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Isolation of Auxotrophs of *Bacillus cereus*[1]

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Auxotrophs of various species of *Bacillus* have been isolated by several workers using a variety of techniques (Iyer, J. Bacteriol. **79**:309, 1960; Zamenhof, Genetics **46**:101, 1960; Wachsman and Mangalo, J. Bacteriol. **83**:35, 1962; Nester, Schafer, and Lederberg, Genetics **48**:529, 1963). Bott and Lundgren (Can. J. Microbiol. **8**:281, 1962) were the first to report the isolation of auxotrophs of *B. cereus*. Their technique, utilizing the antibiotic methicillin, yielded only auxotrophs requiring amino acids containing sulfur. This report is concerned with the isolation of auxotrophs of *B. cereus* with a variety of nutritional requirements. Nishioka (*personal communication*) has described a simple technique utilizing diethyl sulfate for the isolation of auxotrophs of *Escherichia coli*. We have adapted this technique to several strains of *B. cereus*, including NRRL B-569, ATCC 6464, ATCC 9139, and ATCC 7064 (*B. siamensis*).

Most of our work was performed with a minimal medium of the following composition (grams per liter) prepared in distilled water: $(NH_4)_2SO_4$, 2; KH_2PO_4, 6; K_2HPO_4, 14; sodium citrate·$2H_2O$, 1; $MgSO_4 \cdot 7H_2O$, 0.2; $FeCl_3 \cdot 6H_2O$, 0.04; $MnSO_4 \cdot H_2O$, 0.00025; glucose, 2; L-glutamic acid, 2; agar (Difco), 15; *p*H 7.0. Minimal agar was enriched by the addition of 1 or 2% (v/v) of nutrient broth (Difco), and plates containing 25 ml of medium were spread with 0.1 or 0.2 ml of diethyl sulfate (DES) (Fisher Scientific Co., Pittsburgh, Pa.). The plates were placed at room temperature for 5 hr, after which approximately 1,000 spores were spread on each plate. The inoculated plates were then incubated at 37 C for about 48 hr. Many of the resulting colonies had sectors of poor growth produced by auxotrophs feeding on the small amount of nutrient broth present. The sectors, together with any minute colonies present on the plates, were picked with sterile toothpicks onto plates of nutrient agar supplemented with 0.3% of Difco yeast extract (NBY). These were incubated for 16 hr at 37 C, and the colonies were transferred to minimal agar by means of replica plating (Lederberg and Lederberg, J. Bacteriol. **63**:399, 1952). Those colonies which grew on NBY but not on minimal agar were presumed to be auxotrophs. Specific requirements were determined by use of pools of amino acids and other appropriate substances. With *B. cereus* B-569, 40 to 60% of the plated spores were killed by the DES. Of the survivors, 10 to 20% gave rise to sectored colonies, and 10 to 50% of these sectors yielded auxotrophs. Although the DES technique was not as efficient with the three other strains of *B. cereus* tested, auxotrophs were readily obtained with each of them. We isolated mutants with single requirements for serine, methionine, tryptophan, nicotinamide, arginine, adenosine, glycine, leucine, uracil, or phenylalanine.

The minimal medium which we used for the isolation of auxotrophs gave adequate, but somewhat sparse, growth. More luxuriant growth was obtained when this medium was supplemented with a mixture of the following amino acids, each at a concentration of 160 μg/ml; L-alanine, DL-valine, L-leucine, L-isoleucine, L-serine, and L-threonine. However, the supplemented medium was not as effective as the minimal medium for the isolation of auxotrophs.

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