THE MODE OF ACTION OF POLYKETOACIDOMYCIN ON *BACILLUS SUBTILIS*

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(Received for publication February 18, 1974)

The antibiotic action of polyketoacidomycin (PKAM) on *Bacillus subtilis* was examined. PKAM stimulated the turnover of phosphatidyl glycerol (PG) to cardiolipin (DPG) and reduced the amounts of the normal fatty acids (nFAs) C-14 and C-16 and anteiso (a) C-17. Addition of bacterial PG to cultures treated with PKAM reduced the effect of the antibiotic.

The foregoing changes in lipid composition were associated with changes in cell permeability. Loss of cellular valine, norvaline and phosphates was observed and a significant decrease in the cellular concentration of Na^+ and K^+ ions was noted when the test organism was incubated with PKAM for periods up to 15 minutes. Longer incubation of the bacteria with the antibiotic caused an appreciable increase in RNA content of the cells and a reduction in the DNA and protein content.

Polyketoacidomycin (PKAM), first isolated by SHIMI *et al.*,[1] possesses substantial antimicrobial activity against gram-positive bacteria as well as some gram-negative bacteria. The acid hydrolysate of the antibiotic contains glucose, alanine, and acetone, as well as pyruvic, glyoxylic, α-ketoglutaric, α-ketobutyric and acetoacetic acids.

The present work was conducted to study the effect of PKAM on *Bacillus subtilis* icc.

Materials and Methods

Organism and culture conditions

Unless otherwise mentioned *B. subtilis* icc was grown in 200 ml of nutrient broth of the following composition (g/100 ml): yeast extract, 0.15; meat extract, 0.15; peptone, 0.5 and NaCl, 0.35 in shaken cultures (200 rpm) at 35°C for 16 hours. The cells were harvested at half maximal growth (O.D 1.0 at 660 mμ) by centrifugation at 15,000×g, washed with sterile saline solution and were then used immediately.

Analysis of the lipids of *B. subtilis*

Cells of *B. subtilis* were separately collected at different incubation periods from cultures treated with PKAM and from untreated control cultures. The cellular lipids were extracted from the harvested cells as described by BLIGH and DYER[2] using chloroform-methanol (2 : 1, v/v). Non-lipid substances were removed by shaking with 0.5% NaCl. The extracts were dried under vacuum to constant weight.

Analysis of the phospholipids

Thin-layer chromatography (TLC) was applied using silica gel DO. The plates were prewashed with acetone-petroleum ether (3 : 1, v/v) and then developed with chloroform-methanol-acetic acid-water (80 : 13 : 8 : 0.3, v/v). The phospholipids were located on the chromatogram by spraying the right and left lateral sides of each plate with rhodamin 6G. After locating the zones occupied by the major phospholipids, the corresponding non-stained areas occupying the central portion of the chromatoplate were separately removed and extracted with chloroform-methanol (3 : 1, v/v). The PG present in the control culture was methylated

and analyzed by GLC.

GLC analysis of fatty acids (FAs)

The total lipid extracts were methylated with 2 ml of 6% (v/v) methanol - sulfuric acid mixture in a sealed tube under an atomosphere of nitrogen and left in an oven at 110°C overnight. Fifty micrograms of C-13 fatty acid were included in the sealed tube as an internal standard. The fatty acid methyl esters thus formed were extracted with *n*-hexane and analyzed with a Hewlett Packard gas-liquid chromatography Model 5750 with a stainless steel column packed with ECNSS-S 10% (w/w) coated on a 100/200 mesh, gas chrom. P[3]. Temperature programming (140~170°C) identified the iso and anteiso (i and a) FAs. Standard FA methyl esters were run under the same experimental conditions for comparative purposes.

The amino acids were estimated by the zone strip technique[4,5,6] and the results were frequently checked on the amino acid analyzer. Total proteins were assessed by FOLIN[7] reagent. Fractionation of DNA and RNA was conducted following the technique of SCHMIDT-THANNHAUSER.[8] RNA was determined by the orcinol method,[9] and DNA by the indole method.[10] Inorganic phosphorus was analyzed by the method of BARTLETT[11] while Na^+ and K^+ ions were determined using the Perkin-Elmer atomic absorption spectrophotometer type 403 according to manufacturer's instructions (March 1971).

Results

PKAM decreased the viable counts in antibiotic-treated suspensions when compared with control preparations. Furthermore, a decrease in the viable counts was observed after 6 hours for the control whereas this effect was observed 2 hours earlier in the

Fig. 1. Effect of PKAM on bacterial growth.
The collected cells were resuspended in fresh warmed medium, incubated at 35°C with PKAM (1.0 and 10.0 μg/ml). Growth was assessed by viable counts on nutrient-agar medium.

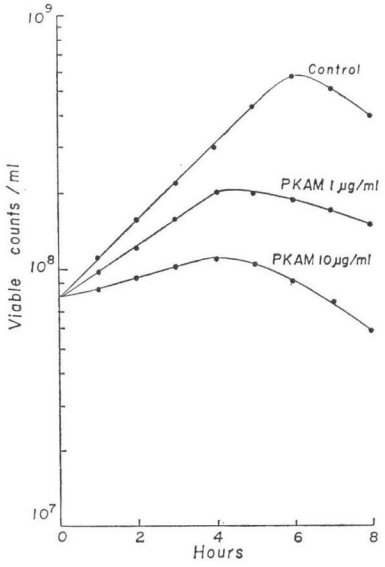


Table 1. Effect of PKAM on the lipid contents of *B. subtilis*

	% of cell dry weight			
	Control		Treated	
	5 min.	10 min.	5 min.	10 min.
Total lipids	5.05	2.23	4.23	3.41
Total phospholipids	1.93	2.06	1.43	1.08
Phosphatidyl glycerol	0.81	0.87	0.25	0.06
〃 ethanolamine	0.52	0.58	0.50	0.59
Cardiolipin	0.36	0.38	0.55	0.94

The harvested cells were grown in nutrient broth. 1 μg/ml of PKAM was added and shaked at 220 rpm for 10 minutes at 37°C. Cultures lacking the antibiotic were run alongside as controls. Samples were withdrawn at 5 and 10 minutes, the cells were collected by centrifugation at 15,000 ×*g* and the lipids were extracted, fractionated as given in the experimental section. The separated zones were eluted, and gravimetrically assessed.

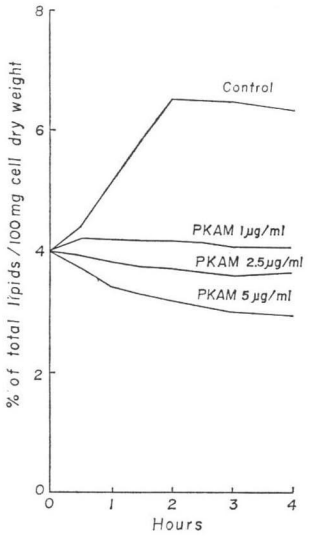
Table 2. Effect of PG on the antimicrobial activity of PKAM

Treatment		MIC (μg PKAM/ml)
PKAM alone		1.0
PKAM	A	12.5
+	B	25.0
PG	C	100.0

A: PKAM+1 μg PG/20 ml of the growing medium
B: PKAM+2.5 μg PG/20 ml 〃 〃
C: PKAM+5.0 μg PG/20 ml 〃 〃

Fig. 2. Effect of PKAM on the total lipids of *B. subtilis* cells.

The experimental steps were conducted as described in legend to Fig. 1, except that one additional concentration of PKAM (2.5 μg/ml) was included.



cultures exposed to the antibiotic (Fig. 1).

The TLC analysis of the phospholipid fractions of *B. subtilis* cells in control cultures revealed the presence of PG, DPG and phosphatidyl ethanolamine (PE) (Table 1). In cells treated with 1 μg of the antibiotic per ml, the PG content decreased rapidly and disappeared completely after 10 minutes of incubation. This rapid decrease in PG was partially offset by an increase in DPG. In contrast the PE content was not affected. GLC analysis of the FAs obtained from the PG fraction revealed the presence of n-C 14, n-C 16, i-C 17 and a-C 17 as major components.

PG collected from the test organism when added to the growth medium partially neutralized the effects of PKAM as indicated by the elevation in the minimum inhibitory concentration (MIC) of the antibiotic for *B. subtilis* (Table 2).

Table 3. Effect of PKAM on the major FAs pattern of *B. subtilis*

Fatty acids	Percent of FAs* concentration of PKAM								
	0.0 μg/ml			1.0 μg/ml			5.0 μg/ml		
	5 min.	10 min.	15 min.	5 min.	10 min.	15 min.	5 min.	10 min.	15 min.
n-C 14	1.0	1.0	1.1	1.0	0.75	0.5	0.95	0.73	0.45
n-C 16	6.3	6.5	6.1	6.0	5.8	5.0	5.90	4.4	4.1
i-C 14	3.3	3.5	3.5	3.1	3.3	3.3	3.1	3.1	3.4
i-C 16	3.3	3.6	3.8	3.4	3.5	3.7	3.2	3.2	3.5
i-C 15	34.9	35.2	35.2	35.0	35.4	35.5	35.3	35.6	35.6
i-C 17	15.3	15.4	15.4	15.0	15.3	15.3	15.2	15.0	15.3
a-C 15	30.8	31.0	31.2	30.4	30.3	31.0	31.4	31.4	31.5
a-C 17	2.3	2.4	2.5	2.4	2.0	1.5	2.0	1.6	1.3

n=normal i=iso a=anteiso
* Expressed as % of total FAs
The bacterial cells were harvested and resuspended in fresh medium (25~30 mg/30 ml) supplemented with various amounts of the antibiotic. The cultures were incubated at 35°C and the cells were collected at 5 minutes intervals for 15 minutes.

Table 4. Effect of PKAM on the release of valine and norvaline in the culture broth of *B. subtilis*

Concentration of PKAM μg/ml	Amino acids	Concentration of amino acids μg/ml			
		15 min.	30 min.	40 min.	60 min.
0.0	Valine	—	2.0	4.5	6.2
	Norvaline	—	—	1.2	2.00
1.0	Valine	3.1	11.3	15.3	21.3
	Norvaline	1.6	5.5	6.8	13.9
5.0	Valine	4.5	16.3	23.1	30.2
	Norvaline	2.06	8.4	7.9	18.8

The collected cells were resuspended in 30 ml of the synthetic medium given by KNIGHT and BROOM.[12] 1 and 5 μg/ml of PKAM were added and the flasks were shaken at 35°C. Samples were withdrawn, centrifuged and the supernatants were analyzed for their contents of amino acids.

Fig. 3. Effect of PKAM on the utilization of phoshorus by *B. subtilis*.
The experimental steps as legend to Table 3.

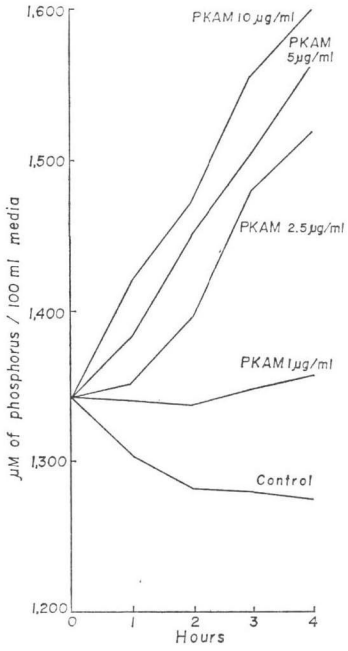
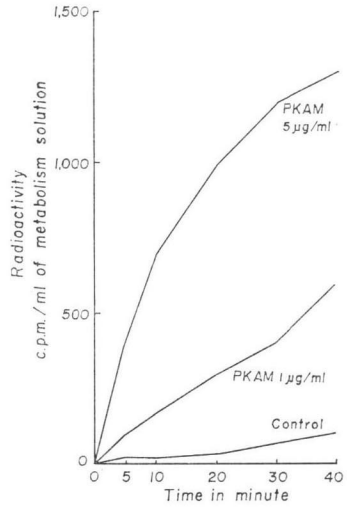


Fig. 4. Effect of PKAM on the release of P^{32} from pre-labelled *B. subtilis* cells.
Experimental steps conducted as described by SHIMI[13]



Shortly after the addition of PKAM to the culture suspension a substantial decrease in total lipids was observed. The higher the concentration of the antibiotic the more pronounced was the effect (Fig. 2). Furthermore PKAM reduced the percentages of the n-C 14, n-C 16 and a-C 17 fatty acids present in the lipid cell-extracts of the treated cultures (Table 3).

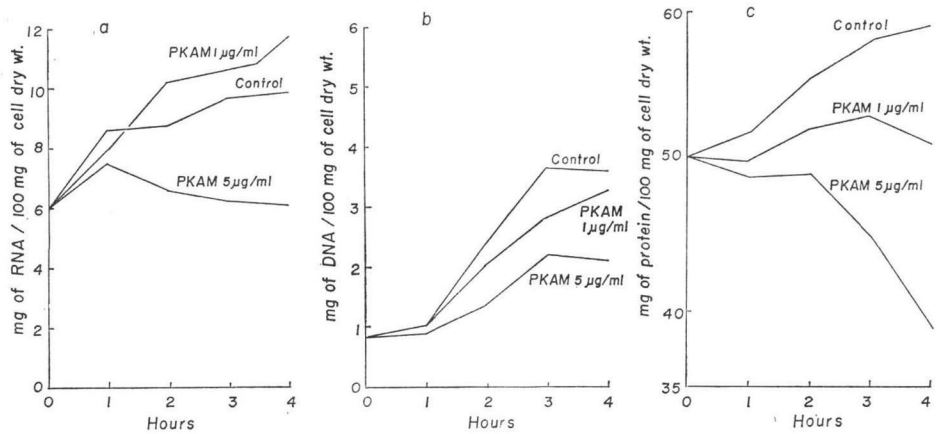
Valine and norvaline accumulated in the culture broth of *B. subtilis* after 15 minutes of exposure to PKAM. The higher the concentration of PKAM the more pronounced was the effect (Table 4). At the MIC level the antibiotic arrested the uptake of phosphorus by the test organism whereas higher levels caused marked leakage of phosphorus from the cells into the metabolism solution (Fig. 3). These results were further confirmed when *B. subtilis* cells

Table 5. Effect of PKAM on the intracellular concentration of Na^+, K^+ and Mg^{++}

Time in min.	Cations concentrations* μg/mg of cell dry weight								
	Control			Plus 1 μg/ml PKAM			Plus 5 μg/ml PKAM		
	Na^+	K^+	Mg^{++}	Na^+	K^+	Mg^{++}	Na^+	K^+	Mg^{++}
0	32.6	18	6	32.6	18	6.0	32.6	18	6
5	33.0	18.3	6.1	33.5	17.8	6.0	31.9	17.8	6
10	35.0	18.5	6.3	37.3	17.4	6.0	30.0	17.0	6.2
15	36	19	6.4	40	16.8	6.0	30	15	6.4
30	90	31.2	6.9	50.6	15.3	6.1	30	16.2	6.0
60	118	46	7.2	60	14	6.2	30	17	6.0
120	100	40.2	7.5	51.3	9	5.6	28	10.3	5.1
180	56	29	7.6	39	6	4.2	27	9.0	4.3

* Medium concentration μg/ml: Sodium 190, Potassium 60, Magnesium 25.
Determinations were carried out as described in the text.

Fig. 5. Effect of PKAM on the RNA, DNA and protein contents of *B. subtilis* cells.
The experimental steps were conducted as described in legend to Table 1. Determination of DNA, RNA and protein was carried out as described in the text.



previously labelled with P^{32} were incubated with PKAM. Detectable increases in the counts attributable to P^{32} were recorded in the extracellular solutions at concentrations of 1 and 2.5 μg PKAM/ml (Fig. 4).

Moreover, as indicated in Table 5, the intracellular concentrations of Na^+ and K^+ in particular were decreased in the presence of the antibiotic with the effect greater for K^+. The effect on Mg^{++} was only detectable 120 minutes after the vital metabolic activities of the bacterial cells had been markedly disturbed.

The presence of antibiotic reduced the cellular contents of DNA and protein when compared with non-treated cultures, whereas it increased the RNA at the level of 1 μg of antibiotic per ml after 1 hour of incubation (Fig. 5). At 5 μg of antibiotic/ml the RNA content of antibiotic-treated cells was appreciably lower than that of the control cells.

Discussion

PKAM at its effective levels stimulated a rapid turnover of PG to DPG in the *B. subtilis* cells. A similar effect of phenethyl alcohol upon *Escherichia coli* has also been recorded.[14]

The patterns of the fatty acid contents of *B. subtilis* in control cultures reported herein are in good agreement with those given in the literature.[15,16,17] PKAM treatment reduced the total lipid content and the total phospholipid content of *B. subtilis* icc. Reduction in the percentages of the FAs n-C 14, n-C 16 and a-C 17 was detectable after 5 minutes of incubation with PKAM. It is speculated that changes such as those described in the total content and composition of the lipid fraction might affect the permeability of the cell, releasing Na^+, K^+, phosphates, valine and norvaline.[18,19] The latter two amino acids might be metabolic products of the corresponding FAs.

BAKER[20] found that small amounts of phospholipids in the medium could protect gram-positive bacteria from the action of gramicidin. A similar finding was recorded during the present work. PG of *B. subtilis* icc when supplemented with PKAM reduced the effect of the antibiotic. This result suggests that the depression in the cellular content of PG might be related to the loss by the cell of the metal ions, phosphate and amino acids.

Finally, changes in the cellular contents of DNA, RNA and protein could only be detected after a relatively long period of incubation with a comparatively high level of the antibiotic. This effect was thus presumed to be an influence secondary to the primary effect of the antibiotic on the cell.

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