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Effect of a New Pyrimidine Analog on *Bacillus subtilis* Growth

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2-Amino-5-ethoxycarbonylpyrimidine-4(3H)-one, a pyrimidine analog, inhibited growth of *Bacillus subtilis*. Data were obtained which suggested that the analog interfered with the methylation process. A mutant resistant to the inhibitor was isolated, and the mutation was mapped.

Amino acid and nitrogen base analogs and mutants resistant to these compounds have been a useful tool in the understanding of metabolic pathways and their regulation (14). Many purine and pyrimidine analogs are known to be inhibitors of nucleotide and nucleic acid biosyntheses as well as possible antagonists of nucleotide coenzymes (3, 8). Moreover, nucleoside analogs interfere with differentiation in animal and plant systems (6, 12).

We have studied the biological activity of six pyrimidine analogs, synthesized by condensation of diethyl-ethoxymethylenemalonate and substituted guanidines (2). The chemical structures of the six analogs are shown in Fig. 1.

The effect of the six new pyrimidine analogs on the growth of cells of *Bacillus subtilis* SB19 (13), *Escherichia coli* CGSC 532, and *Candida utilis* CBS621 was tested on minimal medium (5) supplied with 1 g of asparagine per liter.

Only one of the six analogs, 2-amino-5-ethoxycarbonylpyrimidine-4(3H)-one (Fig. 1a), herein referred to as ECP, inhibited the growth of *B. subtilis* at a concentration of 200 μg/ml. The inhibition resulted in a very reduced growth in liquid culture and in failure to form colonies on solid medium. None of the six analogs showed an inhibitory effect on *E. coli* and on *C. utilis* at a concentration of 500 μg/ml.

To elucidate the mechanism of action of ECP, experiments of incorporation of [^{3}H]thymidine, [^{14}C]tyrosine, and [^{3}H]uracil into macromolecules of growing cells of *B. subtilis* were carried out as described (4). Data (not shown) indicated that ECP (500 μg/ml) had no inhibitory effect on nucleic acid and protein biosyntheses.

Then we assayed, on solid minimal medium, the ability of all natural amino acids and nucleic acid bases to remove the growth inhibition of *B. subtilis* by ECP. We found that only methionine was able to remove the inhibition; the inhibitory effect of 450 μg of ECP per ml was completely removed by 15 μg of methionine per ml.

Other compounds involved in methionine metabolism or in the methylation process were also tested. They were *S*-adenosylmethionine, methionine sulfoxide, *N*-5-methyl-tetrahydrofolate, and homocysteine. The first three compounds, at a concentration of 15, 30, and 100 μg/ml, respectively, were able to remove the growth inhibition by ECP (at 400 μg/ml). These data suggest that the analog could be involved in some step of the methylation process.

The investigation was then extended to the isolation and characterization of mutants resistant to ECP. Spontaneous resistant mutants of *B. subtilis* SB19 were selected on minimal medium plates containing 300 μg of ECP per ml. One of these mutants, designated FB75 *ecp-1*, was resistant up to a concentration of 800 μg of ECP per ml (the maximum of solubility of this compound in the medium used).

To determine whether the resistance was connected to overproduction of methionine, cross-feeding experiments were carried out between the resistant strain FB75 and a methionine auxotrophic strain (BD70 *metC trpC*, obtained from J. Lepesant), using a procedure previously described (11). Cross-feeding was not observed, suggesting that the resistance to ECP of strain FB75 was not due to overproduction of methionine.

Moreover, the content of methionine in the amino acid pool and in the proteins of cells grown in the presence or absence of ECP was examined both for parental strain SB19 and resistant mutant FB75. Cells were disrupted by sonication, and the lysate was precipitated with 10% trichloroacetic acid. After centrifugation, the acid-soluble pool of amino acids was determined by analysis on an Optica Aminolizer (Optica Milan, Italy), operating with a single-column system. The pellet was also analyzed after hydrolysis in 6 N HCl at 110°C for 24 h. No difference was found in all samples analyzed for methionine content. Furthermore, the mutant

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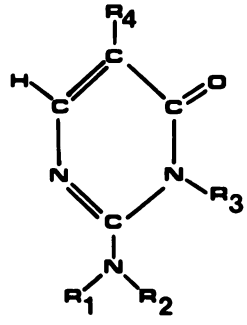

a : $R_1, R_2, R_3 = H$ $R_4 = CO_2Et$

b : $R_1 = H$ $R_2, R_3 = Me$ $R_4 = CO_2Et$

c : $R_1, R_2 = Me$ $R_3 = H$ $R_4 = CO_2Et$

d : $R_1, R_3 = H$ $R_2 = Me$ $R_4 = CO_2Et$

e : $R_1, R_2, R_3 = H$ $R_4 = CO_2H$

f : $R_1, R_2 = Me$ $R_3 = H$ $R_4 = CO_2H$

FIG. 1. *Chemical structure of six analogs tested.*

FB75 did not show any cross-resistance to ethionine and methionine sulfone, two methionine analogs.

The *ecp-1* marker of resistant mutant FB75 was mapped. Preliminary mapping by PBS1 phage transduction, carried out as described (9) using FB75 as the donor and SB202 *aroB2 hisB2 tyrA1 trpC2* as the recipient strain, showed that the *ecp-1* mutation was cotransferred with the *trpC2* marker. To better localize the *ecp-1* marker, three-point transformation crosses, using as recipient strain FB84 *aroB2 hisB2* (derived from SB202 [13]), were performed as described (9).

Data reported in Table 1 and Fig. 2 show that *ecp-1* is cotransferred with *aroB* and *hisB* markers.

TABLE 1. *Mapping of ECP resistance by transformation*[a]

Selected marker	Recombinant classes	No. of colonies scored
Aro^+	$Aro^+ His^+ Ecp^r$	27
	$Aro^+ His^+ Ecp^s$	37
	$Aro^+ His^- Ecp^r$	19
	$Aro^+ His^- Ecp^s$	17
His^+	$His^+ Aro^+ Ecp^r$	21
	$His^+ Aro^+ Ecp^s$	54
	$His^+ Aro^- Ecp^r$	1
	$His^+ Aro^- Ecp^s$	39

[a] Donor strain, FB75 *ecp-1*; recipient strain, FB84 *aroB2 hisB2*. Implied order, *ecp-1-aroB2-hisB2*.

During mapping experiments, when the recipient strain was PB202 and selection was for His^+ or Aro^+, we never found recombinant colonies with a Trp^- Ecp^r phenotype. This observation prompted us to test the effect of the presence of tryptophan in the medium on the expression of resistance to ECP. As expected, the addition of tryptophan to the medium (25 μg/ml) (Table 2), considerably reduced the resistance of strain FB75 to ECP; in fact, cells of this strain, when streaked on plates containing ECP plus tryptophan, formed very small colonies only after 2 days of incubation. The effect was not found when indole, anthranilate, tyrosine, or phenylalanine was added to the growth medium. (Presumably indole is not effective because it is not converted to tryptophan in a sufficient amount.)

By assuming that enzymes of aromatic acid biosynthesis could be involved in the mechanism of resistance to ECP, we also tested the effect of trytophan on strain FB81 *ecp-1 ftr-21*, resistant to ECP and to fluorotryptophan and derepressed for tryptophan synthetase. In *B. subtilis*, *ftr* mutations are known which cause derepression of tryptophan biosynthetic enzymes (1), and, likely, they also concern the methyltryptophan resistance gene (*mtr*) described by Hoch (10). Strain FB81 *ecp-1 ftr-21* was obtained by trans-

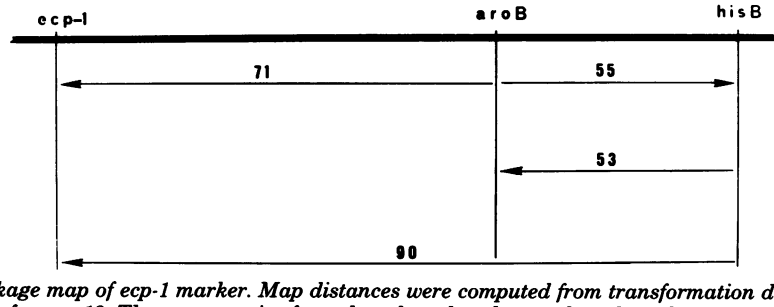

FIG. 2. *Linkage map of ecp-1 marker. Map distances were computed from transformation data of Table 1 according to reference 13. The arrows point from the selected to the unselected marker.*

TABLE 2. *Effect of medium composition on the expression of resistance to ECP in B. subtilis*

Strain	Resistance[a] in minimal medium plus[b]:							
	No addition	ECP	ECP + Trp	ECP + Trp + Met	ECP + Trp + PABA	ECP + Trp + folate	ECP + Trp + FH_2	ECP + FH_2
SB19 (wild type)	+	−	−	+	−	−	−	−
FB75 *ecp-1*	+	+	±	+	±	±	+	+
FB81 *ecp-1 ftr-21*	+	+	+	+	+	+	+	+

[a] −, No growth; ±, very reduced growth; +, growth.
[b] PABA, *p*-Aminobenzoic acid; FH_2, dihydrofolic acid.

ferring, by transformation, the marker *ecp-1* from FB75 to strain FB21 resistant to fluorotryptophan (1). Cells from FB81, seeded on plates containing ECP or ECP plus tryptophan (25 μg/ml), showed the same level of resistance to ECP in the absence or presence of tryptophan; the addition of methionine to medium containing ECP plus tryptophan allowed complete growth of strains FB75 and FB81 (Table 2). The lack of an inhibitory effect of tryptophan on strain FB81 supports the hypothesis that some enzyme of aromatic acid synthesis could be responsible for the resistance to ECP, in particular enzymes of the biosynthetic pathways of folic acid, important for the methylation process. Therefore, assuming that tryptophan could act by reducing the synthesis of tetrahydrofolate precursors, we checked the effect of tryptophan (25 μg/ml) on the growth of FB75 in a medium supplemented with ECP (400 μg/ml) and with one of the following precursors: *p*-aminobenzoic acid, folic acid, or dihydrofolic acid (each 5 μg/ml). Results (Table 2) indicated that only the addition of dihydrofolic acid removed the inhibitory effect of tryptophan on the growth of FB75 in the presence of ECP. These precursors did not abolish the inhibition by ECP of the parental strain SB19. Data obtained so far indicate that ECP interferes with the methylation process, but they are not sufficient to completely explain its mechanism of action. Experiments to test the hypothesis that ECP could be a methyl group acceptor are in progress. Data on the resistance to ECP seem to indicate that the *ecp-1* mutation concerns a gene whose activity is involved in the folic acid pathway. The interference of tryptophan with the expression of resistance could be explained assuming that it acts by reducing, as a repressor, the synthesis of dihydrofolic acid, owing to the presence of a precursor, chorismic acid, common to the tryptophan and folic acid pathway (7).

More investigation is needed in order to better define the mechanism of action and resistance to ECP.

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