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## ACCUMULATION OF PROLINE AND POLY-β-HYDROXYBUTYRATE IN HIGH TEMPERATURE-RESISTANT STRAINS OF *AZOSPIRILLUM*

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WE have recently described the occurrence of high temperature-resistant strains (HTR) of the nitrogen-fixing associative symbiotic bacterium, *Azospirillum*[1,2], and suggested their use as biofertilizer for summer crops. In the tropics high soil temperature appears to limit biological nitrogen fixation (often soil temperature exceeds 50°C). Although the mechanism of thermophilism in bacteria has been fairly well understood[3], no information is available for *Azospirillum*, which is a typical mesophilic bacterium. Proline and poly-β-hydroxybutyrate (PBHP) have been implicated in response to temperature and moisture stresses in organisms[4,5]. We are reporting here the possible mechanism of high-temperature resistance in *Azospirillum*.

Of fifteen HTR strains from 70 isolates of *Azospirillum*, AZ.Ht.1 and AZ.Ht.2 were chosen for the study as these isolates registered fairly high nitrogen-fixing potential. A standard mesophilic strain, *A. brasilense* Sp. 7(ATCC 29145) (supplied by Dr J. Dobereiner, Brazil), was also included in the study for comparison.

To determine PBHB, the method of Zevenhuizen[6] was followed. *Azospirillum* strains were grown in 100 ml quantities of yeast extract glucose broth in 250 ml Erlenmeyer flasks at 30 and 50°C over a temperature-controlled environmental shaker. After five and ten days of incubation cells were harvested by centrifugation at 10,000 *g* for 15 min at 4°C. The cells were suspended in sterile water and one ml of the cell suspension was digested in one ml of 2 N hydrochloric acid at 100°C for 2 h in a steam chamber. After cooling, the digest was extracted with 2.5 ml of chloroform. Samples, containing lipids, were transferred to test tubes after evaporating the chloroform over a water bath. Five ml of 96% sulphuric acid was added and the samples were heated at 100°C for ten min in a steam jacket. The absorbance of the clear solution was recorded at 235 nm in a double-beam spectrophotometer. The PBHB content was calculated from the formula

Extinction coefficient $E_{235} = 0.35$ per 100 μg of PBHB.

To determine proline, the cells were similarly grown in yeast extract glucose broth at 30 and 50°C as described earlier and the procedure of Bates *et al.*[7] was followed for the determination. Colour development was achieved by the addition of acid–ninhydrin mixture (a mixture of 1.25 g of ninhydrin dissolved in 30 ml of glacial acetic acid and 20 ml of 6 M phosphoric acid).

The results are presented in tables 1 and 2. PBHB granules in *Azospirillum* are considered to be a storehouse of energy-rich material usable by the

**Table 1** *Poly-β-hydroxybutyrate in Azospirillum isolates*

| | 5 Days' growth | | 10 Days' growth | |
|---|---|---|---|---|
| Isolate | 30°C | 50°C | 30°C | 50°C |
| AZ.Ht.1 | 0.387 | 1.908 | 0.990 | 3.085 |
| AZ.Ht.2 | 0.789 | 2.163 | 1.209 | 5.835 |
| Sp.7 | 0.859 | 0.834 | 0.886 | 0.709 |
| Mean for 15 HTR isolates | 0.944 | 1.654 | 1.107 | 3.421 |

PBHB in mg per g dry weight of cell. Each value is mean of three determinations.

Table 2 *Proline accumulation in Azospirillum isolates*

| | 5 Days' growth | | 10 Days' growth | |
|---|---|---|---|---|
| Isolate | 30 C | 50°C | 30 C | 50°C |
| AZ Ht 1 | 64.0 | 104 0 | 89 6 | 136 0 |
| AZ Ht.2 | 32 0 | 192.0 | 69.1 | 284.0 |
| Sp.7 | 68.0 | 78 0 | 74.2 | 91.2 |
| Mean for 15 HTR isolates | 61.8 | 96.8 | 74.6 | 112 8 |

Proline in μg per mg dry weight of cells. Each value is mean of three determinations.

organism at times of stress. PBHB content of HTR isolates AZ.Ht.1 and AZ.Ht.2 was higher than that of the standard mesophilic strain Sp.7. Also, PBHB content of the HTR strains was higher when the cells were grown at 50°C, while that of strain Sp.7 tended to decrease, presumably owing to the utilization of PBHB as a source of energy during the prolonged incubation. In general the 15 HTR isolates showed a spectacular increase in PBHB content at elevated temperature.

The HTR strains AZ.Ht.1 and AZ.Ht.2 strains also recorded sharp increase in proline content after growth at 50°C. In Sp.7 strain there was a slight increase in proline content at 50°C. All the 15 HTR isolates tended to accumulate proline at high temperature. The results suggest the role of PBHB and proline in temperature resistance of *Azospirillum* spp.

The problem of temperature sensitivity has been realized in the case of the root nodule bacterium *Rhizobium*. Eaglesham and Ayanaba[8] have indicated the occurrence of several strains of *R. trifolii* and *R. japonicum* which exhibited temperature tolerance up to 70–80°C. They have also stressed the need for developing HTR strains in *Rhizobium*. The occurrence of higher ratios of high-melting-point, saturated fatty acids to branched-chain fatty acids was reported to confer temperature tolerance to bacteria[9,10]. Also, intracellular accumulation of amino acids like proline and hydroxyproline[11], presence of thermostable proteins, and synthesis of heat shock proteins[12] have been suggested as possible mechanisms of high-temperature tolerance in microorganisms.

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## *IN VITRO* EFFECTS OF THREE ORGANOPHOSPHORUS INSECTICIDES ON KINETIC CONSTANTS OF ACETYLCHOLINESTERASE IN A FRESHWATER TELEOST, *CLARIAS BATRACHUS* (LINN.)

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It is well known that acetylcholinesterase (AChE, E.C. 3.1.1.7) is the target enzyme of both organophosphorus (OP) and carbamate pesticides[1,2]. The inhibition of AChE disrupts the transmission of the nerve impulse in the central and peripheral nervous system in vertebrates[3–5]. Several attempts were made to correlate the toxic action of OP pesticides with AChE inhibition and thereby to estimate their anticholinesterase activity[6–8]. OP and carbamate compounds inhibit cholinesterases by binding cova-