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# NOTES

## Developmental Block in Citric Acid Cycle Mutants of *Bacillus subtilis*

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Mutants deficient in different enzymes of the citric acid cycle can be subdivided into two groups according to the frequency at which they produce heat-resistant spores in nutrient sporulation medium. However, the majority of cells can develop in this medium only to the axial filament stage I of sporulation; aconitase and isocitrate dehydrogenase mutants need the addition of glutamate to reach this stage.

Mutants of *Bacillus subtilis*, deficient in enzymes of the citric acid cycle, can grow vegetatively but do not sporulate in nutrient sporulation medium (1, 2, 4, 5). Electron microscope observations of standard *B. subtilis* throughout the developmental period had shown that normal sporulation consists of sequential stages ending with a complete finished spore. Ryter had designated these stages as stage I through VIII (8). We have here investigated at which stage the sporulation of mutants blocked in various enzymes of the citric acid cycle is arrested. For an aconitase and an isocitrate dehydrogenase (DHG)-deficient mutant, development was earlier found to stop at stage 0 or I of sporulation (9). In nutrient sporulation medium, the citric acid cycle mutants grew at a slower rate and to a lower maximal absorbancy at 600 nm than did our standard 60015 type (Table 1), and they produced small pale colonies, easily distinguishable from strain 60015, on tryptose blood agar plates. In addition, the titer of spores produced by mutants blocked in the first half of the cycle was much lower than that produced by mutants in the second half. Whereas aconitase, isocitrate DHG, and α-ketoglutarate DHG mutants produced less than 10 spores/ml, mutants deficient in succinic DHG, fumarase, and malic DHG produced up to $10^5$ to $10^6$ spores/ml. (All strains were inoculated at the same initial titer of 0.1 at absorbancy at 600 nm [$A_{600}$] and grown for the same times.) The number of spores produced increased slowly from 4 to 8 h after cessation of growth, and the large burst of spore production observed in the standard strain from 5 to 7 h after cessation of growth (3) was not observed in any of these mutants. Attempts to understand the difference in sporulation by investigating the dissimilation of $^{14}$C-malate did not reveal a common accumulation intermediate for the mutants of the first or second half of the cycle.

Samples for electron microscopy were taken 5 to 7 h after cessation of growth by methods described previously (3). At this same time, almost all cells of strain 60015 contained endospores (3, 8). The two aconitase mutants and the isocitrate DHG mutants available showed in the electron microscope large, deformed cells and debris of lysed cells (Fig. 1a). Mutant 61113 was analyzed statistically as described by Oh et al. (7). Of 300 cells investigated, 18% were extremely deformed, 64% were wider and shorter than normal, 17.7% looked like dividing cells, and 0.3% had axial filaments. Since these mutants cannot produce glutamate, we also grew them with the addition of 20 mM L-glutamate. In the presence of glutamate, these mutants grew to a higher $A_{600}$ of 1.8. The cells were rarely deformed and proceeded to stage I (Fig. 1b) of sporulation. A statistical analysis of strain 61113 showed that under these conditions 70% of the cells had axial filaments. The mutants listed in Table 1 that are deficient in α-ketoglutarate DHG, succinic DHG, fumarate, or malic DHG and grown in nutrient sporulation medium for the same time all showed many cells with axial filaments, indicating that they

TABLE 1. *Growth and sporulation of citric acid cycle mutants of Bacillus subtilis in nutrient sporulation medium (NSMP)*

| Mutant no. | Source of mutant | Citric acid cycle defect | Maximal $A_{600}$ (NSMP) | Spores/ml (after 24 h) |
|---|---|---|---|---|
| 60 015 | Freese | None | 2.4 | $5 \times 10^8$ |
| 60 871 | Freese | Aconitase | 1.2 | $\leq 10$ |
| 61 113 | Freese | Aconitase | 1.2 | $\leq 10$ |
| 60 991 | Freese | Isocitrate DHG | 1.1 | $\leq 10$ |
| 60 705 | Freese | α-Ketoglutarate DHG | .7 | $\leq 10$ |
| 60 813 | Freese | α-Ketoglutarate DHG | .6 | $\leq 10$ |
| 60 818 | Freese | α-Ketoglutarate DHG | .6 | $\leq 10$ |
| 61 288 | Freese | Succinic DHG | 1.2 | $10^3$ to $10^6$ |
| 61 425 | Hanson (1A1) | Succinic DHG | 1.2 | $10^5$ to $10^6$ |
| 61 422 | Hanson (1A6) | Fumarase | 1.2 | $10^3$ to $10^5$ |
| 61 423 | Hanson (1A8) | Fumarase | 1.3 | $10^3$ to $10^5$ |
| 61 461 | Hanson (1A21) | Malic DHG | 1.6 | $10^4$ to $10^6$ |

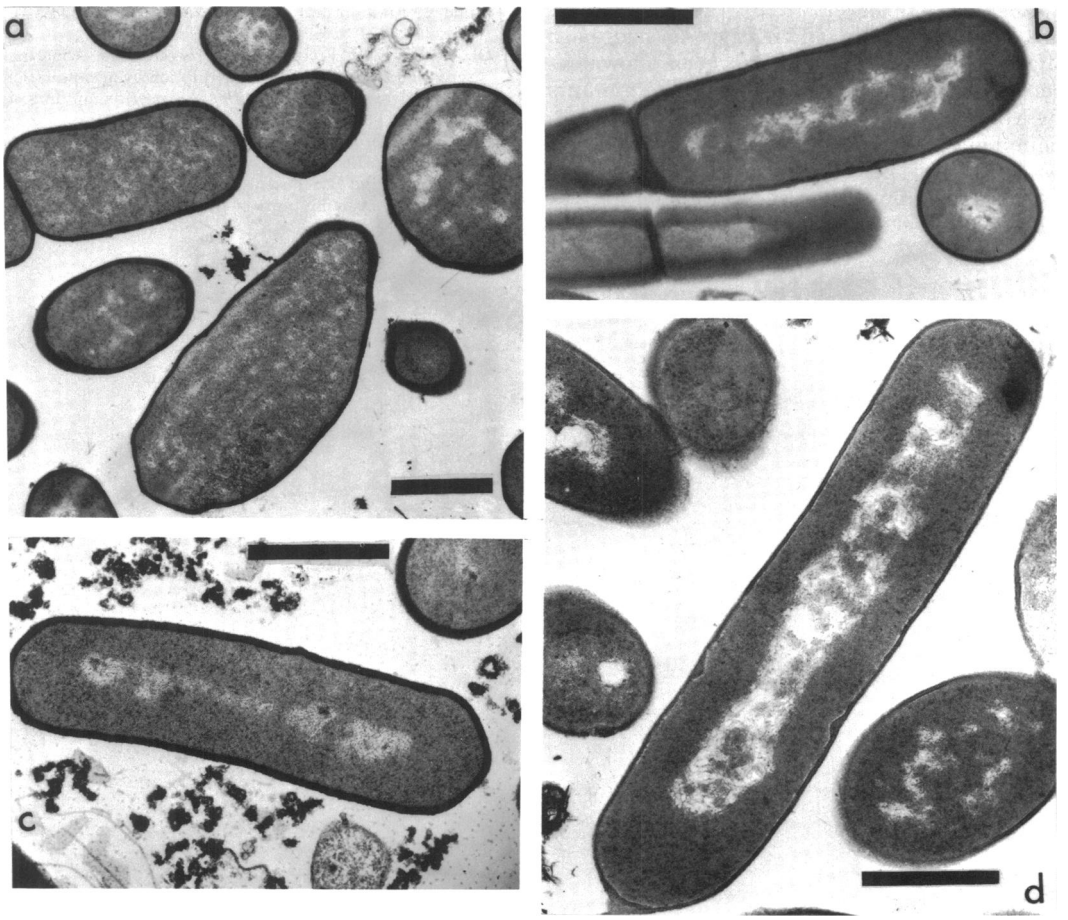


FIG. 1. *Electron micrographs of thin sections of citric acid cycle mutants 5 to 7 h after cessation of growth in nutrient sporulation medium. a, Isocitrate DHG mutant (60991) with large, deformed cells, stage 0 of sporulation; b, the same mutant grown with 20 mM L-glutamate, showing axial filament, stage I; c, α-ketoglutarate DHG mutant (60818) at stage I; d, malic DHG mutant (61461) blocked also at stage I. Horizontal bars: a, b, c = 1 μm; d = 0.5 μm.*

were arrested at stage I of sporulation (Fig. 1c, d). Statistical analysis of strain 60813, deficient in $\alpha$-ketoglutarate DHG, showed 84% of the cells in stage I, 14% in cell division, and 2% deformed cells. Strain 61288, deficient in succinic DHG, had 74% cells in stage I, 25.5% cells in cell division stage, and 0.5% in sporulation stages III-V. In the presence of gluconate, the mutants grew to a higher $A_{600}$ but did not proceed further than stage I.

We have shown that citric acid cycle mutants are blocked at stage I of sporulation. To reach this stage, aconitase and isocitrate DHG mutants even need the addition of glutamate. The arrest at stage I, however, cannot be explained by an insufficient energy supply. Although the adenosine triphosphate could be maintained during the developmental period by addition of various carbon sources (6, 9), the mutants did not sporulate more efficiently. As had been suggested earlier (4) and has been shown recently for aconitase or isocitrate DHG mutants by Yousten and Hanson (9), a suppressor compound may have accumulated preventing development, with possibly different repressors or amounts of repressors accumulating for the first and the second halves of the cycle.

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