ISOLATION AND CHARACTERIZATION OF A MOCIMYCIN RESISTANT MUTANT OF *ESCHERICHIA COLI* WITH AN ALTERED ELONGATION FACTOR EF-Tu

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1. Introduction

Mocimycin [1,2], an antibiotic identical to kirromycin [3], is known to block protein synthesis in an *E. coli* cell-free system [4], by preventing the release of the elongation factor Tu (EF-Tu) from the ribosome during the elongation cycle [5–8].

In this paper we describe the isolation of a mocimycin resistant mutant of *E. coli*. The resistant character of the mutant is caused by an alteration in the target protein, EF-Tu. Genetically this resistance appears to reside in at least two genes at 72′ and 88′ of the new *E. coli* linkage map [9].

2. Materials and methods

2.1. *Materials*

Cell-free extracts, [^{14}C]phenylanyl-tRNA and EF-Tu were prepared as described by Lupker et al. [10]. Ribosomes from *E. coli* MRE 600 were prepared as described by Voorma et al. [11] and washed once with 1 M NH_4Cl. They depend completely on the addition of EF-Tu for poly(U)-directed polyphenylalanine synthesis.

Mocimycin was provided by Dr R. Beukers (Gist-Brocades NV, Research and Development, Delft, The Netherlands).

Ethylmethane Sulfonate (EMS) was purchased from Eastman Kodak Co., the culture media were from Difco Laboratories, the radiochemicals from the Radiochemical Centre, Amersham. Bacterial strains used are listed in table 1.

Table 1
Escherichia coli K12 strains used[a]

Strain	Relevant markers	Origin
KMBL 1001	F^-su^-	W 1485
LBE 2012	mocimycin resistant	KMBL 1001 this paper
LBE 1310	*rps*L(= *str*A)	KMBL 1001
LBE 1431	*met*A	KMBL 1001

[a] All strains are from the stock of the Laboratory of Molecular Genetics, State University, Leiden, The Netherlands

Bacteriophage P1 was used in the transduction experiments. LC-Medium contains per liter: tryptone, 10 g; yeast extract, 5 g; NaCl, 8g. M9 medium [12], was supplemented as indicated in the text.

2.2. *Mutagenesis and selection*

A culture of *E. coli*, KMBL 1001 was mutagenized with EMS according to Miller [12]. The mutagenized culture was diluted ten-fold in M9 medium, supplemented with casamino acids and glucose to a final concentration of 0.5% and 0.2% respectively, and incubated overnight at 37°C. From this overnight culture 0.2 ml was used to inoculate 20 ml of a fresh culture which was incubated at 37°C, to reach a cell density of 3.10^8 cells/ml. From this suspension 0.05 ml was spread on a LC medium agar plate, containing 100 μg/ml of mocimycin. This plate was screened for visible colonies after 18 h incubation at 37°C.

2.3. *[^{14}C]Amino acid incorporation in vivo*

Cells were grown in LC medium at 37°C to a cell density of 3.10^8 cells/ml. They were washed once with 0.12 M Tris–HCl, pH 8.0 and resuspended in this buffer at a concentration of 3.10^9 cells/ml. From this suspension 1.8 ml was added to 0.2 ml 0.02 M EDTA and incubated for 4 min at 37°C [13]. Samples of 10 μl were then diluted ten-fold in M9 medium containing casamino acids (0.01%), glucose (0.2%), [^{14}C]amino acid mixture (0.5 μCi/ml; 54 mCi/matom C) and mocimycin (dissolved in ethanol) as indicated.

The samples were incubated for 15 min at 37°C and assayed for incorporation of the ^{14}C-label into hot TCA precipitable material.

2.4. *Polyphenylalanine synthesis in vitro*

Polyphenylalanine synthesis in vitro was tested according to Lupker et al. [10] except that the TCA precipitate was heated for 15 min at 95°C instead of digested with alkali.

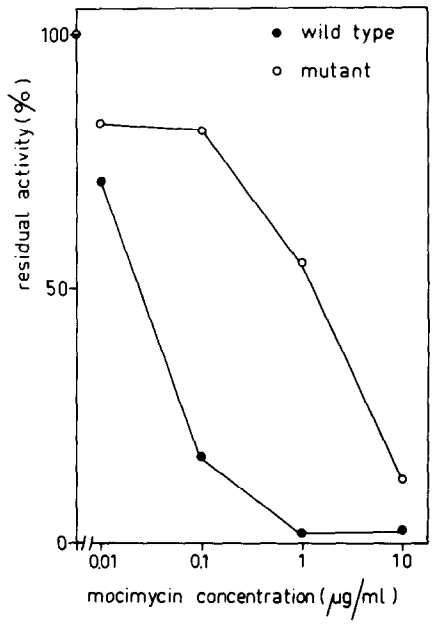

Fig.1. [^{14}C]Amino acid incorporation into hot TCA-precipitable material as a function of the mocimycin concentration, in cells made permeable by EDTA treatment (see Methods).

3. Results

3.1. *Isolation and biochemical characterization of the mutant*

Mocimycin resistant colonies were isolated (see Methods) and purified on plates containing mocimycin. Suspension cultures were grown of each of the purified strains and assayed for [^{14}C]amino acid incorporation in vivo.

In order to exclude in this test the possibility that the results are influenced by membrane mutations, cells were pretreated with EDTA as described by Leive [13] with some minor modifications.

One of the mutant strains (designated LBE 2012) thus showing a decreased sensitivity to mocimycin was chosen for further analysis. Figure 1 illustrates that the antibiotic concentration required to cause a 50% inhibition of amino acid incorporation is about 60 times higher in the mutant, than it is in the parental strain.

That reduced cell-wall permeability is not responsible for the decreased in vivo effect of mocimycin is corroborated by the effect of the antibiotic on in vitro polypeptide synthesis.

In experiments not illustrated here, MS_2-RNA and poly(U)-directed polypeptide synthesis was examined in S30 extracts and in cell-free systems consisting of a S100 fraction from either mutant or wild type cells supplemented with ribosomes from *E. coli* MRE 600. Here again a very similar response towards the antibiotic was recorded.

The next step was the deomstration that the lowered inhibition of protein synthesis by mocimycin is to be ascribed to an alteration of the target protein of the antibiotic, EF-Tu.

Isolation, purification and crystallization of this protein follows the same pattern starting from either wild type or mutant cells. Both immunologically and electrophoretically (on SDS–polyacrylamide gels) mutant and wild type EF-Tu behave identically.

Figure 2 shows the difference in mocimycin sensitivity between the two protein factors when studied in a poly(U)-directed polyphenylalanine synthesizing system. The difference amounts to a factor of about 70 when the antibiotic concentrations causing 50% inhibition are compared. Since mocimycin interferes

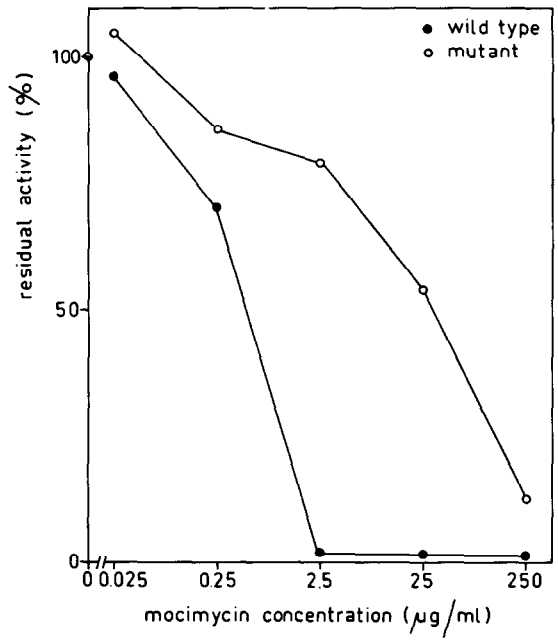

Fig.2. In vitro [^{14}C]polyphenylalanine synthesis as a function of the mocimycin concentration. Reaction mixtures of 100 μl contain: pure EF-Tu, mutant or wild type, 50 pmol; 1 M NH_4Cl-washed ribosomes, 20 pmol; poly(U), 0.2 μg; [^{14}C]phe-tRNA, 27 pmol; GTP, 0.12 mM; phosphoenolpyruvate, 5 mM; pyruvate kinase, 1 μg; Tris–HCl, pH 7.8, 50 mM; magnesium acetate, 8 mM; ammonium chloride 80 mM, mocimycin (dissolved in 5 μl ethanol) as indicated. After 10 min at 37°C the samples were assayed for incorporation of radioactivity into hot TCA-precipitable material.

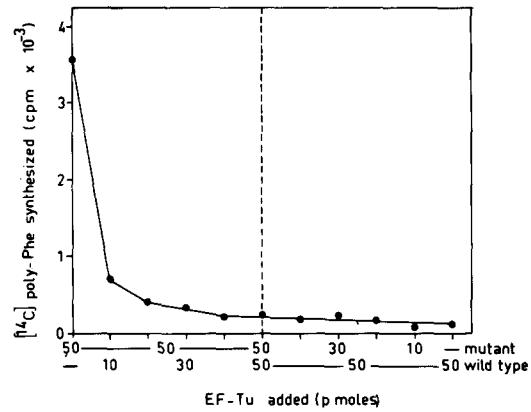

Fig.3. Dominance of mocimycin sensitivity over resistance. Poly-[^{14}C] phenylalanine synthesis was examined as is indicated in the legend to fig.2, except that the mocimycin concentration was kept constant (2.5 μg/ml), whereas the EF-Tu concentration varied. In the left-hand panel 50 pmol of mutant EF-Tu was supplemented with increasing amounts of wild type EF-Tu. In the right-hand panel the wild type EF-Tu was present in a constant concentration and the mutant EF-Tu varied as is indicated.

with the elongation cycle by causing a retention of EF-Tu on the ribosome [8], a block of protein synthesis may be expected in the presence of the antibiotic when sensitive EF-Tu is added to a system containing resistant EF-Tu.

This expectation is borne out by the following experiment:

Polyphenylalanine synthesis was studied at a mocimycin concentration of 2.5 μg/ml, sufficient to block the wild type system completely, but permitting at least 80% polypeptide synthesis in the mutant system (cf. fig.2). Increasing amounts of sensitive EF-Tu were added to the system containing a fixed amount (50 pmol) of mutant EF-Tu.

In a complementary experiment the wild type EF-Tu concentration was fixed and that of the mutant EF-Tu varied.

The results of both series of experiments are combined in one curve (fig.3). They show clearly that mocimycin sensitivity dominates mocimycin resistance as was to be expected considering the mode of action of the antibiotic.

3.2. *Genetic characterization*

Jaskunas et al. [14] reported that two genes code for EF-Tu, one (*tuf*A) at 72′ the other (*tuf*B) at 88′.

Cotransduction of mocimycin resistance with auxotrophic markers, located close to either *tuf*A or *tuf*B was attempted without success. Direct selection of mocimycin resistance after P1 transduction from the mutant to a number of sensitive strains, including the parental strain, was equally unsuccessful.

This implies that resistance is caused by an alteration in at least two genes. To establish the position of these two genes the mutant was made sensitive again by P1-transductions from sensitive strains. In one case the donor strain (LBE 1310) was steptomycin

Table 2
Frequency of cotransduction

EF-Tu gene	Selected marker	Position[a]	Frequency of co-transduction[b]
*tuf*A	*rps*L (= *str*A)	72′	0.89
*tuf*B	*met*A	89′	0.36

[a] Position on the recalibrated linkage map of *E. coli* K12 [9]
[b] Number of colonies tested is 200 in both cases – for details see text

resistant, and 178 out of 200 streptomycin resistant transductants examined appeared to be mocimycin sensitive.

In the other case the *met*A marker was introduced in the mutant via P1 transduction, using LBE 1431 as a donor.

Transductants were isolated after two rounds of penicillin treatment. One of the *met*A transductants, that had become mocimycin sensitive, was used as a recipient in the next P1 transduction in which LBE 2012 acted as the donor. Seventy two out of 200 colonies, selected on medium lacking methionine, appeared to be mocimycin resistant again (table 2).

4. Discussion

Theoretically a number of mechanisms may be envisioned that causes mocimycin resistance. Besides mutations affecting the permeability of the cell membrane for the antibiotic or the detoxification of the compound, two types of mutations are of great interest since they may shed new light on the mechanism of protein synthesis. The first is a mutation which has altered the target protein EF-Tu to such an extent, that the interaction with the antibiotic is impaired. The second type is characterized by alterations in reaction partners of the target protein, e.g., ribosomal proteins, such that the action of the antibiotic is counteracted. The experiments reported in this paper show, that in this case, EF-Tu itself is affected. The discovery by Jaskunas et al. [14] of two genes coding for EF-Tu raises the question whether one or both genes are mutated. Since our in vitro experiments with pure EF-Tu show that mocimycin sensitivity dominates resistance, a double mutation affecting both genes is to be expected.

This has been confirmed by our genetic experiments. They still leave open the question, however, whether both genes are coding for an active resistant EF-Tu or that possibly one gene is completely silenced. The data presented here confirm the occurrence in the intact *E. coli* chromosome of two genes coding for EF-Tu as was previously established by Jaskunas et al. [14] on the basis of transcription studies with transducing phages.

It is to be expected that the mutant described above will be of considerable value in answering questions concerning the functioning and the regulation of expression of the two EF-Tu genes, the distributions of the two gene products in the bacterial cell [17–19], the role of EF-Tu in Qβ-RNA replication [20–22] and other biosynthetic processes [23].

Recently we learned that Drs E. Fisher and A. Parmeggiani have isolated a kirromycin mutant of *E. coli*, which genetically and biochemically resembles our mutant.

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