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Isolation and characterization of thymidylate synthetase mutants of *Xanthomonas maltophilia*

Thomas P. West*

Department of Biological Sciences, Institute of Genetics, University of Southern Mississippi, Hattiesburg, MS 39406, USA

Abstract. Thymidylate synthetase mutants of *Xanthomonas maltophilia* ATCC 13270 were isolated on a solid minimal medium containing 50 mg/l thymidine and a high concentration of trimethoprim (500 mg/l). It was found that a high concentration of trimethoprim was required to prevent background growth of the wild-type strain. The isolated mutants could grow on thymidine or dTMP at a concentration of 50 mg/l while they were unable to grow on 1000 mg/l thymine or 50 mg/l deoxyuridine. Thymidylate synthetase activity was assayed in the wild-type cells and in the mutant cells but only the wild-type cells contained measurable enzyme activity.

Key words: Thymidylate synthetase – Trimethoprim – Thymidine auxotrophy – *Xanthomonas maltophilia* – Thymidine phosphorylase

Thymidylate synthetase (EC 2.1.1.45) is known to catalyze the conversion of dUMP to dTMP in the presence of tetrahydrofolate and magnesium ions (Wahba and Friedkin 1962). The loss of this enzyme due to mutation is known to result in the inability of a bacterial cell to synthesize dTTP and ultimately, DNA (O'Donovan 1978). Bacterial thymidylate synthetase mutants have been shown to exhibit a thymine or thymidine auxotrophy (Kelln and Warren 1973; O'Donovan 1978).

With respect to the *Pseudomonadaceae*, few obligate thymidine auxotrophs have been previously reported. A marine *Pseudomonas* species was utilized to isolate the first thymidine auxotrophic strain. This strain required a low concentration of thymidine for growth but it was not assayed for its thymidylate synthetase activity (Espejo et al. 1971). In contrast, thymidine-requiring mutants of *Pseudomonas acidovorans* were discovered to grow only if high concentrations of thymidine were present. It was confirmed that these mutants did not contain thymidylate synthetase activity (Kelln and Warren 1973). Such mutants have not been isolated in any species of *Xanthomonas*. In this study, a procedure for the isolation of thymidine-requiring mutants in the clinically significant microorganism *Xanthomonas maltophilia* is presented. These mutants were subsequently found to be deficient for thymidylate synthetase activity.

* *Present address and address for offprint requests:* Station Biochemistry, South Dakota State University, Box 2170, Brookings, SD 57007, USA

Materials and methods

Xanthomonas maltophilia ATCC 13270 was the strain used in this study (Levenberg and Hayaishi 1959). A minimal medium of Stanier (1947) was modified to contain 7.35 mM KH_2PO_4, 5.74 mM K_2HPO_4, 17.12 mM NaCl, 1.7 mM sodium citrate, 28.4 mM $MgSO_4$ and 30.27 mM $(NH_4)_2SO_4$. The pH of the medium was adjusted to 7.0–7.2 prior to sterilization. For solid medium, 2% agar was included. After autoclaving, glucose (22.2 mM) was added to the medium as a carbon source. All minimal medium was supplemented with 0.34 mM methionine since methionine is required for *X. maltophilia* growth (Stanier et al. 1966).

To increase the frequency of thymidine auxotrophs, bacterial cells were subjected to ethylmethane sulfonate mutagenesis at 30°C (Watson and Holloway 1976). The mutagenized cells were grown in nutrient broth overnight at 30°C. After washing the cells with 14.55 mM NaCl, approximately 10^8 cells were spread onto minimal medium agar plates containing 500 mg/l trimethoprim and 50 mg/l thymidine. The agar plates were incubated at 30°C for a minimum of 72 h. The resultant colonies were screened for thymidine auxotrophy by picking colonies onto minimal medium agar plates and onto minimal medium agar plates containing 50 mg/l thymidine. Only those colonies which exhibited a growth requirement for thymidine were further investigated.

Studies examining growth of the wild-type strain or the mutant strains were performed using liquid medium cultures. All cultures (5 ml) were aerated at 30°C on a rotary shaker (200 rpm) and their resultant turbidity was measured at 600 nm. To examine trimethoprim inhibition of wild-type strain growth, minimal medium cultures containing 20, 40, 60, 80, 200 or 500 mg/l trimethoprim as well as unsupplemented minimal medium cultures, serving as the control, were inoculated with approximately 10^8 cells and shaken for 72 h. These experiments were performed in triplicate. To investigate the growth of each mutant strain in minimal medium containing a pyrimidine supplement, each culture was inoculated with approximately 10^8 cells and then was shaken for 48 h. Dry weight of mutant cells (g/l) was

estimated from previously determined A_{600} versus dry weight calibration curves.

For enzyme assays, bacterial cultures (60 ml) were grown on a rotary shaker (200 rpm) at 30° C to approximately 10^9 cells/ml and were harvested. After washing the cells with 14.55 mM NaCl, the pellet was resuspended in 2.5 ml of 50 mM KH_2PO_4 buffer (pH 7.4) containing 0.1 M 2-mercaptoethanol. The cell suspension was sonically disrupted at 0° C for a total of 5 min. The cell extract was centrifuged for 20 min at 12,100 × *g* at 4° C. The cell debris was discarded and the supernatant was assayed for enzyme activity.

Thymidylate synthetase activity was assayed at 25° C by modifying prior protocols (Wahba and Friedkin 1962; West et al. 1983). The assay mix contained in 1 ml: 50 mM KH_2PO_4 buffer (pH 7.4), 20 mM $MgCl_2$, 15 mM HCHO, 2 mM EDTA, 2 mM 2-mercaptoethanol, 0.33 mM tetrahydrofolic acid, 0.4 mM dUMP and cell-free extract. The reaction was followed spectrophotometrically by observing the change at 340 nm where an increase in absorbance of 6.4 is equivalent to the formation of 1 µmol dTMP from dUMP (Wahba and Friedkin 1962). A unit of thymidylate synthetase activity was defined as 1 nmol dTMP formed per min under the assay conditions used. Thymidine phosphorylase activity was measured at 30° C, for 40 min, using 0.24 mg protein (Munch-Petersen 1968). Protein was measured according to the method of Bradford (1976) with lysozyme as the protein standard.

Results and discussion

The folate analogue trimethoprim was used in this study as a selective agent to isolate thymidylate synthetase mutants of *Xanthomonas maltophilia*. During the course of the thymidylate synthetase reaction, the oxidation of tetrahydrofolate has been observed (Wahba and Friedkin 1962). Tetrahydrofolate is required for a number of metabolic processes including purine biosynthesis, amino acid metabolism and protein synthesis initiation. When trimethoprim is present, tetrahydrofolate synthesis by the enzyme dihydrofolate reductase is serverely diminished (O'Donovan 1978). In a wild-type bacterial cell, a functioning thymidylate synthetase will deplete the level of available tetrahydrofolate and this will cause a cessation of its growth due to the blockage of cellular RNA, DNA and protein syntheses. In contrast, cells containing depressed thymidylate synthetase activity will continue to grow in the presence of trimethoprim and thymidine since a low level of tetrahydrofolate will remain.

To isolate thymidylate synthetase mutants of *X. maltophilia*, it was first necessary to investigate trimethoprim inhibition of wild-type strain growth. Increasing concentrations of trimethoprim (20 – 500 mg/l) were tested for their effect upon *X. maltophilia* growth after 72 h at 30° C. The minimal inhibitory concentration of trimethoprim that prevents bacterial growth was determined to be 60 mg/l. During mutant isolation, a high concentration of trimethoprim (500 mg/l) was included in the solid medium, in addition to thymidine, to ensure minimal background growth on the agar plates. Using this medium, all colonies screened proved to be trimethoprim resistant. Two thymidine-requiring mutants were isolated at a frequency of 0.14% (1432 colonies screened).

The thymidine-requiring mutants, strains PT101 and PT102, were characterized for their ability to grow on thymidine, dTMP, thymine and deoxyuridine after 48 h at 30° C (Table 1). Thymidine supported the growth of these strains but the amount of growth was dependent upon its concentration. As little as 1 mg/l thymidine in the medium supported growth of the mutants but their level of growth continued to elevate in the presence of increasing thymidine concentrations (Table 1). This contrasts to the 1000 mg/l thymidine requirement of the *Pseudomonas acidovorans* auxotrophs or the 1 mg/l thymidine requirement of the marine pseudomonad auxotroph (Espejo et al. 1971; Kelln and Warren 1973). Both xanthomonad mutants also grew in the presence of dTMP (Table 1). Unfortunately, the ability of the pseudomonad thymidine auxotrophs to grow on dTMP was never determined (Espejo et al. 1971; Kelln and Warren 1973). Such utilization of exogenous dTMP has been observed in the enterobacteria, though, which readily incorporate it into the bacterial DNA (Schwan and Holldorf 1975). Neither mutant strain studied here could grow on thymine or deoxyuridine (Table 1). Similarly, high concentrations of thymine did not permit growth of the thymidine-dependent *P. acidovorans* strains (Kelln and Warren 1973). This can be attributed to the absence of the enzyme thymidine phosphorylase (which can catalyze the conversion of thymine and deoxyribose-1-phosphate to thymidine and P_i) in some species of *Pseudomonas*, including *P. acidovorans* and *Pseudomonas aeruginosa* (Kelln and Warren 1974; O'Donovan 1978; Potter et al. 1982). In the present study this enzyme was not detectable in extracts of wild-type *X. maltophilia* grown in minimal medium containing 50 mg/l

Table 1. Ability of thymidine and related pyrimidines to support mutant strain growth in minimal medium after 48 h at 30° C. Mean dry weight of each mutant strain grown in unsupplemented minimal medium was 0.01 g/l. Each dry weight value represents the mean of 3 separate determinations

Pyrimidine addition	Concentration (mg/l)	Strain dry wt. (g/l)	
		PT101	PT102
Thymidine	1	0.20	0.20
	5	0.26	0.31
	10	0.35	0.38
	25	0.44	0.50
	50	0.59	0.62
dTMP	50	0.42	0.53
Thymine	1000	0.01	0.01
Deoxyuridine	50	0.01	0.01

Table 2. Thymidylate synthetase activity in *Xanthomonas maltophilia* strains. Strains were grown in minimal medium at 30° C and when added, thymidine was present at a concentration of 50 mg/l. Cells were harvested, sonically disrupted, centrifuged and the extract assayed for enzyme activity. The results are the average of 2 separate determinations within 10% error

Strain	Medium	Specific activity (U/mg protein)
Wild-type	None	0.30
Wild-type	Thymidine	0.14
PT101	Thymidine	< 0.01
PT102	Thymidine	< 0.01

thymidine. The inability of strains PT101 and PT102 to utilize 1000 mg/l thymine for growth would seem to be explained by this lack of phosphorylase activity. The most probable route of dTTP synthesis in this microorganism would seem to involve three enzymatic steps (O'Donovan 1978). Initially, thymidine is phosphorylated by thymidine kinase to dTMP. Next, dTMP is converted to dTDP in a reaction catalyzed by thymidylate kinase. Finally, dTTP is produced from dTDP by the action of nucleoside diphosphate kinase.

Since thymidine-requiring bacterial mutants have been found to lack thymidylate synthetase activity (O'Donovan 1978), this enzyme was assayed in the wild-type strain and in both mutant strains (Table 2). In wild-type *X. maltophila*, thymidylate synthetase activity was detectable in cells grown in minimal medium. When thymidine was added to the minimal medium, thymidylate synthetase activity decreased by more than 50%. In strains PT101 and PT102 grown in thymidine-containing minimal medium, thymidylate synthetase specific activity was very low. Therefore, both *X. maltophilia* auxotrophic strains can be characterized as thymidylate synthetase mutants.

These mutants should facilitate a detailed investigation of DNA synthesis in *X. maltophilia*. Such an analysis could be important, since this microorganism is known to be involved in a variety of opportunistic infections in humans, from which it is being isolated at increasing frequency.

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