucrose-labelled vegetative cells were mixed with cysts and applied to the density gradient; radioactivity was not found to be present in the cyst band or in any of the intermediate fractions. This is evidence that the cysts and vegetative cells segregate according to density and do not agglutinate to form intermediate bands.

To study the kinetics of this density shift, a 1.0 ml sample was removed from an 18 h glucose-grown culture and applied to a 50 to 35 % modified Renografin gradient. The remain-

Fig. 1. Buoyant density changes during encystment of *Azotobacter vinelandii*. Bacteria were transferred into the encystment medium at time 0. The vertical bars represent the range of cell densities in Renografin at each sampling time. The closed circles designate the density of the fraction with the highest absorbance.

ing cells were immediately washed, resuspended in 0.2% BHB, and another 1.0 ml sample was removed, applied to another gradient and centrifuged. The BHB-*Azotobacter vinelandii* culture was then incubated on a rotary shaker and further samples were removed at selected intervals and the density of each cell-containing fraction was determined (Fig. 1). The vertical bar shows the range of cell densities at each sample time and the closed circle shows the density of the fraction containing the greatest number of cells.

After the absorbance of each fraction had been determined, the contents of the tube showing the greatest absorbance were observed directly under the phase-contrast microscope. The samples taken immediately before and after transfer to BHB medium contained actively motile vegetative cells. The sample taken at 8 h contained no motile cells, but many were rounded and contained refractile granules. After 24 h all the cells were rounded and some had begun to develop a bright halo-like appearance, while at 48 h almost all of the cells from the peak fraction had the characteristic bright appearance of mature cysts except that the central body was not clearly visible. The samples at 3, 4, 5, 6, 10 and 14 days showed what appeared to be mature cysts. The proportion of these mature-appearing cysts increased between the third and sixth days, after which nearly all the cells were encysted. These observations are similar to those made by Wyss *et al.* (1961) and by Hitchins & Sadoff (1970).

There are no immediately apparent reasons for the density differences between the cysts and vegetative cells reported in this study. Although there is no other published evidence to support the idea, the desiccation resistance of cysts would tend to support the concept of a lower water content in cysts than vegetative cells if a comparison is drawn between spores and cysts in this regard (Black & Gerhardt, 1962). The data of Lin & Sadoff (1969*b*), describing certain chemical differences between vegetative cells and cysts, showed an increase in carbohydrate from 28 to 45% dry weight during encystment. Lipid increased from 9.2 to 16.0% and ash from 7.1 to 8.8% during encystment. Calcium increased nearly fourfold

from 1.24 to 4.85% of dry weight, another similarity between cysts and spores (Warth, Ohye & Murrell, 1963). It is interesting to note that carbohydrate and calcium, two of the components of *Azotobacter vinelandii* which showed the greatest increase during encystment, are reported to be localized predominantly in the intine of the mature cyst.

Using a formula for the volume of a prolate ellipsoid, $V = \frac{4}{3} x^2 y$ (Scientific Tables, 1963), where x is the short axis and y the long axis of the ellipsoid, and regarding the layers of cysts as concentric ellipsoids, the volume of the intine layer in published electron micrographs of thin sections of cysts is 22% of the total volume of the cyst. The relative volumes of exine and central body are 43 and 35% respectively. When cysts and vegetative cells reach their buoyant densities in the Renografin gradient, the contribution of each cyst part to the total density of the intact cyst must be proportional to the density and the amount of each cyst part present (Brakke, 1968). Thus, based on our determination of the densities of exine (1.063 g/ml) and central body (1.102 g/ml), and their respective volumes, the intine must have a density of 1.407 g/ml in Renografin. Since the intact cyst has a buoyant density of 1.152 g/ml in Renografin, it might be expected that increases in density during encystment would correspond to increases in the amount of intine in the cyst.

Electron micrographic studies established that intine material was not deposited as a separate layer in the cyst until the third to the fifth day (Wyss *et al.* 1961; Lin & Sadoff, 1968) after about 80% of the density change had already occurred (Fig. 1). It may be therefore that intine material is actually formed in the cyst before it becomes deposited as a recognizable layer.

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