Biosynthesis of Kitasamycin(Leucomycin) by Leucine Analog-Resistant Mutants of *Streptomyces kitasatoensis*

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The biosynthesis of kitasamycin in *Streptomyces kitasatoensis* B-896 was profoundly influenced by the addition of precursors to complex and defined media: L-valine and L-leucine directed biosynthesis towards the pairs A₄/A₅ (R₂ = butyryl) and A₁/A₃ (R₂ = isovaleryl), respectively, and total kitasamycin titers were doubled and quadrupled, respectively. *S. kitasatoensis* B-896 was very resistant (>20 mg/ml) to α -aminobutyric acid, an analog of L-valine, but very susceptible to L-leucine analogs 5', 5', 5'-trifluoroleucine and 4-azaleucine (5 to 10 μ g/ml). The inhibition by 4-azaleucine could be reversed by L-leucine, but by none of the other amino acids of the pyruvate family or the amino acids of the aspartate pathway. 4-Azaleucine-resistant mutants were isolated which in the absence of any precursors overproduced L-leucine and a kitasamycin complex mainly consisting of the pair A₁/A₃. These 4-azaleucine-resistant mutants are presumed to be regulatory mutants in which α -isopropylmalate synthase, the first enzyme of the L-leucine pathway, has become either derepressed or desensitized to leucine feedback inhibition. L-Leucine-regulatory mutants have economic value: in the absence of expensive precursors, they produce a kitasamycin complex in which the most potent pair A₁/A₃ is dominant and the least active components are absent.

Kitasamycin (leucomycin [10]) is a family of 14 antibiotics which differ by the acyl substituent in position 4'' (R₂) of the mycarose moiety and by the alternation of an hydroxyl and an acetyl group on carbon 3 (R₁) of the 16-membered lactone (17). The structures and specific antibiotic activities of kitasamycin members are reported in Table 1: the most potent pair is A₁/A₃ (R₂ = isovaleryl), followed by A₄/A₅ (R₂ = butyryl); moreover the hydroxylated member (R₁ = OH) of each pair is more active than its acetylated partner. The composition of the kitasamycin complex produced by *Streptomyces kitasatoensis* is about 10% A₁/A₃, 60% A₄/A₅, and 30% other components. All kitasamycin members exhibit the same mode of action, and the lipophilic acyl group (R₂) merely aids transport into target cells (24); thus, compound A₁₁ (R₂ = H) inhibits protein synthesis by cell-free extracts of *Escherichia coli* as efficiently as compound A₁ (R₂ = isovaleryl); yet, A₁ is 30 times more active than A₁₁ in inhibiting growth.

The biosynthesis of kitasamycin can be inferred from studies on magnamycin (8), a closely related macrolide, and the work of Ōmura et al. (18) on kitasamycin. Mycaminoase and mycarose would originate from glucose; N-methyl groups

on mycaminoase and O-methyl groups on the lactone would derive from the S-methyl of methionine. ¹³C precursor studies (18, 21) have shown that carbons 1 and 2 and 9 to 16 are from acetate, carbons 5, 6, 17, and 18 are from butyrate, and carbons 7, 8, and 19 are from propionate. The origin of carbons 3 and 4 remains unknown. Carbons 20 and 21 in 3-acetylated compounds (R₁ = acetyl) also are from acetate; Ōmura et al. (15, 16) have recently observed that the 3-acetylated compound A₃ is derived from its 3-hydroxylated partner, A₁, and that this acetylation is induced by glucose and valerate and repressed by butyrate. The biosynthesis of acyl groups at R₂ is not firmly established. However, it has been known for many years (6) that isovaleryl and butyryl groups, present in the most desirable pairs A₁/A₃ and A₄/A₅, are derived from leucine and valine, respectively, in microorganisms, and it is known that the isovaleryl side chain of magnamycin is derived from leucine (8).

From the foregoing, it appears feasible to modify kitasamycin biosynthesis for improved composition of the complex and increased potency by favoring the formation of A₁/A₃ or A₄/A₅ in the producing organism, and by repressing the synthesis of the acetylated partner in either pair through the addition of butyrate. This paper mainly deals with the first objective: the action of leucine and valine and the overproduction of

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TABLE 1. Structure^a and potency of kitasamycin

Kitasamycin	R ₁	R ₂	Potency ^b (U/mg)
A ₁	H	COCH ₂ CH(CH ₃) ₂	1,701
A ₃ ^c	COCH ₃	COCH ₂ CH(CH ₃) ₂	1,104
A ₄	COCH ₃	COCH ₂ CH ₂ CH ₃	984
A ₅	H	COCH ₂ CH ₂ CH ₃	1,528
A ₆	COCH ₃	COCH ₂ CH ₃	872
A ₇	H	COCH ₂ CH ₃	881
A ₈	COCH ₃	COCH ₃	233
A ₉	H	COCH ₃	288
A ₁₀ =(U) ^d	COCH ₃	H	86
A ₁₁ =(V) ^d	H	H	167
A ₁₂ ^e	COCH ₃	COCH ₂ CH ₂ CH ₂ CH ₂ CH ₃	<528
A ₁₃ ^e	H	COCH ₂ CH ₂ CH ₂ CH ₂ CH ₃	528
A ₁₄ ^e	COCH ₃	COCH ₂ CH ₂ CH(CH ₃) ₂	—
A ₁₅ ^e	H	COCH ₂ CH ₂ CH(CH ₃) ₂	—

^b Components were separated by countercurrent distribution, and their potency was determined by comparison to a standard of 1,204 U/mg (D. Kluepfel and G. Schilling, personal communication).

^c Identical to josamycin (14).

^d Omura et al. (19).

^e D. Kluepfel and G. Schilling (unpublished data).

leucine and preferential formation of A₁/A₃ by leucine analog-resistant mutants of *Streptomyces kitasatoensis* is reported. The action of butyrate is also described.

A preliminary account of this work was presented by Vézina (31).

MATERIALS AND METHODS

Kitasamycin-producing strains. *S. kitasatoensis* B-896 is the ancestor of all the mutants described in this study. It was obtained by two rounds of mutagenesis (ultraviolet irradiation) and selection from the original strain NRRL 2486. Strains B-896 produced ca. 500 U of kitasamycin per ml in chemically defined medium (DM) and up to 3,000 U/ml in complex medium (CM), whereas ancestor strain NRRL 2486 yielded 100 U/ml under optimal conditions (CM). All cultures were propagated on solid medium at 30°C and 70% relative humidity for 12 to 14 days on tomato paste-oatmeal agar (TPO; 22). Conservation was by lyophilization (25) and by drying conidial suspensions in sterile soil (7). The inoculum was prepared as follows: surface growth (spores and mycelial fragments) on TPO from a Roux bottle culture was suspended in 30 ml of sterile, distilled water; the suspension was homogenized by agitation for 15 min at 250 rpm in the presence of glass beads in a 250-ml Erlenmeyer flask

and standardized by adding sterile water until a 1/10 dilution displayed a transmittance (1-cm cuvette, Coleman Junior 6A spectrophotometer) of 65%.

Kitasamycin fermentation. Fermentations were done in 500-ml Erlenmeyer flasks containing 50 ml of medium; flasks were plugged with nonabsorbent cotton, sterilized at 121°C for 20 min, cooled to 30°C, and inoculated with 1 ml of the inoculum. Incubation was at 30°C on a rotary shaker at 250 rpm (2-inch [ca. 5.08-cm] stroke) for 6 to 8 days. For growth determination during fermentation, 500-ml Erlenmeyer flasks equipped with optical glass side arms (light path, 1 cm) were used. DM consisted of the following (in grams per liter): L-methionine, 2.5; L-arginine, 2.5; (NH₄)₂SO₄, 5; "cerelose" (Corn Products Inc., New York), 10; soluble starch, 70; K₂HPO₄, 5; MgSO₄·7H₂O, 0.5; NaCl, 0.5; CaCl₂, 0.2; ZnSO₄·7H₂O, 0.05; MnSO₄·7H₂O, 0.01; FeSO₄·7H₂O, 0.02; distilled water to 1 liter; pH 7.2 to 7.4. CM was made by adding the following (in grams per liter) to DM medium: N-Z Case (Sheffield Chemicals, Memphis, Tenn.), 0.5; maltose, 5; and sperm oil, 10, pH 7.0 to 7.2; cerelose was increased to 15, and CaCl₂ was replaced by CaCO₃, 3; tap water was used instead of distilled water.

Stock solutions of L-leucine, L-valine, isoamyl alcohol, and sodium butyrate were sterilized by membrane filtration (Millipore, 0.45-μm porosity) and added to the media before inoculation to final concentrations of

1, 1, 0.2, and 0.1%, respectively. In preliminary experiments (not reported), these concentrations were found to give maximal effects.

Susceptibility to leucine and valine analogs. The valine analog tested was L- α -amino-*n*-butyric acid (General Biochemicals, Chagrin Falls, Ohio); the leucine analogs were 5', 5', 5'-trifluoroleucine, and 4-azadL-leucine (2-amino-3-dimethylaminopropionic acid) (ICN Pharmaceuticals Inc., Cleveland, Ohio). Crystals of the analog were deposited 1 cm from the edge of petri plates, 10 cm in diameter, containing 20 ml of modified Hopwood (11) minimal medium (MMHM) previously inoculated by spreading 0.5 ml of the inoculum (above) by means of a sterile glass rod. MMHM consisted of the following (in grams per liter): L-asparagine, 0.5; sodium citrate, 1; dextrose, 10; K_2HPO_4 , 0.5; $MgSO_4 \cdot 7H_2O$, 0.2; $FeSO_4 \cdot 7H_2O$, 0.01; agar (Difco), 20; distilled water to 1 liter; pH 7.4 to 7.6 (KOH). Dextrose was sterilized separately as a 50% solution and added to the medium after sterilization. Inhibition by amino acid analog could be detected after 3 to 4 days of incubation at 30°C. Reversibility of inhibition was determined by depositing test compounds (crystals) in the center of plates. For precise determination of susceptibility, inhibitory analogs at various concentrations were incorporated into the medium.

Isolation of 4-azaleucine-resistant mutants. A search was made for 4-azaleucine-resistant mutants in *S. kitasatoensis* B-896. These mutants were very rare; colonies developing after prolonged incubation in the inhibition zone around crystals of the analog and those present on gradient plates (the Szybalski-Bryson method [29]) were generally found to have reverted to susceptibility on re-testing. Mutagenesis (ultraviolet irradiation to 99% kill) increased mutant frequency somewhat. The following method yielded satisfactory results: 250-ml Erlenmeyer flasks with optical side arm, containing 45 ml of liquid MMHM medium, were inoculated and incubated as usual. At early stationary phase (ca. 48 h), 5 ml of a 4-azaleucine solution was added to a final concentration twice as high as that to which the parent was resistant; 0.1-ml samples taken daily were spread on solid MMHM containing the same concentration of 4-azaleucine as that present in the flask. Resistant colonies developed after 1 to 2 weeks of incubation; they were purified twice and preserved on MMHM containing the same concentration of the analog. Resistant mutants were retested by replication (Steers device [27]) on MMHM containing various concentrations of 4-azaleucine.

Kitasamycin determination. The production abilities of resistant mutants and parent B-896 were compared by culturing on MMHM (10 ml/10-cm petri plate) at 30°C for 5 days, cutting out 7-mm-diameter cylinders of agar, and transferring to glass plates (12 by 12 inches [ca. 30.48 by 30.48 cm]) containing 100 ml of Mycin assay agar (Difco Laboratories, Detroit, Mich.), previously inoculated with *Bacillus subtilis* ATCC 6633 (9). Inhibition zones were measured after 18 h of incubation at 37°C.

For quantitative determinations, the standard cylinder plate assay was used. Standard solutions containing 0, 5, 20, 30, and 40 U of kitasamycin per ml in pH 7.6 potassium phosphate buffer (0.01 M) were used to construct standard curves. Dilutions of the "unknown" samples were made in the same buffer.

Kitasamycin composition. Approximate composition was determined by thin-layer chromatography. Broth samples were adjusted to pH 9 with NaOH and extracted with butyl acetate to give an extract of ca. 0.4 mg of kitasamycin per ml. A 10- μ l amount was applied to silica gel plates. Kitasamycin complex (0.5 mg/ml of methanol) and pure A₁, A₃, A₄, and A₅ (1 mg/ml of methanol) were similarly applied. Thinlayer chromatography plates were developed in chloroform:methanol:glacial acetic acid:water (79:11:8:2), dried, sprayed with sulfuric acid (10%), and heated at 160°C for 15 min.

Quantitative determination of kitasamycin composition was made by HPLC by the method of Omura et al. (20).

Leucine determination. L-Leucine excretion was determined auxanographically by the method of Calvo (2). *Salmonella typhimurium leuBCD39*, the test organism, bears a deletion which prevents reversion to prototrophy and is resistant to kitasamycin, which allows the determination of L-leucine in the presence of the antibiotic. The test organism was grown on Calvo medium (2) enriched with 50 mg of L-leucine per ml. After 24 h of growth at 37°C, cells of strain *leuBCD39* were washed thrice with saline by centrifugation at 9,000 rpm for 15 min and resuspended in saline. The washed suspension was finally standardized to read 70% transmittance (1-cm cuvette, Coleman Junior 6A spectrophotometer). It was prepared fresh daily. Calvo medium was inoculated with *S. typhimurium leuBCD39* inoculum and poured into petri plates or glass plates (12 by 12 inches). Agar cylinders (7 mm diameter) from growth plates of streptomycetes under test were deposited on the surface of the inoculated medium. After 18 h of incubation at 37°C, growth could be detected around the cylinders of leucine excretors. A crystal of leucine deposited on each plate served as a positive control.

For the quantitative determination of leucine excretion, a cylinder plate assay (1) was first tried. The method was found of limited use because of the difficulty of precisely reading the diameters of growth. In subsequent experiments, a turbidimetric method (26) was used. Double-strength Calvo medium (without agar) was distributed into culture tubes (5 ml/tube [16 by 150 mm]). A standard solution of L-leucine was distributed into a series of tubes to give final concentrations of 0, 10, 20, 40, 60, 80, and 100 μ g/10 ml (per tube). Dilutions of "unknown" samples (1, 3, and 5 ml) were similarly distributed into another series of tubes. Volume was completed to 10 ml, and all tubes were sterilized at 121°C for 5 min, and then rapidly cooled to 37°C. Each tube was inoculated with one drop of *S. typhimurium leuBCD39* inoculum and incubated at 37°C for 12 h. Turbidity was determined at 660 nm in a Coleman Junior 6A spectrophotometer. A standard curve was drawn, and the concentration of leucine in "unknown" samples was read by interpolation from the standard curve.

RESULTS

Effect of L-valine and L-leucine on kitasamycin titer and composition. The addition of L-valine or L-leucine to DM had a profound effect on titer and composition of the kitasamycin complex. In preliminary experiments (31),

the maximal effect was found at a concentration of 1% of either amino acid: L-valine, added to DM, increased the proportion of A_4/A_5 mainly at the expense of minor components A_6 to A_{15} ; addition of L-valine also had the effect of doubling potency (1,000 instead of 480 U/ml). On the other hand, addition of L-leucine shifted the ratio of components in favor of A_1/A_3 and increased titer to 1,700 U/ml. The combined addition of 1% L-leucine and 0.2% isoamyl alcohol, a product of L-leucine degradation in many microorganisms (6), led to further increase in potency (2,500 U/ml).

Other presumptive precursors of kitasamycin acyl side chains had some effect, but their toxicity precluded their use in subsequent experiments. α -Ketoisovalerate (0.2%), the branch point intermediate of the valine and leucine pathways, favored the production of A_1 and A_5 , but had no influence on potency. α -Ketoisocaproate (0.1%), the immediate precursor of leucine, favored the formation of A_1 and slightly increased titer. L-Isoleucine (1%) greatly decreased titer. Leucine degradation products, added singly to DM, only showed a modest effect (isoamyl alcohol at 0.2% or isovaleric acid at 0.1%), or no effect at all (isovaleraldehyde at 0.1%); higher concentrations were toxic, and antibiotic production was decreased.

The time course of fermentation in complex and defined media is illustrated in Fig. 1 and 2, respectively. In CM (Fig. 1), the effect of L-leucine was twice as large as that of isoamyl alcohol; when added together, they showed an additive increase in production. In DM (Fig. 2), L-leucine was also more effective than isoamyl alcohol, but a synergistic effect was observed when

FIG. 2. Effect of L-leucine (1%) and isoamyl alcohol (IAA, 0.2%), added to DM, on production of kitasamycin by *S. kitasatoensis* B-896.

both compounds were added. DM and CM gave similar distributions of kitasamycin components.

The foregoing results prove the feasibility of producing kitasamycin complexes of high potency by adding to the production medium either L-valine, which mainly leads to A_4/A_5 , or L-leucine and isoamyl alcohol, which mainly give A_1/A_3 . The high cost of these amino acids, however, precludes their use in the production plant. The preparation of amino acid regulatory mutants, a concept used successfully by Elander et al. (5) to produce pyrrolnitrin with tryptophan overproducers, was attempted in order to circumvent this economic difficulty.

Susceptibility of *S. kitasatoensis* B-896 to analogs of L-valine and L-leucine. The susceptibility of *S. kitasatoensis* B-896 to two L-leucine analogs, trifluoroleucine and 4-azaleucine, was tested auxanographically (Fig. 3): the organism was much more susceptible to 4-azaleucine than to trifluoroleucine. When incorporated into agar (data not shown), 4-azaleucine inhibited growth completely at 5 to 10 $\mu\text{g/ml}$, whereas more than 100 μg of trifluoroleucine per ml was necessary to observe the same effect. On the other hand, the organism was very resistant (>20 mg/ml) to α -aminobutyric acid, a specific inhibitor, or L-valine in *Serratia marcescens* (12).

The inhibition of the kitasamycin producer by 4-azaleucine was reversed by L-leucine, as shown in Fig. 4; L-valine, L-isoleucine, L-threonine, and L-methionine had no effect. Because the organism is more susceptible to 4-azaleucine than to trifluoroleucine and because the former analog

FIG. 1. Effect of L-leucine (1%) and isoamyl alcohol (IAA, 0.2%), added to CM, on production of kitasamycin by *S. kitasatoensis* B-896.

FIG. 3. Inhibition of *S. kitasatoensis* B-896 by 4-azaleucine and 5',5',5'-trifluoro-leucine on medium MMHM. Crystals of inhibitors were deposited 1 cm above the lower edge of plates. Plate on top contained no inhibitor (control).

FIG. 4. Reversal of 4-azaleucine inhibition of *S. kitasatoensis* B-896 on medium MMHM. Crystals of 4-azaleucine were deposited on each plate 1 cm below the upper edge. Crystals of L-leucine (+LEU), L-isoleucine (+ILE), L-valine (+VAL), L-methionine (+MET), and L-threonine (+THR) were then deposited in the center of indicated plates.

has been shown in *S. typhimurium* (B. I. Stieglitz, Ph. D. thesis, Cornell University, Ithaca, N.Y., 1971) to be more specific to leucine than the latter, it was decided to search for 5-azaleucine-resistant mutants which might overproduce L-leucine which might direct biosynthesis to the pair A₁/A₃.

Isolation of 4-azaleucine-resistant mutants. 4-Azaleucine-resistant mutants are very rare, and their expression and isolation require the presence of citrate in the medium (J. M.

Calvo, personal communication). This was confirmed in *S. kitasatoensis* B-896. Moreover, colonies that developed in the inhibition zone around a 4-azaleucine crystal and those appearing on gradient plates were all unstable and reverted to susceptibility upon retesting. The organism was thus grown in shake flasks containing liquid MMHM, and 4-azaleucine was added after 48 h of incubation; thereafter, daily samples were spread on solid MMHM containing the same concentration of the inhibitor. Only

after 2 weeks did the first resistant colonies appear; altogether, 66 resistant mutants were isolated and purified. The mutants (and parent B-896) were tested auxanographically for L-leucine excretion; 29 of the 66 mutants excreted L-leucine, whereas parent B-896 yielded no zone. The 29 mutants were grown in DM in shake flasks, and the excretion of L-leucine and production of antibiotic were determined quantitatively; their resistance to 4-azaleucine was also tested in MMHM plates. One mutant, designated 10/6, was selected which was more resistant to the inhibitor, excreted more L-leucine, and produced more kitasamycin than the other mutants and the parent (31). Mutant 10/6 was inhibited by 500 μ g of 4-azaleucine per ml. On plates containing the latter concentration of the inhibitor, some colonies developed on prolonged incubation (15 days). One hundred resistant colonies were isolated, purified, and retested for their resistance to 4-azaleucine incorporated into agar. They were also grown in DM medium in shake flasks, and L-leucine excretion and kitasamycin production were determined. One mutant, designated 250/7, was superior to all others for the three parameters studied (31). As resistance to 4-azaleucine increased in the mutants selected, L-leucine excretion and kitasamycin production also increased; furthermore, the composition was shifted in favor of the pair A_1/A_3 (R_2 = isovaleryl). The chromatogram in Fig. 5

FIG. 5. Composition (thin-layer chromatography) of kitasamycin produced by *S. kitasatoensis* B-896 and its *azl* mutants 10/6 and 250/7 in DM in the absence of precursors. Fermentation broths were extracted after 6 days of incubation. Pure A_1 and pure A_5 were added to the broth extract, as indicated, to clearly identify certain components. Mixt., Commercial product containing all kitasamycin components.

shows that *azl* 10/6 produced less of the minor components than did parent B-896 and that mutant *azl* 250/7 yielded the pair A_1/A_3 almost exclusively. The time course of fermentation by *azl* 250/7 in DM and CM is reported in Fig. 6. Kitasamycin was extracted after 6 days of incubation and found to consist mainly of A_1/A_3 .

Isolation of highly resistant mutants from *azl* 250/7. Mutant 250/7 was grown in shake flasks for 48 h in MMHM, and 4-azaleucine was added to give final concentrations of 3.75 and 20 mg/ml. Incubation was continued, and mutants were isolated as described above. Twelve mutants were isolated, and all 12 upon retesting had lost the capacity to sporulate, excrete L-leucine, or produce kitasamycin. All attempts to improve their growth by enriching DM and CM media were unsuccessful. The severe selection imposed by abrupt increase in 4-azaleucine concentration was probably responsible for the "crippled" nature of these resistant mutants. Therefore, the experiment was repeated with more gradual increases in 4-azaleucine concentration.

Plates of MMHM containing 1.0, 1.5, 2.0, and 3.0 mg of 4-azaleucine per ml were inoculated by spreading on the surface 0.1 ml of *azl* 250/7 inoculum. Another series of plates were inoculated with the survivors of ultraviolet irradiation (>99% kill). The resistant colonies that developed after several days of incubation were tested as done previously (Table 2). Several mutants excreted more L-leucine than parent 250/7. The highest excreter, which yielded more than twice as much L-leucine as did the parent, was resistant to 3 mg of 4-azaleucine per ml. However, none of the high leucine excreters (spontaneous or induced) yielded as much kitasamycin as the parent.

FIG. 6. Time course of kitasamycin fermentation by *azl* mutant 250/7 in CM and DM in the absence of precursors.

Resistant mutants were also isolated from liquid MMHM in shake flasks. Flasks were inoculated as usual, and 4-azaleucine was added at 0, 4, 18, and 42 h of incubation at various concentrations. Added at time 0, 4-azaleucine inhibited growth completely; it had no effect when added at 42 h (early stationary phase). The effect of various concentrations of 4-azaleucine added at 4 and 18 h is shown graphically in Fig. 7 and 8, respectively. In Fig. 7, growth was severely inhibited by all concentrations, but could resume after 125 to 150 h of incubation in the presence of the lowest concentration (0.5 mg/ml). Added at 18 h (Fig. 8), 4-azaleucine was also inhibitory, but growth resumed much earlier (50 to 75 h) and at all concentrations. At the end of incubation, samples from all flasks were spread on solid MMHM containing corresponding concentrations of 4-azaleucine; resistant mutants were isolated, purified, and tested as usual for resistance, L-leucine excretion, and kitasamycin production. All the 70 mutants tested were more resis-

TABLE 2. Excretion of L-leucine and production of kitasamycin by spontaneous and ultraviolet-induced mutants from *azl* 250/7.

Resistance of mutants (mg/ml)	Relative production ^a			
	Spontaneous mutants		Induced mutants	
	L-Leucine	Kitasamycin	L-Leucine	Kitasamycin
1.0	1.27	0.81	1.44	0.81
1.5	1.52	0.67	1.42	0.85
2.0	1.12	0.77	1.46	0.96
3.0	2.24	0.93	2.23	0.89
5.0	1.08	0.81	1.04	0.91

^a DM; incubation for 6 days at 30°C; 100 mutants per class. Production of parent *azl* 250/7 taken as 1.00.

FIG. 7. Effect of 4-azaleucine, added after 4 h of incubation at the concentrations indicated, on growth of *azl* mutant 250/7 in medium MMHM.

FIG. 8. Effect of 4-azaleucine, added after 18 h of incubation at the concentrations indicated, on growth of *azl* mutant 250/7 in medium MMHM.

tant than *azl* 250/7 and most excreted more L-leucine; 3 mutants excreted five times more L-leucine than the parent. One mutant, resistant to 5 mg of 4-azaleucine per ml, excreted 4 times as much L-leucine and produced 1.5 times as much kitasamycin as the parent.

Effect of butyrate on kitasamycin composition. Mutant *azl* 250/7 was cultured in shake flasks in DM medium in the presence and absence of sodium butyrate (0.1%). Kitasamycin was extracted after 4 and 6 days of incubation, and its composition was determined by thin-layer chromatography (Fig. 9). The effect of butyrate was detected only after 6 days of incubation (column S_{2a} in Fig. 9): A_1 was dominant over A_3 ; however, A_3 was not completely eliminated.

DISCUSSION

The complex nature of the kitasamycin family and the unequal potency of its components require us to define the composition as well as the specific activity of the final product to assure its uniformity. These requirements complicate strain improvement programs which aim for isolation of strains which produce a complex with an increased proportion of desirable compounds such as A_1/A_3 or A_4/A_5 . The physical separation of these pairs by countercurrent distribution and the elimination of the others are tedious and uneconomical. Therefore, to simplify the mixture, the best solution lies in directing the biosynthesis towards desirable components either environmentally or genetically in the producing microorganisms. Mutation and random selection can lead to the isolation of strains with altered spectra of antibiotics (13, 31). Among other methods for altering the spectrum of metabolites (4, 23), the addition of precursors often is useful.

FIG. 9. Composition (thin-layer chromatography) of kitasamycin produced by *azl* mutant 250/7 in absence (S_1) and presence (S_2) of butyrate (0.1%). Fermentation broths were extracted after 4 (S_1 and S_2) and 6 days (S_{1a} and S_{2a}) of incubation. M, Mixture of pure A_3 , A_4 , A_1 , A_5 .

Foster (6) reported that isovaleryl and butyryl groups, present in kitasamycins A_1/A_3 and A_4/A_5 , respectively, are derived from L-leucine and L-valine, and Grisebach and Achenbach (8) proved that L-leucine is the precursor of the isovaleryl side chain of magnamycin. The same pathways seem to operate in *S. kitasatoensis*, because L-valine or L-leucine added to defined or complex media drastically shifted kitasamycin composition in favor of the pairs A_4/A_5 and A_1/A_3 , respectively, and appreciably increased the total titer of antibiotic. Because the addition of these amino acids is too expensive in the production plant, we decided to search for regulatory mutants which would overproduce either L-valine or L-leucine (see Demain [3] for a review). L-Valine and L-leucine belong to the pyruvate family of amino acids (30). The branch point intermediate is α -ketoisovalerate which is transaminated to L-valine or acetylated to α -

isopropylmalate by α -isopropylmalate synthase, the first enzyme of the leucine pathway; α -isopropylmalate is isomerized to β -isopropylmalate by α -isopropylmalate isomerase, and β -isopropylmalate is transformed into α -ketoisocaproate by β -isopropylmalate dehydrogenase. The three enzymes are exclusive to the leucine pathway; finally, α -ketoisocaproate is converted to L-leucine by transamination. On the other hand, degradation of L-leucine (6) would take place through α -ketoisocaproate, isovaleraldehyde, and isoamyl alcohol, the immediate precursor of isovaleryl group. Therefore, it was not surprising that the addition of α -ketoisovalerate increased titers of $A_1 + A_5$, whereas that of α -ketoisocaproate greatly favored A_1 at the expense of A_5 , and increased total titers. Isovaleric acid and isoamyl alcohol increased total production but had no significant effect on the ratio $A_1:A_5$; isovaleraldehyde had no effect. L-Isoleucine, which belongs to the aspartate family and is synthesized by a pathway parallel to that of L-valine, inhibited the formation of kitasamycin.

In *S. kitasatoensis*, the valine pathway seems to be derepressed, because (i) the organism is very resistant to α -aminobutyric acid, an inhibitor of acetohydroxy acid synthase (the first enzyme of the L-valine and L-isoleucine pathway), and (ii) it makes sufficient valine for the accumulation of the A_4/A_5 pair in the complex. The leucine pathway, however, is probably under more stringent regulation, because the organism is susceptible to trifluoroleucine and 4-azaleucine, two inhibitors of α -isopropylmalate synthetase (28). The organism also makes only limited amounts of leucine, and the components A_1/A_3 constitute only a minority pair. The action of added leucine appears to involve not only an increase in the concentration of isovaleryl groups for the formation of A_1/A_3 , but also repression or inhibition of the valine pathway, because the pair A_4/A_5 is very strongly decreased in the presence of L-leucine.

The 4-azaleucine-resistant (*azl*) mutants of *S. kitasatoensis* expectedly overproduced L-leucine and yielded a kitasamycin complex which mainly consisted of the pair A_1/A_3 . The concentration of L-leucine excreted increased with the increase in resistance up to ca. 3 mg/ml; kitasamycin production increased similarly (Table 2). The relationship failed above this concentration. In conclusion, knowledge of the biosynthesis and regulation of the branched-chain amino acids was used empirically to modify the biosynthesis of kitasamycin. The interpretations given here, however, are mainly speculative, and their correctness must await the isolation and assay of the responsible enzymes.

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