Stimulation of sporulation by ppApp in a conditionally asporogenous rifampin-resistant mutant of *Bacillus subtilis*[1]

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Summary. Addition of ppApp to Sterlini-Mandelstam medium stimulates sporulation of a conditionally asporogenous rifampin-resistant mutant of *Bacillus subtilis* to the same extent as the effect of 4 amino acids. Mutant cells sporulating in the presence of amino acids also produce 2 phosphorylated nucleotides one of which comigrated with ppApp on PEI thin layer chromatogram.

Bacterial sporulation proceeds sequentially with biochemical and morphological changes[3]. It has been viewed as a model system of cellular differentiation. Unique sporulation genes in *Bacillus subtilus* are transcribed at specific times during the sporulation process whereas the transcription of certain vegetative genes is turned off when the bacteria sporulate[4]. The findings that transcribing specificity of RNA polymerase during sporulation may be altered[5,6] and that mutants bearing lesions in the enzyme had lost their ability to sporulate[7] suggested that this enzyme may regulate the sporulation process. A rifampin-resistant, conditionally asporogenous mutant of *B. subtilis* which sporulates poorly in Sterlini-Mandelstam[8] (SM) sporulation medium but sporulates normally in modified Difco sporulation medium[9] was found to regain its ability to sporulate in the SM medium by the addition to the medium of arginine, methionine, valine, and isoleucine[10]. The rifampin-resistant lesion has been shown to be located in the gene encoding RNA polymerase[10]. Several highly phosphorylated adenine nucleotides (HPN) were found in sporulating cells of *B. subtilis*. In vitro studies showed that only ribosomes from sporulating but not vegetative cells are able to synthesize HPN[11,12]. The implication that HPN are involved in regulating the initiation of sporulation is supported by the isolation of asporogenous mutants defective in the synthesis of HPN[13,14]. This conclusion, however, was challenged by recent evidence implicating HPN in the stringent control of RNA synthesis upon amino acid starvation but not in the control of sporulation in *B. subtilis*[15]. This paper presents direct evidence that ppApp is involved in the regulation of sporulation in *B. subtilis* in support of earlier conclusions made by Rhaese and co-workers[11–14].

Experimental part. Bacterial strains used, conditions for sporulation and method of determining sporulation frequency have been previously reported[10]. The only modification of the procedure was the use of double strength MLY medium to grow exponential cultures. The extraction and quantitation of the highly phosphorylated nucleotides were done as described by Rhaese and co-workers[11,14]. Radioactive labelling of cell extracts was carried out by incubating $H_3{}^{32}PO_4$ (carrier free, ICN Radiochemicals, Irvine, Cal., USA) with the cells at a final concentration of 25 µCi/ml at the time of resuspension in SM medium. Polyethyleneimine (PEI) cellulose sheets were pre-equilibrated in 1 M LiCl for 3 h and then placed in deionized water for 3 h and air dried. 3–6 µl of samples were spotted

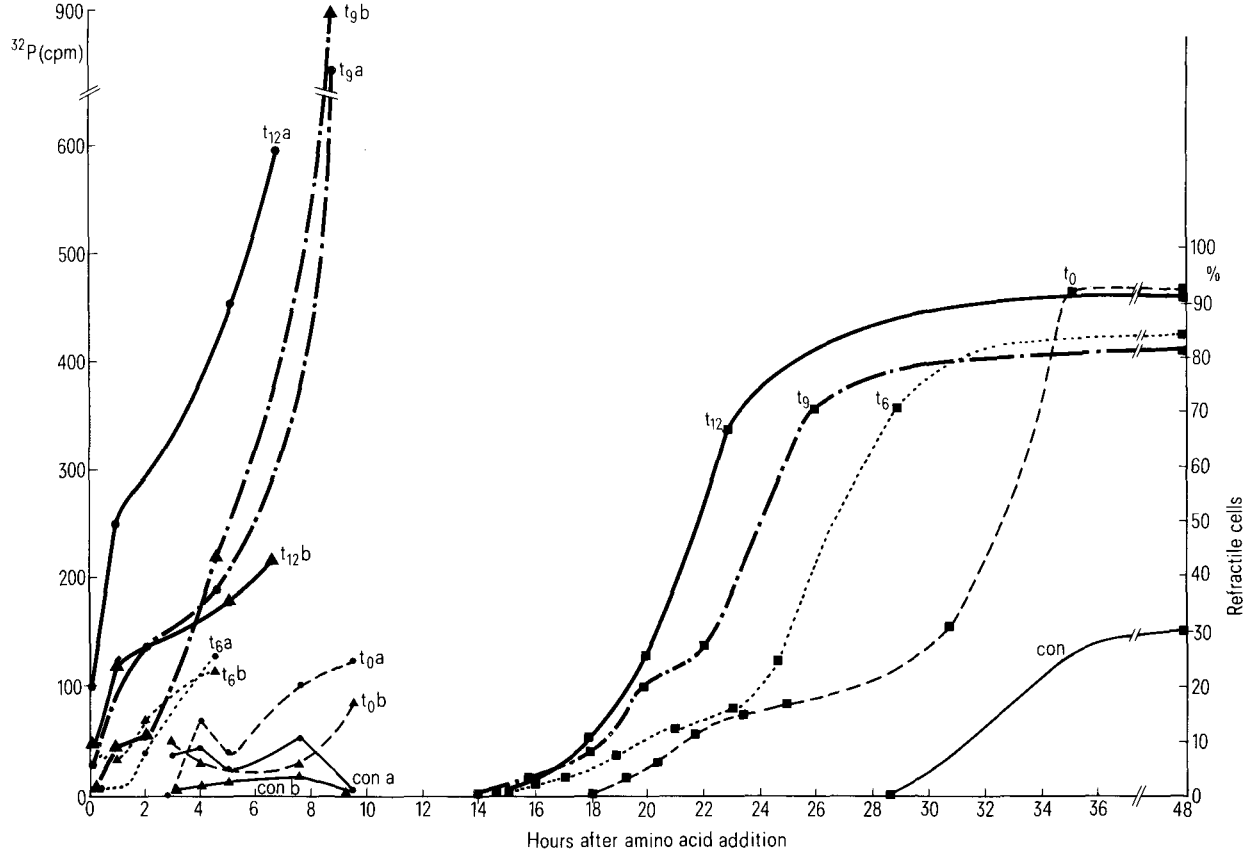
Sporulation profile in SM medium supplemented with amino acids or various nucleotides

Bacterial strain	Experiment	Addition	Per cent sporulation determined at (h after resuspension in SM medium) 0	24	29	48	54	58	72	90	100	120
NS321	I	-	0	9			52					
		Amino acids[a]	0	59			83					
		ppApp[c]	0	38			74					
NS321	II	-	0			18						
		Amino acids[a]	0			51						
		ppApp[d]	0			87						
NS22	III	-	0		16			12			19	
		Amino acids[a]	0		84			52			80	
		ppApp[b]	0		33			38			69	
168wt		-	0		45			88			95	
NS22	IV	-	0									< 1
		Amino acids[a]	0									43
		Adenine[b]	0									< 1
		ATP[b]	0									< 1
NS22	V	-	0							17		
		Amino acids[a]	0							80		
		AMP[b]	0							5		
		pAp[b]	0							5		
		ppGpp[b]	0							< 1		
		pppGpp[b]	0							< 1		
NS22	VI[e]	-	0						10			
		Amino acids[a]	0						90			
		ppApp[b]	0						80			
NS22	VII	-	0									28
		Amino acids[a]	0									76
		ADP + ATP[b]	0									19

[a] Amino acids: arginine, valine, methionine, and isoleucine at a final concentration of 50 µg/ml. [b] All base and nucleotides were at a final concentration of 50 µg/ml. ppApp from ICN Pharmaceuticals was used. [c] 20 µg/ml. [d] Obtained from H.J. Rhaese. [e] Values of duplicate cultures under each condition agreed closely in experiment VI. The variation in the basal levels of sporulation in the mutant strains in the other experiments was probably due to the residual amino acids left over from the pregrowth medium since washing the cells before resuspension did affect sporulation frequencies.

on the pre-equilibrated chromatograms and they were developed in 1.5 M KH_2PO_4 at pH of 3.4. The developed chromatograms were packed with Kodak No-Screen X-ray film for 24–48 h. After developing the film, the radioactive spots on the TLC sheets as indicated on the autoradiograms were cut out and counted in the scintillation counter according to Bray[16]. PEI cellulose sheets were supplied by Brinkmann Instruments, Inc., Westbury, N.Y., USA. ppApp (adenosine 3′,5′-bis-diphosphate), pppApp (adenosine 3′-diphosphate-5′-triphosphate), ppGpp (guanosine 3′,5′-bis-diphosphate) and pppGpp (guanosine 3′-diphosphate-5′-triphosphate) were purchased from ICN Pharmaceuticals, Cleveland, Ohio, USA. Adenine, ATP (adenosine-5′-triphosphate), AMP (adenosine 5′-monophosphate), ADP (adenosine-5′-diphosphate) and pAp (adenosine 3′,5′-diphosphate) were obtained from Sigma Chemicals, St. Louis, Missouri, USA.

Results and discussion. As shown in the table, ppApp restored the sporulation capacity of the mutant strains NS321 and NS22 to an extent comparable to the level restored by amino acid supplement and that of the wild type cells. The results were verified by using 2 strains of *Bacillus subtilis*, NS321 and NS22, both having the same mutation[10] and ppApp derived from 2 sources (ICN Pharmaceuticals, and the laboratory of Dr H.J. Rhaese[11–14]). The variation in the basal levels of sporulation in the mutant in the absence of amino acid supplement was probably due to the residual amino acids left over from the pregrowth medium prior to resuspension in SM medium. In all cases tested, the sporulation frequencies of cultures supplemented with amino acids or ppApp were significantly higher than the unsupplemented cultures. Addition of ppGpp, pppGpp, 2 of the phosphorylated nucleotides involved in the stringent control of RNA synthesis in *B. subtilis* upon amino acid starvation, did not stimulate sporulation in the mutant strain NS22. Addition of adenine and other possible adenine containing precursors of ppApp also did not show enhancement of sporulation frequencies. These results were highly suggestive that the stimulatory effect of sporulation of ppApp is specific. The involvement of ppApp in sporulation is further suggested by the analysis of the appearances of HPN in the cells in relation to the onset of sporulation. The figure shows that there is a good correlation over time between the addition of amino acids, the appearances of the HPN represented by the ^{32}P incorporation into formic acid extract resolved by PEI TLC, and the appearance of the refractile spores in the SM medium. Since spot a comigrated with ppApp, it is possible that intracellular ppApp rises to initiate the onset of sporulation, as implied by the somewhat similar kinetics of appearance of HPN and the sporulation profile. Spot b migrated in phosphate buffer at a R_f value (0.39) smaller than that



Increase of HPN synthesis and sporulation frequencies in strain NS321 on relative time scale. An exponential culture of strain NS321 was washed and resuspended into SM medium to which amino acids were added at zero time (t_0), 6 h (t_6), 9 h (t_9), and 12 h (t_{12}) after resuspension. A culture with no amino acid supplement was also included as a control (con). HPN was extracted and quantitated as described by Rhaese et al.[11,14]. The ^{32}P activities in t_0a, t_6a, t_9a, $t_{12}a$, and con a comigrated with ppApp (adenosine-3′,5′-bis-diphosphate). The ^{32}P cpm was corrected for the background radioactivity of the thin layer chromatogram as well as for the volume of the cell extracts. Since spores did not appear in the t_0 culture until 18 h after the addition of amino acids which was 3 h later than the t_6, t_9, and t_{12} cultures, samples from the t_0 and con cultures were withdrawn starting at 3 h after amino acid addition so as to synchronize with t_6, t_9, and t_{12} cultures. Samples collected at 6.5 h after amino acid addition in the t_6 culture were inadvertantly lost. Note the correlation between the increases of HPN synthesis and the percentages of refractile bodies. Similar results were also obtained with strain NS22. Symbols: ■, % Refractile cells; ●, cpm of ^{32}P obtained in spot a; ▲, cpm of ^{32}P obtained in spot b.

of ppApp (R_f=0.51) but larger than that of pppApp (R_f=0.34) and its identity was unknown. Works are carried out at present to identify spots a and b. Preliminary data also suggested that there is a correlation between the rate of instantaneous RNA synthesis in the mutant as well as the wild type cultures with the appearance of HPN and their sporulation frequencies. Experiments to refine these date are in progress.

In conclusion, we have presented evidence in support of the involvement of ppApp in the initiation of sporulation in *Bacillus subtilis*. Since ppApp is known to alter transcriptional selectivity of *Escherichia coli* RNA polymerase[17] and it restores the sporulation capacity of the conditional asporogenous rifampin-resistant mutant of *B. subtilis* to the same extent as 4 amino acids, this system is useful in the possible illumination of the involvement of amino acids in the transcriptional control of sporulation[18].

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Biosynthesis of roquefortine and 3,12-dihydroroquefortine by the culture *Penicillium farinosum*

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Summary. A new culture *Penicillium farinosum* synthesizing roquefortine and 3,12-dihydroroquefortine was found. Unlike *P. roqueforti*, a known producer of these compounds, the culture under study does not synthesize clavine alkaloids. The maximal roquefortine content was observed in the late logarithmic and early stationary growth phases while the maximal 3,12-dihydroroquefortine content was obtained in the beginning of the stationary phase.

Roquefortine and 3,12-dihydroroquefortine are basic components of alkaloid fractions of several strains of the fungus *Penicillium roqueforti* used in the production of many varieties of blue cheese[2-5]. Besides the compounds mentioned, this culture synthesizes an appreciable amount of clavine alkaloids such as isofumigaclavine A and isofumigaclavine B, found earlier in *Claviceps*[6] and *Aspergillus fumigatus*[7].

We found that the culture of *Penicillium farinosum* also performs the biosynthesis of roquefortine and 3,12-dihydroroquefortine. This culture was obtained from the All-Union Collection of Microorganisms.

Methods. The cultures were grown in shaking flasks in medium containing mannitol and succinic acid[2]. Extraction of alkaloids from the mycelium and filtrate of the culture liquid, chromatographic separation and analysis were performed as described earlier[5]. With the aim of determining the quantity of roquefortine and 3,12-dihydroroquefortine, a sample, dissolved in chloroform, was banded on a Silufol plate (UF-254, ČSSR) washed beforehand with methanol. After development, the UV-absorbing regions, corresponding to roquefortine and 3,12-dihydroroquefortine, were scraped off and eluted with ethanol. Concentrations of substances were determined spectrophotometrically at the wavelength of 328 and 301 nm for roquefortine and 3,12-dihydroroquefortine, respectively, using calibrating straight lines constructed with the aid of individual compounds isolated earlier from *P. roqueforti*. Identification of substances was performed by the TLC method[5] with the help of UV-, IR-spectroscopy and mass-spectrometry.

Results and discussion. Dynamics of changes in the contents of roquefortine and 3,12-dihydroroquefortine in the culture under study are given in figures 1 and 2. The maximal intracellular concentration of these compounds was observed on the 4th-5th day of the growth. During the stationary growth phase, their contents diminish and are minimal by the 12th day. The maximal contents of roquefortine and 3,12-dihydroroquefortine in the culture liquid are 24 mg/l, and 2.0 mg/l, respectively. The maximal

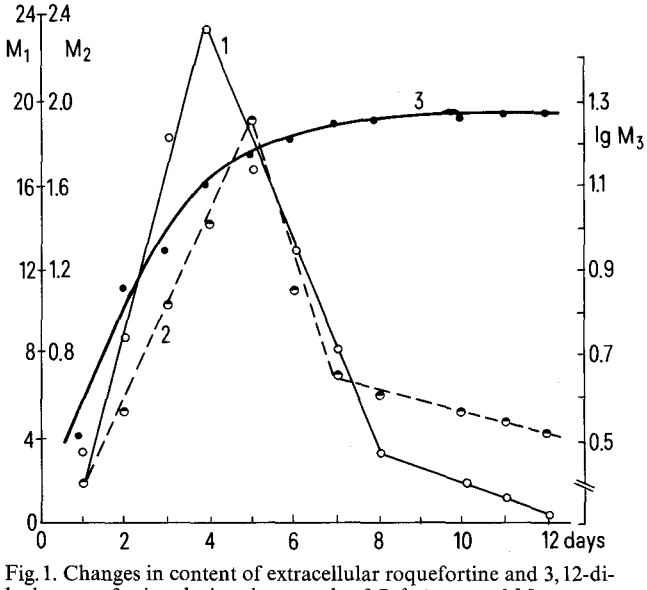

Fig. 1. Changes in content of extracellular roquefortine and 3,12-dihydroroquefortine during the growth of *P. farinosum*. *1* M_1, roquefortine (mg/l); *2* M_2, 3,12-dihydroroquefortine (mg/l); *3* M_3, biomass (g dry mycelium/l).