J. Gen. Appl. Microbiol., **14**, 305–310 (1968)

CHEMICAL BASIS OF STREPTOMYCIN RESISTANCE IN *BACILLUS SUBTILIS*

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(Received April 5, 1968)

Cytological studies on the cellular components of streptomycin-sensitive strain B_3 of *Bacillus subtilis* and its two resistant strains $2R_2B_3$ and $2R_3B_3$ reveal that development of streptomycin resistance is accompanied by an increase in both DNA and RNA content of cells. The resistant strains are able to concentrate in higher amounts 13 different amino acids in the free state in the internal environment. The levels of all the amino acids and amino sugars of cell walls are higher in the resistant strains.

Limited investigation is available about the chemical aspects of streptomycin resistance, although a large number of investigators have employed streptomycin-resistant bacterial strains in numerous genetic and biochemical studies. GROS *et al.* (*1*) observed that streptomycin-fast cells of *Staphylococcus aureus* contained higher amount of ribonucleic acid than sensitive cells. BELJANSKI (*2*) reported that streptomycin-resistant *Salmonella enteritidis* were richer in deoxyribonucleic acid than the sensitive one. BEZBORODOV *et al.* (*3*) observed lower deoxyribonucleic acid content in streptomycin-resistant *Alcaligenes faecalis*. CHERTKOVA *et al.* (*4*) demonstrated that streptomycin sensitivity was associated with a reduction of total nucleic acid content mostly at the expense of ribonucleic acid content of cells. COSTIN-PODHOVSKY *et al.* (*5*) observed less marked alteration in cellular contents of nucleic acid in *M. tuberculosis* var. *bovis* (B.C.G.) on development of resistance to streptomycin. FEDOROVA (*6*) observed no significant differences of nucleotide composition of DNA and RNA in streptomycin-sensitive and resistant strains of *Erwinia carotovora Citrullis*. TERESHIN (*7*) also did not find any difference of DNA level in streptomycin-sensitive and resistant strains of *Salmonella gertneri*. There are only very few reports on the observations of free amino acid composition of streptomycin-sensitive and resistant strains. MIZUNO *et al.* (*8*) observed less free aspartic and glutamic acids in streptomycin-resistant *Staphylococcus aureus*. HASHIMOTO *et al.* (*9*) reported no difference in the

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free amino acid content of streptomycin-sensitive and resistant strains of *Mycobacterium tuberculosis*. There is also little information about the variation of cell wall composition on streptomycin resistance. YAMAZI *et al.* (*10*) observed that the ratio of rhamnose to hexosamine was reduced in the cell walls of *Streptococcus pyogenes* resistant to chloramphenicol, penicillin and streptomycin.

In the present paper are recorded the results of a study on nucleic acid and free amino acid composition of the cells of streptomycin-sensitive and resistant strains of *B. subtilis* and of the amino acid and amino sugar content of their cell walls.

METHODS AND MATERIALS

The bacterial strains were grown in 50 ml of glucose broth (nutrient broth+1% glucose) in 250 ml Erlenmeyer flasks placed on a rotary shaker (120 rev/min) at 30°. After cultivating the organism to the logarithmic phase of growth, the cells were harvested by centrifugation in the cold and washed thrice with cold phosphate buffer (pH 7.0) and then suspended in the same buffer.

Extraction and estimation of nucleic acids

Nucleic acids were extracted from the bacterial cell suspension by the procedure of SCHNEIDER (*11*). RNA was determined by the procedure of MEJBAUM (*12*). The intensity of the colour developed was measured at 660 mμ in Klett-Summerson photoelectric colorimeter and the RNA concentration read off from the standard curve obtained with yeast RNA.

DNA was estimated by the procedure of DISCHE (*13*). The colour intensity developed was measured at 560 mμ in Klett-Summerson photoelectric colorimeter and the standard curve prepared with polymerized calf thymus DNA. The protein content of the nucleic acids was estimated by biuret method (*14*).

Extraction of free amino acids from cells

The cells after cultivating to the logarithmic phase of growth were centrifuged at 5°, washed twice with distilled water and immediately freeze-dried. 0.15 g of dried cells was extracted with 80% aqueous ethyl alcohol (*15*). The extract after separation of cell debris was evaporated to dryness and the residue taken up in 5 ml of 10% isopropyl alcohol.

Chromatographic technique for estimation of amino acids

The concentration of different samples was determined by descending two-dimensional paper chromatography, using phenol-water as the first solvent and *n*-butanol-acetic acid-water (4 : 1 : 1) as the second solvent. After development with ninhydrin, the coloured spots were identified and separately eluted with 5 ml of 75% ethyl alcohol containing $CuSO_4 \cdot 5H_2O$ (5 mg/100 ml) (*16*). The

concentration of amino acids was then determined colorimetrically. The amounts of amino acids present in the sample were calculated from the standard curve of individual amino acid chromatographed under identical conditions.

Preparation of cell walls

The method used for preparing the cell wall was that of CUMMINS and HARRIS (*17*). Bacterial cells as prepared above were disrupted with Ballotini No. 12 glass beads in a Mickle disintegrator for 30 min. The suspension was centrifuged at 3,000 rpm for 10 min and the supernatant was then centrifuged at 10,000 rpm for 30 min and the sediment containing the crude cell wall fraction was washed once with cold distilled water and then suspended in 0.05 M phosphate buffer (pH 7.6). The wall suspension was digested with ribonuclease (0.5 mg/ml) at 37° for 3 hr. It was then centrifuged and washed once with distilled water and then suspended in the same buffer. The crude wall preparation was next digested with trypsin (0.5 mg/ml) at 37° for 3 hr and then centrifuged. The sediment was washed twice with distilled water and then suspended in 0.02 N hydrochloric acid and the suspension was digested with pepsin (1 mg/ml) at 37° for 24 hr. The wall preparation was finally washed several times with distilled water.

Hydrolysis of the cell wall preparation

Cell wall preparation was hydrolysed in 6 N HCl in a sealed tube at 105° for 24 hr. The material was then evaporated to dryness in vacuo over NaOH and finally dissolved in distilled water.

Estimation of amino acids and amino sugars

Amino acids and amino sugars were separated by two-dimensional chromatography on Whatman No. 1 filter paper (56×46 cm) with *n*-butanol : acetic acid : water (4 : 1 : 1) as the first solvent and water-saturated phenol as the second solvent. Amino acids were estimated quantitatively after development of the paper with 0.2% ninhydrin. Amino sugar content was determined using Elson-Morgan reagent according to PARTRIDGE (*18*).

RESULTS AND DISCUSSION

Development of streptomycin resistance in *B. subtilis* is accompanied by a change in both RNA and DNA (Table 1) content of resistant cells. The levels of RNA and DNA in the resistant cells are about 1.4–1.5 times higher than in the sensitive cells. Table 2 indicates that streptomycin resistance is accompanied by a change in the free amino acid composition of cells of *B. subtilis* strain B_3. The resistant strains $2R_2B_3$ and $2R_3B_3$ differ from the sensitive strain B_3 in that the former are able to concentrate in higher amounts 13 different amino acids in the free state in the internal environment.

Table 1. Nucleic acid content of streptomycin-sensitive and resistant strains of *B. subtilis*.

(Results expressed as μg/mg of protein)

Strains	RNA	DNA
Sensitive B_3	48.6	46
Resistant $2R_2B_3$	73	61
$2R_3B_3$	66	58

Table 2. Concentration of free amino acids in the cells of streptomycin-sensitive and resistant strains of *B. subtilis*.

(Results expressed in mg/100 mg dry cells)

Amino acids	Sensitive strain	Resistant strains	
	B_3	$2R_2B_3$	$2R_3B_3$
Aspartic acid	0.048	0.145	0.090
Glutamic acid	0.760	1.028	0.975
Serine	0.065	0.150	0.120
Glycine	0.110	0.278	0.200
Lysine	0.107	0.165	0.231
Threonine	0.036	0.120	0.690
Arginine	0.149	0.225	0.200
Alanine	0.524	0.717	0.662
Histidine	0.290	0.371	0.385
Valine	0.066	0.200	0.180
Methionine	0.042	0.120	0.108
Leucines	0.042	0.200	0.142
Cystine	0.141	0.124	0.128
Proline	0.195	0.351	0.249

As the free amino acid content of the cells represents the difference in amounts of amino acids assimilated and metabolized, either a higher rate of assimilation of amino acids or a lower rate of incorporation of amino acids into peptide (the organism is a producer of polypeptide antibiotic, mycobacillin (*19*)) and protein may account for the increase in the size of free amino acid pool in the resistant cells.

Streptomycin resistance results in the changes in amino acid and amino sugar composition of the cell wall (Table 3). The cell walls of sensitive and resistant strains contain glutamic acid, diaminopimelic acid, alanine, aspartic acid, glycine, glucosamine and one unidentified amino sugar, the first three amino acids and glucosamine being present in relatively higher proportions.

Table 3. Amino acid and amino sugar composition of the cell wall of streptomycin-sensitive and resistant strains of *B. subtilis*.

(Expressed as μmole/mg dry cell wall wt.)

Amino acid or amino sugar	Sensitive strain	Resistant strains	
	B_3	$2R_2B_3$	$2R_3B_3$
Aspartic acid	0.007	0.012	0.015
Glutamic acid	0.204	0.300	0.298
Glycine	0.010	0.038	0.036
Alanine	0.450	0.615	0.595
Diaminopimelic acid	0.194	0.290	0.288
Glucosamine	0.210	0.318	0.300
Unidentified	+	++	++

+, Low concentration; ++, Higher concentration.

It is further observed that the levels of all the amino acids and amino sugars are higher in the walls of resistant cells. The present study shows that there is a significant difference between streptomycin-sensitive and resistant cell walls in regard to their amino acid and amino sugar composition. The differences in the amino acid and amino sugar composition of the cell walls of sensitive and resistant strains seem to suggest a structural change. As the composition and structure of the bacterial surface play an important part in the biological reaction to environmental effects, the difference in the structure of the cell wall may be associated with altered biological properties characterizing the strains resistant *in vitro*.

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