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Translocation of mycobacillin synthetase in *Bacillus subtilis*

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The extracellular release of mycobacillin from *Bacillus subtilis* first occurred in the medium at the onset of stationary phase and continued at a high rate even after 6 days. Mycobacillin synthetase activity appeared earlier than late-exponential phase in the cytosol of producer cells and was not sedimentable even at 105000***g***. The activity then quickly reached the maximum late in the stationary phase. With further increase in the age of the culture, the activity gradually disappeared from the cytosol, to reappear concomitantly in the membrane in an insoluble particulate form, even in absence of protein synthesis. The membrane-bound synthetase activity was sedimentable at 10000***g*** and was fairly active even after 5 days.

The biosynthesis of the antibiotic peptides gramicidin S and tyrocidine produced by different strains of *Bacillus brevis* has been elucidated by several groups (Roskoski *et al.*, 1970; Lipmann, 1980; Kurahashi, 1981; Kleinkauf & Von Dohren, 1983). It was observed that the synthesizing-enzyme system, which happened to be present in the soluble supernatant in the early stage of growth, changed from a soluble to a membrane-bound form with the age of the culture in the case of tyrocidine (Lee, 1974), gramicidin S (Vandamme & Demain, 1976; Vandamme, 1981; Nimi *et al.*, 1982) and bacitracin (Frøyshov, 1977), whereas in case of polymyxin (Balakrishnan *et al.*, 1980; Vasantha *et al.*, 1980) the reverse phenomenon occurred. However, in the case of edeine, the synthesizing-polyenzyme system has been shown to be associated with a membrane–DNA complex (Kurylo-Borowska, 1975) in the post-exponential-phase cells of *Bacillus brevis* Vm 4.

Mycobacillin (Majumder & Bose, 1958) is an antifungal cyclic tridecapeptide antibiotic whose synthesizing-enzyme system has been shown to be present in the soluble supernatant of the producer *Bacillus subtilis* B_3 during the early phase of growth (Sengupta & Bose, 1971). The synthesizing enzyme, mycobacillin synthetase, has been purified and appears to be a polyenzyme system that resolves itself into three fractions, which cannot carry out the synthesis unless added together (Ghosh *et al.*, 1983). We therefore decided to study the localization of the three-fraction enzyme in relation to the age of the culture.

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Materials and methods

Chemicals and radiochemicals

Lysozyme from egg white, ATP and sucrose were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). U-^{14}C-labelled *Chlorella* protein hydrolysate (sp. radioactivity 42mCi/mg-atom of C) was purchased from Bhabha Atomic Reserach Centre (Trombay, India). Other chemicals used were from commercial sources.

Strain, media and growth conditions

Bacillus subtilis B_3 producing mycobacillin was grown in a shaking incubator at pH7.2 and at 30 ± 1°C in nutrient broth as reported previously (Ghosh *et al.*, 1983).

Measurement of growth

The growth under agitation was monitored by measurement of A_{660} in a photoelectric colorimeter.

Microbial assay

Extracellular and intracellular mycobacillin production under agitation was assayed, by a cup-plate method, by its activity against *Aspergillus niger* G_3Br (Banerjee & Bose, 1969; Ray & Bose, 1971). The concentration of the antibiotic was

determined from the standard curve of mycobacillin.

Preparation and fractionation of sonic extract

A homogenized washed cell suspension of *B. subtilis* in 50mM-Tris/HCl buffer was sonicated (Mukhopadhyay *et al.*, 1984). Portions of the sonicated extract were then separately centrifuged at 10000*g* for 30min in a Sorvall RC-5B refrigerated Superspeed centrifuge and also at 105000*g* for 90min in the ultracentrifuge below 5°C. The pellet and supernatant at each step were assayed for enzyme activity. For characterization, the 10000*g* pellet as obtained from 80h-old cells was suspended in buffer and centrifuged first at 3000*g* and then at 10000*g* to give a heavy and a light pellet respectively. The light pellet was further fractionated by discontinuous 20–60%-(w/v)-sucrose density-gradient centrifugation and its activity assayed at each step.

Preparation of protoplasts

Protoplasts were prepared by the method of Sengupta & Bose (1971).

Assay of mycobacillin synthetase activity

The incubation mixture and the assay procedure was the same as described by Ghosh *et al.* (1983). The concentration of the enzyme and the period of incubation in the assay procedure were so adjusted as to maintain a linear relationship during the process. In practice, the specific activity of the soluble enzyme preparations was 405c.p.m./h per mg of protein, and the enzyme synthesized mycobacillin at enzyme-protein concentrations of 3mg/ml for at least 120h, whereas at higher concentrations (4 and 5mg/ml) a slight decline was observed after 90h; the pellet preparations had a specific activity of 320c.p.m./h per mg of protein and produced mycobacillin for at least 120h.

Protein determination

Protein was determined by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Results

Kinetics of cellular growth, antibiotic production and synthetase activity in subcellular fractions

The extracellular mycobacillin production started at the onset of stationary phase (Fig. 1) and continued for 6 days or more, the peak value being attained at about 136h (stationary phase). The intracellular accumulation of mycobacillin occurred first at the lower limit of the assay, 40μg/ml, at late stationary phase, and continued during the observed period. Fig. 1 also shows that mycobacillin synthetase activity first appeared in the 105000*g* supernatant at the late-exponential phase

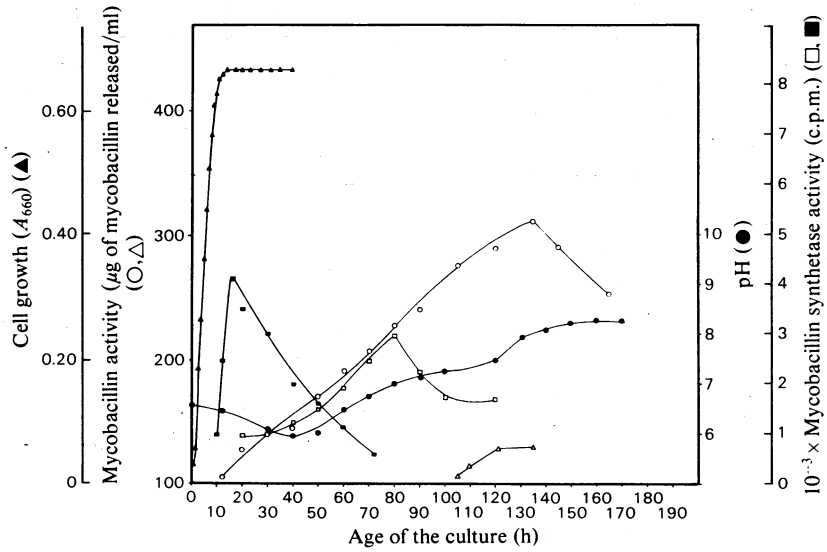

Fig. 1. *Correlation between cell growth, antibiotic production and mycobacillin synthetase activity*
The enzyme activity in the supernatant as well as in the pellet fraction was determined as described in the Materials and methods section. The age of the culture was varied as required. Incubation temperature and period were respectively 30°C and 90min. Key to symbols: ▲, Cell growth; ○, extracellular and △, intracellular, mycobacillin activity; ■, 105000*g*-supernatant and □, 10000*g*-pellet mycobacillin synthetase activity; ●, change in pH of fermentation medium.

of growth, increased abruptly and reached the maximum at the onset of stationary phase (16h). The activity then declined slowly to become almost zero at about 72h. The synthetase activity in the 10000*g* pellet appeared first at about 20h, reached the maximum at about 80h and then slowly declined to attain, at 100h, 50% of its peak activity, which thereafter remained constant all through the observed period.

Distribution of synthetase activity under conditions of inhibition of protein synthesis

Studies on the pattern of distribution of mycobacillin synthetase activity between the 10000*g* supernatant and the pellet over a short period, namely 40–46h, when the activity was present in both the pellet and the supernatant (Table 1) and under conditions where protein synthesis was completely blocked by chloramphenicol, indicate that the total synthetase activity showed a slight downward trend over this period. However, whereas the activity in the pellet fraction increased continuously, that in the supernatant decreased during this period, even in the absence of any protein synthesis.

Localization of the 10000g particulate synthetase activity of stationary-phase cells

Studies on the localization of the particulate enzyme obtained in the 10000*g* pellet of sonicated stationary-phase cells indicate that the activity was absent from the heavy pellet (3000*g*) but present in the light one (10000*g*). On further fractionation by sucrose-density-gradient centrifugation the activity in the light pellet resolved itself into three fractions. The major synthesizing activity was found to be associated with fractions sedimented at 40% (w/v) sucrose (Fig. 2), whereas a minor one was sedimented at 20% (w/v) sucrose. The heavy precipitate sedimented at the bottom of the 60% (w/v) sucrose was devoid of any synthesizing activity.

Characterization of particulate mycobacillin synthetase system

Mycobacillin synthetase activity of the unfractionated 10000*g* pellet was decreased by 13% after lysozyme treatment (Table 2), whereas the fractions obtained from the sucrose density gradient possessed lysozyme-insensitive enzyme activity.

Table 2 further indicates that succinate dehydrogenase activity was present in the 10000*g* pellet and in the fractions sedimented at 40% (w/v) sucrose, but was completely absent from the fractions sedimented at 20% (w/v) sucrose.

The particulate enzyme activity of the 10000*g* light pellet and that of the fractions sedimented at 40% (w/v) sucrose was destroyed almost totally (75%) by treatment with sodium deoxycholate and sodium dodecyl sulphate (Table 2), and this loss of activity was linearly related to detergent concentration. The enzyme activity in the 105000*g* supernatant of exponential-phase cells was unaffected by the treatment (result not shown).

Site of membrane binding of particulate mycobacillin synthetase activity

The mycobacillin synthetase activity was determined not only in the supernatant from lysozyme-

Table 1. *Effect of chloramphenicol on mycobacillin synthetase activity*

Chloramphenicol (50μg/ml) was added to the growing culture (40h) at 30°C, which was further incubated with aeration. The cells of different age groups (200ml) were harvested and the protein concentration as well as the synthesizing activity were determined separately for the 10000*g* pellet and supernatant as described in the text.

Age of the culture (h)	Incorporation of ^{14}C-labelled amino acid into mycobacillin (c.p.m.)		
	Supernatant	Pellet	Total
40	2890	1150	9773
42	2700	1298	9242
44	2545	1430	8836
46	2304	1625	8281

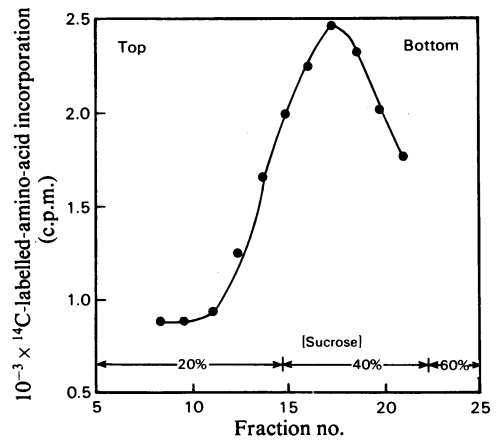

Fig. 2. *Isolation of membrane-associated mycobacillin synthetase by sucrose-density-gradient centrifugation*

The 10000*g* light pellet in Tris/HCl buffer was layered (5ml) on top of a three-step sucrose-density-gradient column consisting of 7ml of 60% (w/v) sucrose overlayered with 7ml of 40%, and then with 13ml of 20%, sucrose. The samples were then centrifuged for 30min at 75000*g* (Beckman model-L-5-50 ultracentrifuge; swinging-bucket type-SW.25.1 rotor) in the cold. Fractions (1ml) were analysed for mycobacillin synthetase activity.

Table 2. *Nature of the 10000g particulate enzyme synthesizing mycobacillin*

Different fractions were treated with lysozyme (1 mg/ml) for 10 min at 30°C and centrifuged at 10000*g* in the cold. The supernatant from each fraction was taken for sugar detection by the anthrone reaction (Gilbert, 1957). The synthetase activity of each fraction before and after lysozyme treatment, expressed as c.p.m., was determined as described in the text. The presence of the enzyme succinate dehydrogenase in different fractions was assayed by the method of Slatter & Bonner (1952). The different fractions were treated with different concentrations of the detergents sodium deoxycholate (DOC) and sodium dodecyl sulphate (SDS) for 10 min in the cold, washed twice in buffer and enzyme activity assayed. (For brevity, only the results for 1% detergent are shown.) The control was run with denatured protein.

System	Lysozyme-sensitivity	Succinate dehydrogenase activity	Effect of 1% (w/v) detergent (c.p.m.)		Incorporation ability (c.p.m.)	
			DOC	SDS	Lysozyme-treated	Lysozyme-untreated
10000*g* pellet	+	+	505	480	1657	1925
Enzyme system at:						
20% (w/v) Sucrose	−	−	1088	1050	1068	1105
40% (w/v) Sucrose	−	+	450	430	2496	2506

treated cell suspension (40 h), but also in the intact protoplast and in the protoplast lysate, and showed that neither the supernatant nor the intact protoplast contain mycobacillin synthetase activity, which was present only in the protoplast lysate.

Discussion

In contrast with tyrothricin, which in the course of its production accumulated within cells of *Bacillus brevis* A.T.C.C. 8185, mycobacillin was rapidly released to the growth medium and only a negligible concentration was found within the cells of the producer at the late stage of growth. This situation was more or less similar to that of edeine A and B produced by *Bacillus brevis* Vm4.

The pattern of distribution of mycobacillin synthetase activity in different subcellular fractions indicate that all the three fractions of the three-fraction mycobacillin synthetase are present, firstly in a soluble form in the cytosol not sedimentable at 105000*g* and secondly in a particulate form sedimentable at 10000*g*, depending on the stages of growth of the producer cell. Interestingly, the increase in synthesizing activity in the particulate form is accompanied by a concomitant decrease in the activity of the soluble form of the enzyme complex with the age of the culture in the absence of protein (and so of mycobacillin synthetase) synthesis. This might be considered as a clear case for the translocation of all the three fractions of the enzyme complex from the cytosol to the particulate fraction with the age of the culture.

Characterization of the particulate enzyme seems to indicate its presence in the membrame, it being associated with the membrane marker enzyme succinate dehydrogenase and completely free from cell-wall components (as indicated by the absence of sugar after lysozyme treatment). This membrane-bound activity persisted up to the observed period of 120 h, although at a gradually decreasing rate, in contrast with tyrocidine, whose membrane-bound synthetase enzyme activity disappeared soon after attachment.

There remains a question regarding the site of membrane binding of the particulate enzyme. To this end we sought the activity in the still-lysozyme-sensitive cells when the enzyme was present in both the soluble and the particulate form. The presence of the enzyme activity in the protoplast lysate and its absence from the intact protoplast or in the supernatant from lysozyme-treated cell suspension might be taken to mean that the mycobacillin synthetase was not a periplasmic enzyme or attached to the exterior surface of the protoplast, but localized within the membrane itself. That the enzyme activity was intimately associated with the membrane was further supported by a 75% decrease in its activity after detergent treatment, which has no effect on the soluble form of the enzyme activity. However, the enzyme, like other peripheral membrane proteins, may not be bound very strongly to the membrane, being partly released during sucrose-density-gradient purification in the 20%-(w/v)-sucrose fraction, which did not contain any membrane marker enzyme.

Thus mycobacillin synthetase activity, which appears to be present in the cytosol of vegetative cells, becomes associated with the membrane of the producer cells in the stationary phase.

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