Formation of Fragile Cysts by a Strain of *Azotobacter chroococcum*

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**SUMMARY**

A strain of *Azotobacter chroococcum* (designated *A. chroococcum* NTS) which produces fragile cysts was isolated from soil. Fragility of the cysts was measured by ultrasonic treatment and compared to the fragility of cysts of a typical strain of *A. chroococcum*. Differences in fragility and resistance to ultraviolet radiation were observed but differences in desiccation resistance were not perceptible between the two strains. Electron micrographs of cysts of the new isolate revealed a structural aberration in the exine area of the cyst coat which may be associated with the physiological differences described. The morphological aberration and the fragility of the cysts are inheritable traits of this strain of *A. chroococcum*.

**INTRODUCTION**

Spores and cysts are dormant forms of some bacteria which are highly resistant to adverse environmental conditions. The cyst has been described as the dormant stage in the life-cycle of some members of the Azotobacteraceae, and Socolofsky & Wyss (1962) have shown it to be resistant to both chemical and physical agents. The basis for this resistance is not known but Parker & Socolofsky (1966) attribute it to the cyst coat: the encystment process, implying the formation of a cyst coat, is correlated with the acquisition of resistance to ultrasonic treatment, ultraviolet radiation, and desiccation; removal of the cyst coat by certain chemical agents makes the surviving central body as vulnerable as the vegetative form to deleterious agents.

This paper describes a strain of *Azotobacter chroococcum* which produces cysts which are as resistant to desiccation and ultraviolet radiation as the typical cysts of *A. chroococcum* but which are mechanically very fragile. These data are presented because they indicate that the structure of the cyst coat may not be the entire basis of the resistant properties of the azotobacter cyst.

**METHODS**

**Organisms and culture conditions.** Two organisms were used in this study. *Azotobacter chroococcum* 75–1 was obtained from the stock culture collection, Department of Microbiology, University of Texas, Austin, U.S.A., and maintained on Burk basal medium (Wilson & Knight, 1952) with 1% glucose as the carbon source and 2% agar as the solidifying agent. This organism produced cysts readily when grown on the same medium with 0.2% n-butanol instead of 1% glucose as the carbon source. The second organism, *A. chroococcum* strain NTS, was isolated from a soil sample obtained...
from the state of Louisiana, U.S.A. It was isolated on Burk medium with glucose and induced to encyst by culturing it on plates of Burk medium with n-butanol. All cultures were incubated at 30°. Purity of all cultures was established by colonial morphology, microscopic examination, and subculture on Tryptic Soy Agar (Difco).

Determination of fragility. Cyst fragility was first noted microscopically when cysts were seen to rupture during preparation of thin wet mounts between cover glass and microscope slide. Subsequently, cysts were ruptured by ultrasonic treatment 20,000 cyc./sec. delivered into an aqueous suspension of cysts at approximately 0°, with a Branson Model S-75 Sonifier (Branson Instruments, Inc., Stamford, Connecticut, U.S.A.). Disruption of cysts was monitored by changes in extinction at a wavelength of 540 mp and confirmed by microscopic examination.

Ultraviolet irradiation resistance. The resistance of cysts to ultraviolet radiation was determined by measuring survival after exposure to radiation of approximately 2537 Å wavelength. Cysts were washed by centrifugation and resuspension in sterile water, placed in Petri dishes to a layer depth of approximately 1 mm., irradiated at room temperature, and kept constantly in motion to minimize exposure differences. All radiation studies were done in a darkened room to avoid photoreactivation. (Recent work in this laboratory shows that cysts of Azotobacter can be photoreactivated: unpublished data.) Radiation survival was determined by spread plate counts using Burk basal agar with 1% glucose and the death curves obtained were not unlike those reported previously (Socolofsky & Wyss, 1962; Vela & Wyss, 1965).

Desiccation resistance. Resistance of cysts to desiccation was determined by the method of Socolofsky & Wyss (1962).

Electron micrography. Cysts were collected from the surface of agar plates and fixed in 3% glutaraldehyde buffered with 0.1 M-sodium cacodylate at pH 7.2 for 1 hr at 3°. They were washed several times in 0.2 M-cacodylate buffer at 3° and suspended in Bouin fluid, pH 3.7, for 15 min. at 3°. They were washed several times with cacodylate buffer at 3°, fixed with 1% OsO₄ buffered with 0.1 M-cacodylate at pH 7.2 for 2 hr at 3°, then dehydrated by passage through a series of graded ethanol solutions terminating in absolute ethanol. Embedding was accomplished by infiltration with a 1:1 mixture of propylene oxide and Epon 812 followed by serial increases of Epon 812. Polymerization was done in a drying oven at 60° overnight. Thin sections were prepared with the Porter–Blum ultramicrotome equipped with a diamond knife (Ivan Sorvall, Inc., Norwalk, Connecticut, U.S.A.). The sections were treated with a saturated ethanolic solution of uranyl acetate for 30 min. and then with an aqueous saturated solution of lead tartrate for 7 minutes. All preparations were examined with an RCA-EMU-3 G electron microscope.

RESULTS AND CONCLUSIONS

Gross observations. The preliminary observations of mechanically fragile cysts were made during routine examinations of soil isolates. Encystment of newly isolated cultures of Azotobacter was determined by microscopic examination of wet mounts using a cyst stain previously described (Vela & Wyss, 1964). Large numbers of cysts from isolate NTS were ruptured but preparations from other isolated cultures of Azotobacter yielded whole normal cysts which, like those from known encysting
Fragile cysts of *Azotobacter chroococcum*

Azotobacter species, were not ruptured by the staining and mounting procedure. The extent of cyst rupture was roughly correlated with the amount of pressure used in attempting to remove excess stain from the coverslip preparations. The newly isolated organism and *Azotobacter chroococcum* 75-1 produced cysts which were indistinguishable when examined with the light microscope, with the cyst stain, and with the phase-contrast microscope. In cultural characteristics, cell morphology, and colonial morphology strain NTS was *A. chroococcum* according to the criteria given in *Bergey's Manual* (1957). The isolate was subcultured every day or two for a total of 47 transfers using Burk medium with 1% glucose without detectable changes in the relative fragility of its cysts as compared to those of *A. chroococcum* 75-1.

*Cyst fragility and desiccation resistance.* Figure 1 records quantitative comparisons of the fragility of *Azotobacter chroococcum* strains 75-1 and NTS to ultrasonic treatment in an aqueous medium; the correlation between changes in extinction and cyst breakage was established by Socolofsky & Wyss (1962). Cysts of *A. chrococcum* 75-1 were much more resistant to sonication than those of *A. chroococcum* NTS. No difference was detected in the desiccation resistance of cysts of *A. chroococcum* strains 75-1 and NTS.

![Fig. 1](image1.png)

**Fig. 1**

Fig. 1. Effect of ultrasonic treatment on the cysts of *A. chroococcum* 75-1 (○) and those of *A. chroococcum* NTS (●). Changes in extinction were indicative of cyst rupture as verified by microscopic examination.

![Fig. 2](image2.png)

**Fig. 2**

Fig. 2. Relative resistance of cysts of *A. chroococcum* 75-1 (○) and those of *A. chroococcum* NTS (●) to ultraviolet radiation. The abscissa indicates the number of seconds of exposure to the flux of ultraviolet light established by a Model R-51 Mineralight at a distance of approximately 15 in. from the cyst suspensions while the ordinate indicates the surviving fraction of bacteria after exposure.

*Ultraviolet radiation resistance.* The data from an experiment typical of several (Fig. 2) indicate that, while there was a difference between the two strains in their response to the bactericidal effect of ultraviolet radiation, the difference was qualitative and not quantitative, since small doses of radiation produced a sigmoidal survival curve in *Azotobacter chroococcum* 75-1 but a parabolic survival curve in NTS. With higher doses of radiation the survival curves of the two strains were essentially identical. Thus the two organisms reacted differently to ultraviolet irradiation, but their resistance was approximately the same.
Ultrastructure of the cysts. Under the light microscope the two strains of Azotobacter chroococcum appeared identical. The morphologies of the cyst coats were different and easily distinguishable when examined by electron microscopy: the cyst coats possessed structurally different exines. The exine of A. chroococcum NTS (Pl. I, fig. 1) appeared to be a loose non-laminar structure. Azotobacter chroococcum 75-1 (Pl. I fig. 2) possessed a well-defined compact exine in agreement with previous descriptions of Azotobacter cysts (Wyss, Newmann & Socolofsky, 1961; Socolofsky & Wyss, 1962; Tchan, Birch-Andersen & Jensen, 1962; Parker & Socolofsky, 1966).

Conclusions. Resistance of Azotobacter chroococcum NTS to various deleterious agents may be attributed to diverse morphological characteristics. Parker & Socolofsky (1966) stated that the resistance of the Azotobacter cyst to deleterious agents resides in the cyst coat. The data presented here indicate that resistances to desiccation, ultraviolet radiation, and ultrasonic treatment are not entirely due to the same structural entity, the cyst wall. There is an ultrastructural difference between the two Azotobacter strains and these two strains show marked differences in their resistance to ultrasonic treatment. It seems reasonable therefore to state that the resistance to desiccation is apparently not entirely a function of the cyst wall structure, that the resistance to ultraviolet radiation is apparently affected by the ultrastructure of the cyst wall, and that the resistance to mechanical stress is apparently due to the configuration of the exine.

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REFERENCES

EXPLANATION OF PLATE
(Magnification, × 25,000)
Fig. 1. Thin section of the cyst of Azotobacter chroococcum NTS.
Fig. 2. Thin section of the cyst of Azotobacter chroococcum 75-1.