J. Biosci., Vol.1, Number 1, March 1979, pp. 91–97. © Printed in India.

Inhibitory effect of penicillin on spore-specific functions in *Bacillus polymyxa* 2459

ANIL K. GOEL and KUNTHALA JAYARAMAN
Department of Molecular Biology, School of Biological Sciences, Madurai Kamaraj University, Madurai 625 021

MS received 26 August 1978; revised 12 December 1978

Abstract. Penicillin at concentrations non-inhibitory to the vegetative growth was found to inhibit sporulation in *Bacillus polymyxa* 2459. The effect of penicillin was shown to be at the level of spore-specific mucopeptide synthesis. Penicillin had no effect on the early events such as DNA and protein synthesis in sporogenesis The sensitive period of inhibition was between T_0 to T_2 hours of sporulation.

Keywords. Spore-specific functions; spore-specific mucopeptide; sporulation; *Bacillus polymyxa* 2459.

Introduction

Penicillin, besides its bactericidal effects can inhibit spore formation in *Bacillus* species. The inhibitory effect of penicillin on sporulation was shown to be at the level of the formation of forespore septum (Hitchins and Slepecky, 1969; Lawrence, 1974) and on the formation of spore cortex (Fitz-James, 1963) The present study with *Bacillus polymyxa* has indicated that penicillin at concentrations non-inhibitory to the vegetative growth, interfered specifically with the formation of spore cortex materials. Since there exist contradictory reports on the nature and duration of synthesis of mucopeptides during sporulation (Vinter, 1963; Pitel and Gilvarg, 1970), the effect of penicillin on other spore-specific events was also monitored to ascertain its inhibitory role in sporulation.

Materials and methods

Microorganism

The organism used in this investigation was *Bacillus polymyxa* 2459, obtained through the courtesy of Pfizer and Company, USA. Stock cultures were maintained as heat-shocked spores.

Chemicals and radioactive compounds

All the chemicals used were from British Drug House or Merck (India) and were of the analytical reagent grade. Benzyl-penicillin was obtained from Alembic Chemicals, Baroda, [^{l4}C]-leucine. [U], (20·9 mCi/mmol), [^{3}H]-thymidine (6·5 Ci/mmol), ^{45}Ca (5 to 10 mCi/mg of Ca^{2+}) were purchased from Bhabha Atomic Research Centre, Bombay, and uniformly labelled [^{3}H]-(DL *meso*)-2,6-diaminopimelic acid dihydrochloride [G] (4·6 mCi/mg) was from the Radiochemical Centre, Amersham, England.

Growth and sporulation medium

The growth and sporulation media used were those of Sterlini and Mandelstam (1969). The growth medium used was of the following composition: for 1000 ml; casein-hydrolysate 10·6 g, L-glutamic acid 3·9 g, alanine 0·14 g, KH_2PO_4 1·44 g, Na_2SO_4 0·11 g, $FeCl_3$ 0·001 g, NH_4N0_3 0·l0g, NH_4C1 0·56g, $MgSO_4$ 0·02g. The last three components were added after sterilising the stock solutions separately. The pH of the medium was adjusted to 7·0 prior to sterilisation. The sporulation medium was of the following composition : for 1000 ml, $FeCl_2$ 0·046 mg, $MgSO_4$ 4·80 g, $MnCl_2$ 12·6 mg, NH_4C1 0·54 g, Na_2SO_4 0·106 g, KH_2PO_4 0·068 g, NH_4NO_3 0·097 g, $CaCl_2$ 0·219 g, L-glutamic acid 2·00 g The pH was adjusted to 7·1 with KOH. The organism was grown at 37° C.

Culture conditions

Spore stocks of Bacillus *polymyxa* (10^8 spores/ml) were used for inoculation in growth medium. When the exponentially growing cells reached a turbidity of 200 klett units, they were harvested and quickly resuspended in the pre-warmed sporulation medium. Penicillin (40 µg/ml) was added at the beginning of the experiment or at specified times.

Assay for spore-specific functions

Samples drawn at different time intervals from the sporulation medium were assayed for: extracellular protease, intr acellular alkaline phosphatase, dipicolinic acid (DPA), Ca^{2+} uptake and heat resistance. Protease was assayed by the method of Rinderknecht *et al.* (1968), using brilliant blue-hide (RBB-hide) as substrate. Alkaline phosphatase was assayed by following the method based on that of Torriani (1960) using *p*-nitrophenyl phosphate as substrate. Dipicolinic acid was estimated by the method of Janssen *et al.* (1958) Ca^{2+} uptake was determined by adding $^{45}Ca^{2+}$ to the medium at the beginning (T_0) of the resuspension period Samples (1 ml) were collected on millipore filters, washed extensively with medium containing unlabelled Ca^{2+}, and the radioactivity was measured in a Liquid Scintillation Spectrometer (Electronics Corporation, Hyderabad). Spores were counted as refractile bodies in a phase contrast microscope and the viable spore counts were determined after plating the heat-shocked spores at appropriate dilutions.

Protein, DNA and mucopeptide synthesis during sporulation

Radioactive precursors, viz., [^{14}C]-leucine (5 µ*C*i/ml) or [^{3}H]-thymidine (10 µCi/ml or [^{3}H]-DAP (4 µCi/ml) were added at the beginning of the suspension of the cells in sporulation medium at time T_0 with or without the addition of penicillin (40 µg/ml). At specific time intervals 1 ml samples were withdrawn and added to tubes containing 1 ml of cold 10% trichloroacetic acid. After 1 h at 0° C, the acid insoluble materials were filtered on millipore filters. They were washed with 15 ml of 5% trichloroacetic acid containing either unlabelled leucine, or thymidine or DPA. They were washed with cold ethanol prior to drying and estimating the radioactivity. The extent of incorporation of DAP into acid-precipitable materials

was used as the criterion of mucopeptide synthesis (Pitel and Gilvarg, 1970) Lysine (20 μg/ml) was added to cultures treated with [^{3}H]-DAP to dilute out the conversion of DAP to lysine.

Results

Effect of penicillin on sporulation in B. polymyxa

Addition of penicillin (40 μg/ml) was not inhibitory to vegetative growth in rich medium but was found to be inhibitory for sporulation under resuspension conditions. Since synchronisation of sporulation has been possible in this system (Sterlini and Mandelstam, 1969), we have investigated the specific period at which penicillin could exert its effect on sporulation. Accordingly, penicillin was added at hourly intervals between T_0–T_4 (figure 1, A). It is clear

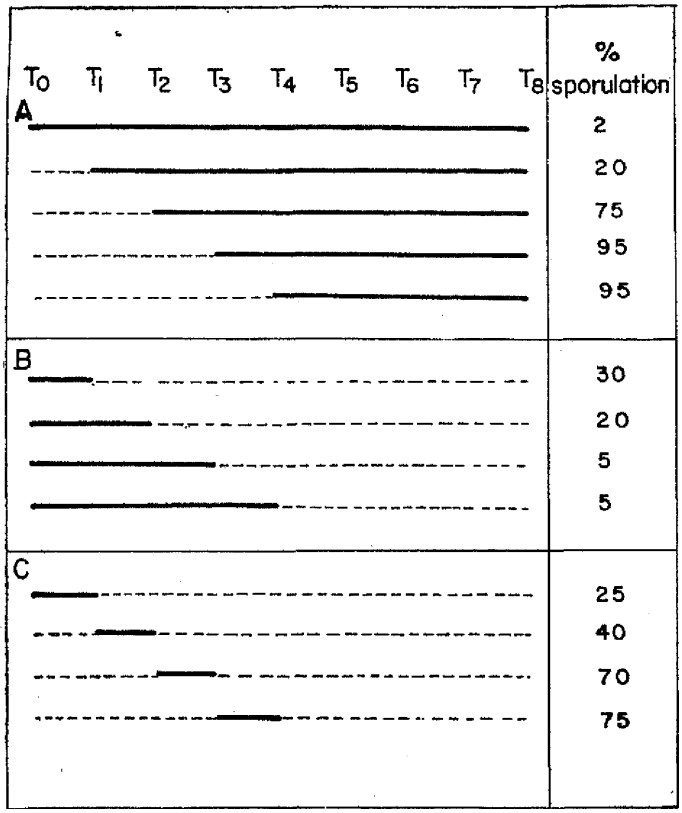

Figure 1. Effect of penicillin on sporulation in *B. polymyxa*.

Cells at mid-log phase of growth were transferred at T_0 to sporulation medium. Cultures were set up without or with penicillin (40 μg/ml). The thick lines represent the period in which penicillin was present in the medium. At T_8, samples were processed for spore counts as outlined in methods. *Panel A* : Penicillin was added at the start of specified time intervals and kept until the period T_8. *Panel B* : Penicillin was added at T_0 and kept until T_1 or T_2 or T_3 or T_4. *Panel C* : Penicillin was present between T_0-T_1 or T_1-T_2 or T_2-T_3 or T_3-T_4.

that if penicillin was not added until T_2, sporulation was not inhibited thus indicating that the T_0–T_2 was the critical period for the effect of penicillin. Addition and withdrawal of penicillin at different time periods also substantiated this observation. Addition of penicillin at T_0 and withdrawal at T_1, T_2, T_3 and T_4 resulted in the inhibition of sporulation (figure 1, B). Further narrowing of the time period in which penicillin exerted maximal effect was possible by the addition of penicillin at the beginning of T_0 or T_1 or T_2 or T_3 and its withdrawal after 1 h (figure 1, C).

Effect of penicillin on spore-specific functions in B. polymyxa

Several stages of sporulation have been characterised by the onset of biochemical events that could form essential or related phenomena (Mandelstam, 1976). We have monitored the effect of penicillin on such spore-specific biochemical events Presence of penicillin during the entire period of sporulation did not prevent formation of extracellular proteases and intracellular alkaline phosphatase (figure 2) However, it inhibited the formation of DPA (figure 2) and totally abolished the capability of the cells to take up Ca^{2+} when added at T_0 (figure 3) Under conditions in which the penicillin could not inhibit sporulation, i.e., addition of penicillin at T_4, both Ca^{2+} uptake (figure 3) and DPA level (data not shown) remained unaffected.

Thus, it was obvious that the inhibition of penicillin was felt at the later stages of sporulation, although the critical period of the effect was between T_0–T_2. Hence, we decided to look at the effect of penicillin on the three major processes involved in the transition from vegetative phase to sporulation. Penicillin neither inhibited the sharp rise in DNA synthesis observed between T_0–T_2, nor did it affect the incorporation of [^{14}C]-leucine into proteins (figure 4). The third parameter, namely, the mucopeptide synthesis was also not inhibited during the very

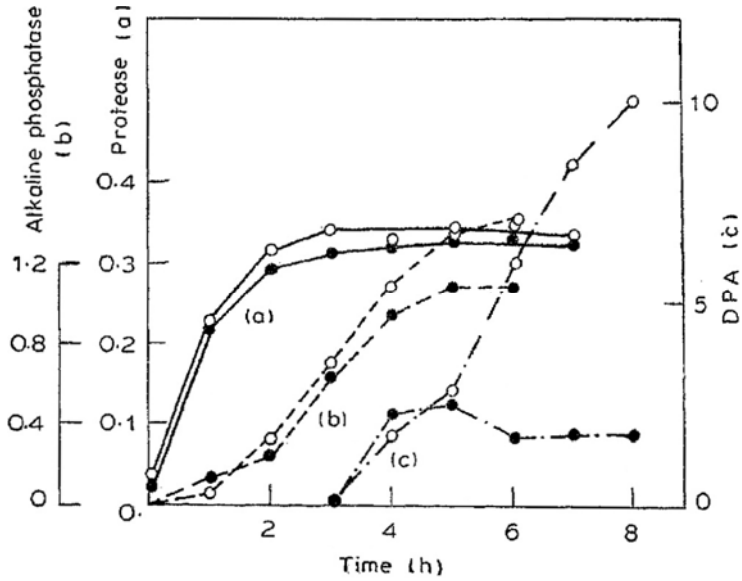

Figure 2. Effect of penicillin on spore-specific biochemical events in *B. polymyxa.*

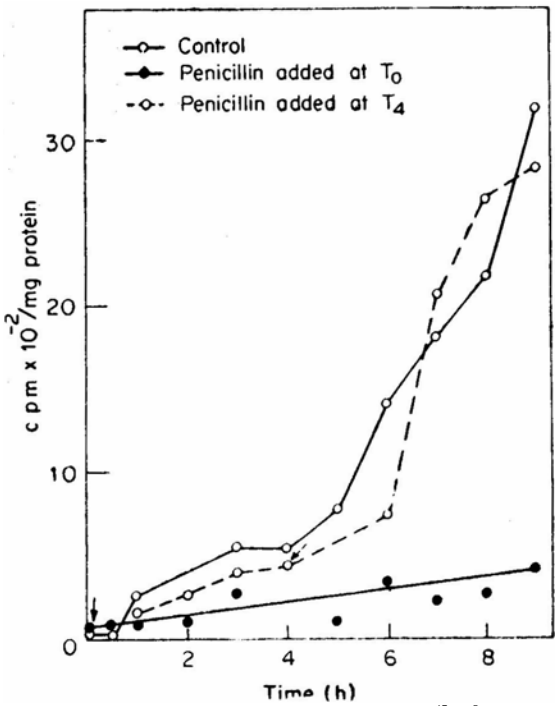

Figure 3. Effect of penicillin on the uptake of $^{45}Ca^{2+}$, in *B. polymyxa.*
Radioactive Ca^{2+} (5 μCi/ml) was added to cultures without and with penicillin (44 μg/ml) at the start (T_0) and at T_4 h of sporulation, as indicated by the arrow.

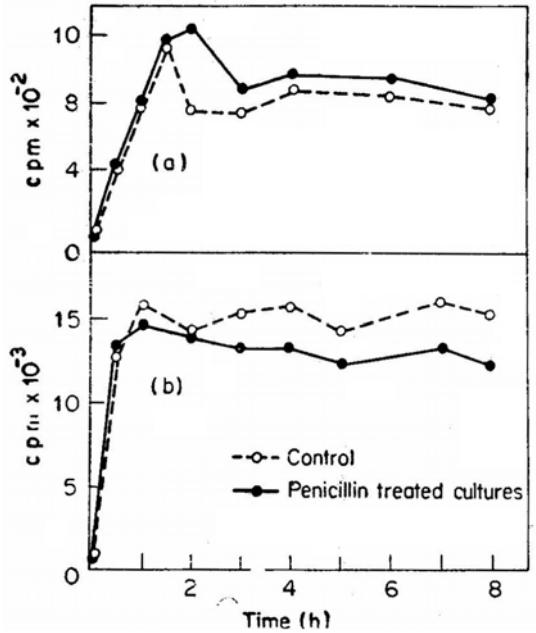

Figure 4. Effect of penicillin on the macromolecular synthesis during sporulation in *B. polymyxa*.
[^{3}H]-Thymidine (10 μCi/ml) (a); or [^{14}C]-leucine (5 μCi/ml) (b), was added at time T_0 to control and penicillin-treated cultures Samples were withdrawn at hourly intervals and acid-precipitable counts were determined.

early periods, i.e., up to T_1 (figure 5). However, the incorporation of the [^{3}H]-DAP in the cross-linked peptides ceased thereafter (figure 5) Thus it was apparent that inhibition of spore-specific mucopeptide formation by penicillin was reflected in the defective formation of spore cortex materials.

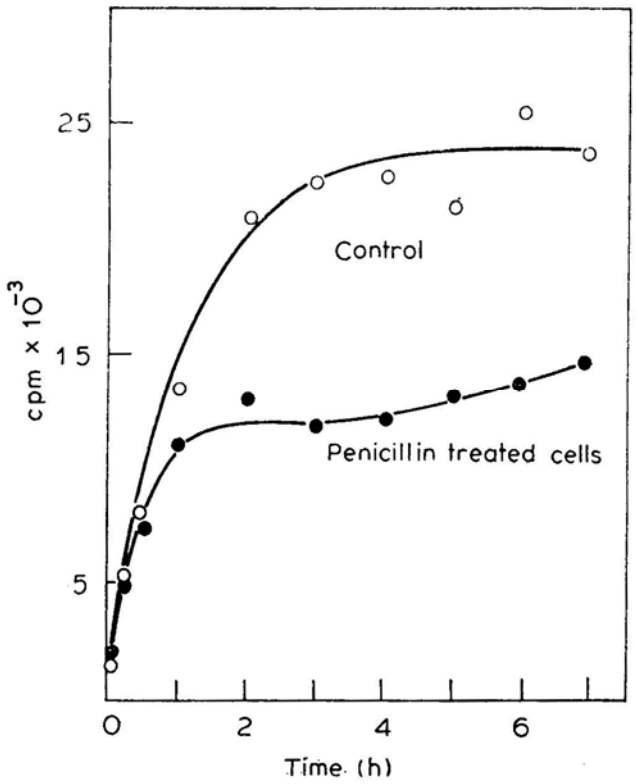

Figure 5. Effect of penicillin on the mucopeptide synthesis during sporulation in *B. polymyxa*.
[^{3}H]-DAP (4 μCi/ml) was added at time T_0 to control and. penicillin-treated cells. At time points indicated in the graph, 1·0 ml samples were withdrawn and the radioactivity of trichloroacetic acid precipitates were measured.

Discussion

Penicillin inhibits the growth of microorganisms by interfering with cell wall synthesis. The synthesis of mucopeptides during sporulation has been studied with reference to its influence on the formation of septum and spore cortex (Pitel and Gilvarg, 1970). Contradicting reports exist on this situation. Vinter (1963 has observed that there exist two specific periods of mucopeptide synthesis during sporulation. The mucopeptides, synthesised during the vegetative phase were not contributed to the formation of the spore cortex (Vinter, 1963). Pearce, and Fitz-James (1971) have also observed that there are two periods of muco-peptide synthesis. However, using more stringent conditions, such as the use of an auxotroph requiring DAP, Pitel and Gilvarg (1970) have established in

B. megaterium that there was only one period of spore-specific mucopeptide synthesis, during later stages of commitment of the cells for sporulation, *i.e.*, after T_3 .

The results of our investigations indicate that the specific inhibitory effect of penicillin was on the mucopeptide formation during later stages of sporulation in *B. polymyxa*. The inhibition of Ca^{2+} uptake (figure 3), DPA synthesis (figure 2), refractility and heat resistance in penicillin-treated cultures (data not shown) clearly argued for its effect on defective cortex formation. Most of the early events of sporulation were not affected by penicillin. The concentration of penicillin used here neither affected the vegetative growth nor the limited growth in the sporulation medium. When higher concentrations of penicillin were used, the initial rise in turbidity up to T_2 was maintained but thereafter a rapid lysis occurred. Thus it may be concluded that the inhibitory effect of penicillin, either on the formation of forespore septum or on spore cortex, is felt at a critical concentration.

The observations made by us, namely that penicillin did not interfere significantly with the incorporation of [^{3}H]-DAP into mucopeptides up to T_1, indicated that penicillin did not interfere with the cross-linking of mucopeptides during the transition between vegetative to sporulating stages. Polymerisation of mucopeptides synthesised during vegetative phase thus appeared to be a different process from that of the mucopeptide polymerisation during sporulation.

Acknowledgements

One of us (A.K.G.) acknowledges the award of a Senior Research Associateship by the University Grants Commission. The technical assistance of Miss S. Padmavathy is gratefully acknowledged. The work was supported by research grants from Council of Scientific and Industrial Research and the Department of Science and Technology.

References

Fitz-James, P. C. (1963) *Regulations* chez *les microorganismes* ed M. J. C. Senez (Paris : Centre National de la Recherche Scientifique), p 529.
Hitchins, A. D. and Slepecky, R. A. (1969) *J. Bacteriol*., **97,** 1513.
Janssen, F. W., Lund A. J. and Anderson, L. E. (1958) *Science*, **127,** 26.
Lawrence, P. J., (1974) *Antimicrobial agents and chemotherapy*, **6,** 815.
Mandelstam, J. (1976) *Proc R Soc*., (*London*), **B193,** 86.
Pearce, S. M. and Fitz-James, P. C. (1971) *J. Bacteriol*., **105,** 339.
Pitel, D. W. and Gilvarg C. (1970) *J. Biol. Chem*., **247,** 6711.
Rinderknecht, H., Geokas, M. C., Silverman, P. and Haverback, B. J. (1968) *Clin. Chim. Acta*, **21**, 197.
Sterlini, J. M. and Mandelstam, J. (1969) *Biochem. J.* **113**, 29.
Torriani, A. (1960) *Biochem. Biophys. Acta*, **38**, 460.
Vinter, V. (1963) *Folia Microbiol. Praha*., **8,** 147.