BIOLOGICAL TRANSFORMATION OF KANAMYCIN A TO AMIKACIN (BBK-8)

Sir:

A method is reported for the biological formation of amikacin(AMK)from kanamycin A(KMA) using a mutant strain of the butirosin (BTR) producing organism, *Bacillus circulans* NRRL B-3313.[1,2] Amikacin is a broad spectrum aminoglycoside antibiotic synthesized from KMA and L(−)-α-hydroxy-γ-aminobutyric acid (L-HABA), (Fig. 1).[3] Butirosin (Fig. 2) has the L-HABA structure as an acylated side chain on its 2-deoxystreptamine molecule. The structural analogies between AMK and BTR suggest that AMK could be formed by feeding KMA to a broth culture of *B. circulans*. Recent reports on the biosynthesis of BTR[4,5] indicate that the coupling of L-HABA to the ribostamycin (RBT) molecule (Fig. 2) could be one of the last steps in the biosynthesis of BTR.

The parent strain of *B. circulans* was found to be sensitive to KMA at 5~10 μg/ml. A KMA resistant strain of *B. circulans* was obtained by stepwise enrichment in liquid culture at KMA concentrations of 600~800 μg/ml. Therefore, the KMA-resistant strain was further mutagenized with NTG to obtain a mutant deficient in the biosynthesis of both BTR and RBT. This was carried out to achieve a more exacting identification of any active compounds and, hopefully, a more efficient transformation due to the absence or to the reduced amount of RBT, the natural substrate.

Fig. 1. Structures of amikacin and kanamycin A.

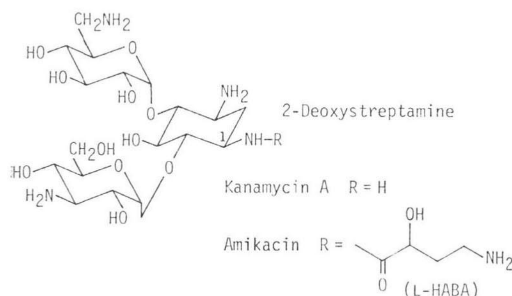
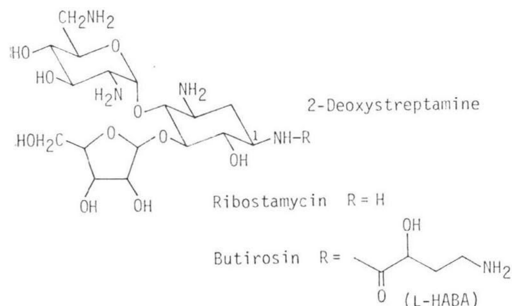


Fig. 2. Structures of butirosin and ribostamycin.



The mutant strain *B. circulans* LMC 1387 (ATCC 31773) produced about 200 μg/ml of BTR compared with about 1,000 μg/ml of the parent strain when grown in a 20-liter jar fermenter containing 10 liters of medium consisting of 4% glycerol, 2% soybean flour, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1004% $CaCl_2 \cdot 2H_2O$, 0.0005% $FeSO_4 \cdot 7H_2O$, 0.00005% $ZnSO_4 \cdot 7H_2O$, 0.01% silicone antifoam, and tap water. The pH of the medium was adjusted to 7.5 prior to sterilization. After inoculation, the culture was incubated at 30°C, with an aeration rate of 50 liters/minute and an agitation rate of 500 rpm for 120 hours. Kanamycin A sulfate was added to the fermenter after 8 hours (200 mg), 24 hours (500 mg), 48 hours (1,500 mg) and 72 hours (2,500 mg).

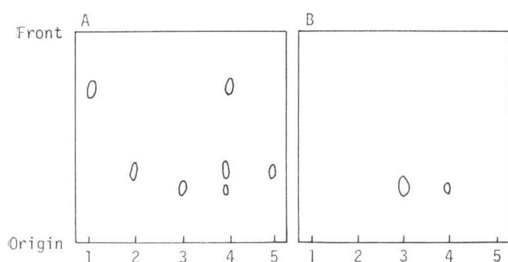
A control fermentation was also run with no added KMA. The harvested whole fermentation broth (9 liters) was passed at pH 6.8 through a column containing 300 ml of Amberlite IRC 724 resin (Rohm and Haas Co.) in NH_4^+ form. After washing with 0.1 M NH_4OH, the resin was eluted with 1.0 M NH_4OH and the eluates were concentrated under reduced pressure to 90 ml. This step was repeated using a smaller column containing 30 ml of resin and the eluates were concentrated to 10 ml.

The concentrated eluates were analyzed by TLC and the results are shown in Fig. 3. Samples from the culture broth supplemented with KMA gave a ninhydrin-positive compound with an Rf identical to authentic AMK. This compound was not present in the culture broth without KMA. The developed TLC plates were also assayed by bioautography against *Pseudomonas aeruginosa* LMC 1487, a strain which is resistant to KMA and BTR (MIC >500 μg/ml), but is sensitive to AMK (MIC 0.4 μg/ml). The extract from the culture broth supplemented with KMA gave an inhibition zone at the same Rf as authentic AMK. Again, this zone was absent in the sample from the culture broth lacking KMA.

The presence of AMK in the transformation culture broth was further confirmed by HPLC

Fig. 3. Alumina thin-layer chromatograms of the products in the culture broth of *B. circulans* LMC 1387.

A=Compounds positive to the ninhydrin reaction. B=Compounds active against *P. aeruginosa* LMC 1487.

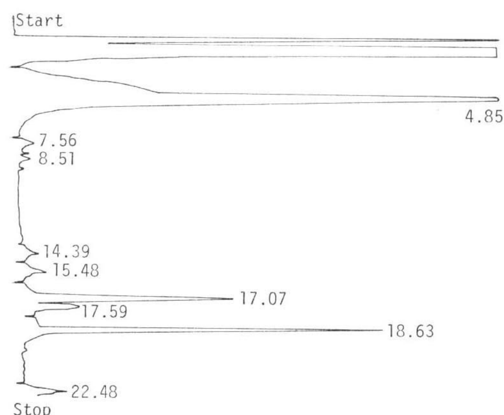


1=Kanamycin A. 2=Butirosin. 3=Amikacin. 4=Culture broth fed with KMA. 5=Culture broth without KMA.

1, 2, 3: Reference standard.

TLC Solvent: Methylethylketone - ethanol - 32% aqueous NH_4OH - water (1:1:1:1).

Fig. 4. High-pressure liquid chromatogram of the products from culture broth of *B. circulans* LMC 1387.



Retention time (Minutes)	Relative peak area (μvolts/second)	Area (%)
14.39	43130	2.678
15.48	74480	4.624
17.07	488500	30.329 Butirosin
17.59	148800	9.238 Amikacin
18.63	783400	48.638 Kanamycin A
22.48	72380	4.494

(Fig. 4). The samples were derivatized with 2,4,6-trinitrobenzenesulfonic acid and then analyzed on a reversed phase Lichrosorb RP8 column (Merck and Co., Darmstadt, W. Germany). A linear gradient of acetonitrile - sodium acetate buffer was used as a mobile phase, followed by detection of the compounds at 254 nm. Addition of authentic AMK to the concentrated eluates did not show differences in either the TLC or HPLC patterns.

Fig. 4 shows that the main peaks present in the HPLC chromatogram are: butirosin (17.07′), amikacin (17.59′) and residual kanamycin A (18.63′) and the respective concentrations in the harvested culture broth were 207, 12 and 411 μg/ml. The low bioconversion yield (~3%) may be attributed to the high sensitivity of *B. circulans* LMC 1387 to AMK (MIC ~1.0 μg/ml). The use of a properly selected AMK-resistant mutant could improve the transformation efficiency. However, we think that the analytical data obtained by TLC, bioautography and HPLC provide evidence of the partial transformation of KMA to AMK by *B. circulans* LMC 1387.

In conclusion, we believe that this biological approach may be a potential alternative to the expensive chemical synthesis of AMK. This process does not require highly pure isolated KMA, and intermediate preparations of the recovery process of KMA from the fermented broth of *Streptomyces kanamyceticus* could be used.

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(Received September 29, 1982)

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