AMINO ACID PRECURSORS OF *MYXOCOCCUS XANTHUS* ANTIBIOTIC TA

S. FYTLOVITCH, P. D. NATHAN, D. ZAFRIRI and E. ROSENBERG

Department of Microbiology, George S. Wise Faculty of Life Sciences, Tel Aviv University, Ramat Aviv, Israel

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The production of *Myxococcus xanthus* antibiotic TA was stimulated by addition of alanine, serine and glycine to Casitone medium. These three amino acids served as the major biosynthetic precursors of the antibiotic. Alanine and serine were incorporated *via* acetate. In Casitone medium supplemented with alanine and serine, 29 to 30 of the 34 carbon atoms of antibiotic TA were derived from these two amino acids. Both carbon atoms of glycine were incorporated into antibiotic TA by a mechanism not involving acetate as an intermediate. Antibiotic TA was split into two fragments by alkaline hydrolysis followed by periodate oxidation. Radioactive alanine was incorporated into both fragments, whereas glycine was incorporated only into the smaller, polar fragment.

Myxococcus xanthus TA produces a broad spectrum antibiotic when grown under nutrionally limiting conditions.[1,2] This antibiotic, referred to as TA, causes lysis of growing bacteria by blocking cell wall synthesis at the stage of polymerization of the lipid-disaccharide-pentapeptide.[3] Recently, the chemical properties of antibiotic TA ($C_{34}H_{57}O_9N$) were reported.[4]

Since concentrations of peptone greater than 1% inhibited antibiotic TA production in *M. xanthus*,[1] a study was made of the effect of individual amino acids on TA production. Alanine, serine and glycine were precursors of the antibiotic and stimulated its production.

Materials and Methods

Radioactive Isotopes

L-[U-^{14}C]Alanine (165 Ci/mol), L-[1-^{14}C]alanine (52 Ci/mol), L-[U-^{14}C]serine (170 Ci/mol), [U-^{14}C]-glycine (114 Ci/mol), [1-^{14}C]glycine (58 Ci/mol) and [2-^{14}C]glycine (54 Ci/mol) were products of Amersham Nuclear Corp. Sodium [1-^{14}C]acetate (59 Ci/mol) was purchased from Nuclear Research Center, Negev, Israel.

Bacteria and Growth Conditions

Myxococcus xanthus TA (ATCC 31046) is an antibiotic producing strain which grows in dispersed state in CT medium: 0.5% Casitone (Difco) and 0.2% $MgSO_4 \cdot 7H_2O$. The strain was maintained by transferring fresh colonies from CT agar to CT liquid medium, incubating for 2 days at 30°C with aeration and then streaking on CT agar. Colonies appeared after 5~7 days. *Escherichia coli* ESS cap^R is a chloramphenicol resistant strain of *E. coli* ESS[5] which was derived by transducing *E. coli* ESS with P1 cap^R (Y. AHARONOVITCH, unpublished). *E. coli* ESS cap^R was maintained on nutrient agar (Difco).

Production and Assay of Antibiotic TA

All antibiotic production experiments were performed in test tubes (inside diameter 12 mm). Starter cultures were obtained by inoculating 2 ml of CT medium with a fresh colony of *M. xanthus* TA and incubating with shaking for 2 days at 30°C. The starter culture (0.1 ml) was then inoculated into 4.4 ml of CT medium (with or without supplements) and incubated for 48 hours at 30°C with gyratory shaking. Culture turbidity was measured at 560 nm using a Gilford Model 240 Spectrophotometer. Antibiotic was extracted with an equal volume of $CHCl_3$ from the cell-free supernatant fluid following

centrifugation at 10,000 × g for 10 minutes at 4°C. Bound antibiotic was extracted directly from the cell pellet fraction with 2 ml absolute ethanol. Antibiotic TA activity was determined by the paper disc assay method using *E. coli* ESS cap^R as the test organism, as previously described.[4] The results reported here are the total antibiotic TA which is the sum of the cellular and extracellular activities.

Incorporation of Radioactive Precursors into Antibiotic TA

M. xanthus was grown in media containing ^{14}C-labelled precursors (1 μCi/ml). When the cells reached stationary phase, the cultures were harvested by centrifugation at 10,000 × g for 10 minutes at 4°C. Antibiotic TA was extracted from the cell-free fluid with an equal volume of $CHCl_3$. The chloroform layer was concentrated in a stream of nitrogen and purified by thin-layer chromatography (TLC) on 0.25 mm thick (10 × 20 cm) silica gel, F-254 type 60 (Merck), using acetone - benzene (1: 1) for development. Antibiotic TA spots were located under the short wave ultraviolet lamp, scratched from the plate and then eluted off the silicic acid with 2 ml ethanol. Samples of the solution were used for determination of radioactivity and antibiotic activity. Samples (0.2 ml) were applied to strips (1 × 2 cm) of Whatman 3 MM filters, dried and put into scintillation vials containing 2 ml of a toluene based scintillation mixture. Radioactivity was determined using a Packard Tri-Carb liquid scintillation spectrometer.

Periodate Oxidation of Radioactive Antibiotic TA

Radioactive antibiotic TA, labelled with either [^{14}C]alanine or [^{14}C]glycine, was purified by 2-dimensional TLC. About 10,000 cpm of each of the radioactive TA samples were hydrolyzed in 1 ml of 0.01 M KOH in 50% ethanol at 30°C for 24 hours. The ethanol was removed by evaporation and the remaining aqueous phase was acidified to pH 1 and mixed vigorously with 1 ml H_2O and 2 ml $CHCl_3$. The chloroform layer, containing all of the radioactivity, was concentrated *in vacuo* and applied to a preparative TLC plate that was developed with acetone - benzene (1: 1). The purified alkaline hydrolyzed products from the glycine-labelled TA (5,880 cpm) and the alanine-labelled TA (8,080 cpm) were then oxidized in 5 mM $NaIO_4$ in 0.2 M acetate buffer in 50% ethanol, pH 4.5, at room temperature for 1.5 hours. After the ethanol was removed by evaporation, the remaining water was extracted with an equal volume of $CHCl_3$ and the two layers were separated and examined for radioactivity.

Results

Amino Acid Precursors of Antibiotic TA

The usual growth media for *M. xanthus* (peptone digests) contain a mixture of amino acids and small peptides. To examine which amino acids served as precursors to antibiotic TA, 1 μCi [U-^{14}C] of each of the naturally occurring amino acids was added to *M. xanthus* growing in CT medium. At the completion of growth, the antibiotic was isolated and its radioactivity determined. Three amino acids (alanine, serine and glycine) were particularly effective in labelling antibiotic TA and were chosen for further investigation.

Table 1 shows the incorporation of radioactive alanine and serine into antibiotic TA. To control the specific radioactivity of the precursor more precisely, unlabelled alanine and/or serine were added to the medium. In CT plus alanine medium, about 7 mol of alanine were incorporated per mol TA. This corresponds to almost 21 of the 34 carbon atoms of the antibiotic. In CT plus serine medium, 3.7 mol of serine were incorporated per mol TA, corresponding to 11.1 carbon atoms per TA molecule. In CT medium plus alanine and serine, antibiotic TA received 14.1 and 15.6 carbon atoms from alanine and serine, respectively. Thus, in this latter medium, 29 to 30 of the 34 carbon atoms of antibiotic TA came exclusively from alanine and serine. The fact that unlabelled serine decreased the incorporation of alanine and unlabelled alanine decreased the incorporation of serine into the antibiotic, indicates that these two amino acids enter the antibiotic after being converted into a common intermediate.

Table 1. Incorporation of alanine and serine into antibiotic TA.*

Precursor	Addition to CT medium	Specific activity (cpm/nmol)		Incorporation of precursor	
		Precursor	Antibiotic TA	(mol/mol TA)	(C-atom/TA molecule)
[^{14}C]Alanine	Ala	181	1,250	6.9	20.7
[^{14}C]Alanine	Ala+Ser	176	819	4.7	14.1
[^{14}C]Serine	Ser	156	573	3.7	11.1
[^{14}C]Serine	Ser+Ala	160	833	5.2	15.6

* Incorporation of L-[*U*-^{14}C]alanine or L-[*U*-^{14}C]serine (2.0×10^6 cpm/ml) into antibiotic TA was performed during growth of *M. xanthus* on CT medium supplemented with 0.89 mg/ml of unlabelled alanine and/or serine, as described in Materials and Methods. Antibiotic TA was extracted from the cell-free culture fluid with $CHCl_3$ and purified on TLC plates.
Radioactivity and antibiotic activity were determined on the purified TA.

Table 2. Decarboxylation of alanine during incorporation into antibiotic TA.*

Precursor	Specific activity (cpm/nmol)		Incorporation of alanine	
	Precursor	Antibiotic TA	(mol/mol TA)	(C-atom/TA molecule)
[*U*-^{14}C]Alanine	144	1,000	6.94	20.9
[1-^{14}C]Alanine	272	75	0.3	0.3

* The experiment was performed as described in Table 2, using either L-[*U*-^{14}C]alanine (1.63×10^6 cpm/ml) or L-[1-^{14}C]alanine (3.1×10^6 cpm/ml). In both cases the CT medium was supplemented with 0.89 mg unlabelled alanine per ml.

Table 3. Role of acetate in biosynthesis of antibiotic TA.*

Precursor	Addition to CT medium	Specific activity (cpm/nmol)		Incorporation of precursor	
		Precursor	Antibiotic TA	(mol/mol TA)	(C-atom/TA molecule)
[*U*-^{14}C]Alanine	Ala+Acetate	187	416	2.22	6.7
[*U*-^{14}C]Serine	Ser+Acetate	128	212	1.66	5.0
[1-^{14}C]Acetate	Acetate	93	1,060	11.4	11.4

* The experiment was performed as described in Table 1, using [*U*-^{14}C]alanine (2.1×10^6 cpm/ml), [*U*-^{14}C]-serine (1.7×10^6 cpm/ml) or [1-^{14}C]sodium acetate (1.4×10^6 cpm/ml). Additions to the CT medium were 0.89 mg/ml of each unlabelled component.

As shown in Table 2, carbon atom 1 of alanine was not incorporated significantly into the antibiotic under conditions in which uniformly labelled alanine was an effective precursor. Thus, decarboxylation of alanine must occur prior to antibiotic formation, suggesting that acetate was the more direct precursor of antibiotic TA. Table 3 demonstrates a large reduction in the incorporation of uniformly labelled alanine and serine into TA in the presence of unlabelled acetate. The conclusion that alanine and serine share a common pathway with acetate during their incorporation into antibiotic TA is further supported by the fact that 11.4 carbon atoms in antibiotic TA came from labelled acetate when cells were grown in CT+acetate medium. Acetate does not enter antibiotic TA *via* mevalonic acid, since radioactive mevalonic acid was not incorporated into antibiotic TA. Mevalonic acid was incorporated into *M. xanthus* pigments, indicating that it was taken up by the cells.

Approximately 2 carbon atoms of uniformly labelled glycine were incorporated into the antibiotic (Table 4). Both carbon atoms of the glycine molecule were incorporated into the antibiotic with the same

Table 4. Incorporation of glycine into antibiotic TA.*

Precursor	Addition to CT medium	Specific activity (cpm/nmol)		Incorporation of glycine	
		Precursor	Antibiotic TA	(mol/mol TA)	(C-atom/TA molecule)
[U-^{14}C]Glycine	None	2,120	2,450	1.15	2.3
[2-^{14}C]Glycine	None	2,230	1,670	0.75	0.75
[1-^{14}C]Glycine	None	2,550	2,030	0.80	0.80
[1-^{14}C]Glycine	Gly	109	128	1.17	1.17
[1-^{14}C]Glycine	Gly+Ala	108	124	1.15	1.15
[1-^{14}C]Glycine	Gly+Ser	117	138	1.18	1.18
[1-^{14}C]Glycine	Gly+Acetate	141	160	1.13	1.13

* The experiment was performed as described in Table 1, using [U-^{14}C]glycine (1.2×10^6 cpm/ml), [1-^{14}C]-glycine (1.5×10^6 cpm/ml) or [2-^{14}C]glycine (1.5×10^6 cpm/ml). Additions to the CT medium were 0.89 mg/ml of each unlabelled component.

Table 5. Incorporation of alanine and glycine into antibiotic TA fragments I and II.*

Precursor	Alkaline hydrolyzed TA (cpm)	Periodate oxidation products	
		Fragment I (cpm)	Fragment II (cpm)
[U-^{14}C]Alanine	8,080	6,410	1,155
[U-^{14}C]Glycine	5,880	680	4,425

* Two samples of radioactive antibiotic TA were prepared: one sample was prepared from *M. xanthus* grown in the presence of uniformly labelled alanine; the other from cells grown in the presence of uniformly labelled glycine. In both cases the radioactive antibiotic was purified from cell-free supernatant fluids by extraction into chloroform and TLC. The antibiotics were then alkaline hydrolyzed (0.1 M KOH in 50% ethanol, 30°C, 24 hours) and again purified by chloroform extraction and TLC. The alkaline hydrolyzed TA was cleaved by periodate into fragment I (chloroform extractable, Rf 0.83) and fragment II (chloroform nonextractable).

Table 6. Stimulation of antibiotic TA production by alanine, serine and glycine.*

Addition to CT medium	Growth (A_{560})	Antibiotic production	
		(units/ml)	(units ml^{-1} A_{560}^{-1})
None	2.56	78	30
Ser	2.62	96	37
Ala	2.70	126	47
Gly	1.45	79	55
Ala+Ser	2.46	155	63
Ala+Gly	1.87	152	81
Ser+Gly	2.01	102	51
Ala+Ser+Gly	2.58	210	81

* Tubes containing 4.4 ml CT medium, supplemented with 0.89 mg/ml of amino acid (s), were inoculated with 0.1 ml of a starter culture (A_{560} 2.0) and incubated with shaking at 32°C. Antibiotic activity was determined after 48 hours.

specific activity. Additions of alanine, serine and acetate did not lower the incorporation of glycine into the antibiotic. Thus, glycine must enter the antibiotic *via* an independent pathway.

Cleavage of Radioactive Antibiotic TA into Fragments I and II

Mild alkaline hydrolysis of antibiotic TA opens the lactone ring with a loss of biological activity.[4] Radiolabelled antibiotic TA ([^{14}C]alanine and [^{14}C]glycine experiments) was hydrolyzed under alkaline conditions (Table 5), and the product or products were then cleaved into two fragments by periodate oxidation. Fragment 1 was extractable into $CHCl_3$, whereas fragment II remained in the aqueous phase. The ultraviolet absorption spectrum of fragment I was identical to the spectrum of TA (λ_{max} 239 nm).[4] Examination of fragment I by TLC showed a single UV-absorbing spot at Rf 0.83 in acetone - benzene (1: 1). All of the radioactivity was coincident with the UV-absorbing spot. Fragment II exhibited no absorption maximum in the visible or ultraviolet region.

When [^{14}C]alanine was used as the precursor, 85% of the recovered radioactivity was found in fragment I and 15% in fragment II. With [^{14}C]glycine as the precursor, only 13% of the recovered radioactivity was in fragment I, while 87% was in fragment II. These data further demonstrate that glycine and alanine (*via* acetate) serve as precursors of antibiotic TA by separate pathways.

Stimulation of Antibiotic TA Production by Ala, Ser and Gly

As seen in Table 6, antibiotic TA production per ml was enhanced by serine (23%), alanine (62%) and alanine plus serine (99%). These amino acids affected growth yields by less than 6%. Higher concentrations of these amino acids either separately or in combination did not stimulate antibiotic production further.

Addition of glycine to CT medium resulted in a partial inhibition of growth, but caused no significant increase in antibiotic production per ml. However, addition of glycine to CT media supplemented with alanine and/or serine resulted in a large increase in the yield of antibiotic. Maximum production of TA (210 units per ml) was obtained in CT medium supplemented with all three amino acids. Under these conditions, antibiotic production was 2.7 times higher than in unsupplemented CT medium.

Discussion

M. xanthus is a proteolytic bacterium that grows best on amino acid and peptide mixtures, such as Casitone.[6] Antibiotic TA production, however, is repressed when the Casitone concentration exceeds 1%.[1] In this report, three amino acids (alanine, serine and glycine) were shown to stimulate TA production when added to Casitone medium. In addition to enhancing production, each of the amino acids served as a precursor for the biosynthesis of the antibiotic molecule. Of the 34 carbon atoms in antibiotic TA, approximately 30 came from alanine plus serine and 3 came from glycine.

The data indicate that serine and alanine are not direct precursors of TA, but are incorporated presumably *via* acetyl CoA. In *M. xanthus*, alanine is deaminated to pyruvate and then decarboxylated to acetate.[7] The fact that acetate did not stimulate antibiotic production as effectively as serine or alanine probably reflects a less efficient uptake system for acetate than for amino acids in *M. xanthus*.

Although the detailed chemical structure of antibiotic TA is unknown, it exhibits very similar UV, IR, ^{1}H NMR and ^{13}C NMR spectra[4] to those of myxovirescin A.[7,8] The chemical structure of myxovirescin A has recently been reported.[8] Hydrolysis of the lactone or periodate oxidation of the vicinal hydroxyl groups opens the ring structure of TA, but does not produce smaller fragments. When the hydrolysis and oxidation reactions were performed sequentially, however, at least two fragments were produced. The larger, nonpolar fragment, containing the ultraviolet-absorbing diene was labelled with alanine, but not with glycine. Both carbon atoms of glycine (and possibly the nitrogen atom) were incorporated into the smaller, polar fragment.

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