Increased translational fidelity caused by the antibiotic kasugamycin and ribosomal ambiguity in mutants harbouring the *ksgA* gene

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The aminoglycoside kasugamycin, which has previously been shown to inhibit initiation of protein biosynthesis in vitro, also affects translational accuracy in vitro. This is deduced from the observation that the drug decreases the incorporation of histidine relative to alanine into the coat protein of phage MS2, the gene of which is devoid of histidine codons. The read-through of the MS2 coat cistron, due to frameshifts in vitro, is also suppressed by the antibiotic. In contrast, streptomycin enhances histidine incorporation and read-through in this system. The effects of kasugamycin take place at concentrations that do not inhibit coat protein biosynthesis. Kasugamycin-resistant mutants *(ksgA)* lacking dimethylation of two adjacent adenosines in 16 S ribosomal RNA, show an increased leakiness of nonsense and frameshift mutants (in the absence of antibiotic). They are therefore phenotypically similar to previously described ribosomal ambiguity mutants *(ram)*.

Kasugamycin *Translational fidelity* ksgA

1. INTRODUCTION

The antibiotic kasugamycin does not, like many other aminoglycosides, cause mistranslation in cell-free systems of *Escherichia coli* [1]. Instead it has been shown to inhibit initiation of protein biosynthesis in vitro [2] without affecting elongation on natural mRNA [3,4]. Sensitivity is a property of the 30 S ribosomal particle and resistance is caused by lack of dimethylation of two adjacent adenosines near the 3′-end of 16 S RNA [5,6]. This is due to a mutation in the *ksgA* gene coding for a methyltransferase that is specific for this site in ribosomal RNA [6]. Since the importance of the 3′-terminus of 16 S RNA in initiation of protein biosynthesis is generally acknowledged [7], the selective effect of kasugamycin on initiation lends further support to the putative function of this ribosomal site.

We re-examined the effect of kasugamycin on coding fidelity in vitro. For this we used a very sensitive system, i.e., the incorporation of histidine into the coat protein of phage MS2, the gene of which does not contain in-phase histidine codons [8]. Our results show that kasugamycin increases accuracy of translation in vitro, an effect opposite to that caused by streptomycin.

We find that *ksgA* mutants display an increased leakiness of certain nonsense and frameshift mutations, and therefore phenotypically resemble ribosomal ambiguity mutants [9–11].

2. MATERIALS AND METHODS

2.1. *Bacterial strains*

E. coli Q13 and its kasugamycin-resistant derivative (compare below) were used as a source of extracts for in vitro protein synthesis. *E. coli* strains carrying nonsense mutations in the I-gene part of F′ lacIZ fusions, originating from [12] were obtained through the courtesy of D. Andersson [13]. These strains are further described

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in table 2. The *E. coli* strain no.37 containing Newton's frameshift mutation ICR38 in lacZ [14], was a gift from J. Gallant [15]. Spontaneous mutants resistant to at least 250 μg/ml kasugamycin (obtained at a frequency of 10^{-6}) or to 100 μg/ml streptomycin were isolated from agar plates. The kasugamycin-resistant strains were tested for the *ksgA* genotype by determining the absence of a functional methyltransferase in cell-free extracts [16].

2.2. *Antibiotics*

Kasugamycin 1/2 H_2SO_4 (M_r 428.4) was purchased from Boehringer, Mannheim. Streptomycin was from Rhone-Poulenc.

2.3. *In vitro protein synthesis*

Extracts (S30) were prepared from *E. coli* either by grinding with Alcoa or by sonication. Cell debris was removed by centrifugation at 30000 × *g*. Assays for in vitro protein synthesis contained in a volume of 50 μl: 40 mM Tris–HCl (pH 7.5), 60 mM NH_4Cl, 9 mM $MgAc_2$, 6 mM 2-mercaptoethanol, 1 mM ATP, 0.12 mM GTP, 5 mM phosphoenolpyruvate, 0.1 μg pyruvate kinase, 20 μg uncharged tRNA, 0.3 A_{260} units of dialyzed S30 extract, 8 μg MS2 RNA, 4×10^{-3} μmol each of all amino acids minus alanine and histidine, 0.27×10^{-3} μmol L-[^{3}H]histidine (8222 dpm/pmol) and 1.10×10^{-3} μmol L-[^{14}C]alanine (168 dpm/pmol). The concentration of antibiotics, which were always added at zero time, is given in tables, figures and legends.

After incubation (25 min at 37°C) the proteins were precipitated with 10% trichloroacetic acid, washed 3 times with 10% trichloroacetic acid and twice with 96% alcohol, and electrophoresed on 15% polyacrylamide gels [17]. In vitro labelled [^{14}C]MS2 coat protein was included in one slot as a marker. The gels were autoradiographed [18], the bands containing the coat protein product were cut from the gels and burned in a Packard Oxidizer to separate ^{3}H and ^{14}C isotopes.

2.4. *β-Galactosidase assay*

All bacteria used except strain no.37 (ICR38) synthesize β-galactosidase constitutively. Cells were cultured in 25 ml minimal medium containing 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in the case of strain no.37. After harvesting and washing the cells were disrupted by sonication in 1 ml buffer (10 mM Tris–HCl (pH 7.5), 60 mM NH_4Cl, 10 mM $MgAc_2$, 6 mM 2-mercaptoethanol). Cells from 25 ml of log-phase cultures were collected by centrifugation, resuspended in 1 ml buffer and sonicated.

Debris was removed and 200 μl of the supernatant was assayed by measuring the hydrolysis of *O*-nitrophenyl-β-D-galactoside (ONPG) [19]. Protein was determined by the Lowry method. Activities were expressed as ΔA_{420}/h per μg protein (cf. table 2).

3. RESULTS

3.1. *In vitro experiments*

A mixture of [^{3}H]histidine and [^{14}C]alanine, an amino acid that appears 14 times in the coat protein of MS2 [8], was added to a MS2 RNA directed cell-free system. The proteins were separated by gel electrophoresis to detect the coat protein product by autoradiography. The coat protein band was then cut from the gel and the slices burned in an oxidizer that separates the $^{14}CO_2$ and [^{3}H]H_2O. By using proper controls the molar incorporation of both amino acids was determined. Table 1 shows that in the absence of drugs roughly one histidine residue is incorporated into the coat protein for every 70–85 alanine residues. With extracts from wild-type *E. coli*, kasugamycin reduces the misincorporation of histidine by a factor of two, while streptomycin enhances the incorporation of the 'wrong' amino acid 2–3-fold. Kasugamycin obliterates the miscoding effect of streptomycin in this system (table 1). With an extract from a kasugamycin-resistant mutant (*ksgA*) of *E. coli*, kasugamycin has no effect on the relative incorporation of histidine, while the effect of streptomycin is the same as in the wild-type extract.

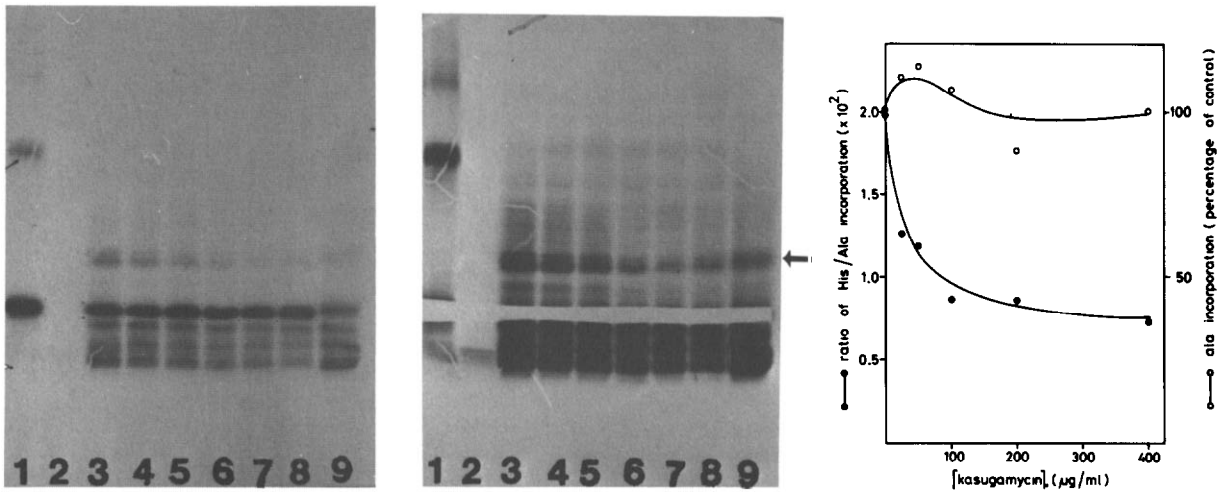
It is important to note that the effect of kasugamycin on the fidelity of in vitro translation is recorded at drug concentrations that hardly influence the amount of synthesized coat protein. This is most clearly shown in fig.1. The incorporation of alanine into coat protein is not affected by kasugamycin concentrations up to 400 μg/ml in this system, but the level of histidine incorporation is reduced 3-fold (compare section 4). The MS2 RNA-directed system allows the detection of an additional effect of streptomycin and kasugamycin

Table 1

Effect of antibiotics on the error frequency in in vitro synthesized MS2 coat protein

Exp.	Genotype	Antibiotic (μg/ml)	Histidine/alanine
1	wild-type	–	1.18×10^{-2} (1/85)
		kasugamycin (200)	0.63×10^{-2} (1/159)
		streptomycin (0.08)	3.70×10^{-2} (1/27)
2	wild-type	–	1.41×10^{-2} (1/71)
		kasugamycin (200)	0.67×10^{-2} (1/149)
		streptomycin (0.08)	2.57×10^{-2} (1/39)
		kasugamycin (200) and streptomycin (0.08)	1.45×10^{-2} (1/69)
3	*ksgA* mutant	–	1.23×10^{-2} (1/81)
		kasugamycin (200)	1.19×10^{-2} (1/84)
		streptomycin (0.08)	3.42×10^{-2} (1/31)

The coat protein bands were cut from gels such as shown in fig.1. The gel slices were burned in an oxidizer (Packard) separating ^{3}H and ^{14}C isotopes. Radioactivities were converted to pmol amino acids and the table shows the molar ratios between incorporated histidine and alanine. The alanine incorporation in the controls (– antibiotics) corresponds to 40.3, 49.1 and 36.3 pmol, respectively. Overall inhibition of protein synthesis in the sensitive system was approx. 35% with kasugamycin and 50% with streptomycin



A B C

Fig.1. Differential effect of kasugamycin on coat protein synthesis and on histidine incorporation. Standard mixtures containing wild-type *E. coli* extracts were incubated with increasing amounts of kasugamycin. (A) Autoradiogram of the products; lane 1: marker ^{14}C-labelled coat protein; lane 2: incubation mixture lacking MS2 RNA; lanes 3–8: containing 0, 25, 50, 100, 200 and 400 μg/ml kasugamycin, respectively; lane 9: containing 0.08 μg/ml streptomycin. (B) Same gel as in (A) after removal of the band of coat protein and prolonged exposure. Arrow indicates read-through proteins. (C) Dependence of alanine incorporation and of histidine/alanine ratio in coat protein product on kasugamycin concentration. 100% alanine incorporation corresponds to 28 pmol.

Table 2

Leakiness of nonsense and frameshift mutants[a]

Designation[b]	*Lac*-genotype	Position of nonsense	'Context'[a]	(β-gal. activity in resistant strain)[c]/ (β-gal. activity in sensitive strain)
F′ UD364 Ksg resistant	F′ *lacIZ* fusion	189	CCA*UAA*AGU	1.3
F′ UD365 Ksg resistant	F′ *lacIZ* fusion	189	CCA*UAG*AGU	1.3
F′ UD366 Ksg resistant	F′ *lacIZ* fusion	189	CCA*UGA*AGU	1.5
F′ UD367 Ksg resistant	F′ *lacIZ* fusion	220	GAC*UAA*AGU	1.2
F′ UD368 Ksg resistant	F′ *lacIZ* fusion	220	GAC*UAG*AGU	1.9
F′ UD368 Str resistant	F′ *lacIZ* fusion	220	GAC*UAG*AGU	0.1
F′ UD471 Ksg resistant	F′ *lacIZ* fusion	220	GAC*UGA*AGU	1.2
no.37 Ksg resistant	*lacZ* ICR38			1.8
no.37 Str resistant	*lacZ* ICR38			0.68

[a] These mutants have been described in detail [12–15,25]

[b] Ksg: kasugamycin; Str: streptomycin

[c] β-gal. activity varied from 1.53×10^{-4} A_{420}/h per μg protein (UD368) to 1.15×10^{-3} A_{420}/h per μg protein (no.37)

related to fidelity. This is illustrated in fig.1B. Two bands in the autoradiogram (arrow in fig.1B) originate from 'read-through' products caused by ribosomal frameshifts during translation of the coat protein cistron [20]. Streptomycin (lane 9, fig.1B and [21]) increases the relative amounts of these two proteins, while kasugamycin strongly reduces these products (lanes 3–8, fig.1). Hence, kasugamycin diminishes ribosomal frameshifts, at least in vitro.

3.2. *Leakiness of nonsense and frameshift mutants in* ksgA *strains*

Streptomycin-resistant mutants (*strA*) show an increased fidelity in vivo while *ram* mutants are less stringent in translation than wild-type cells [22]. Ribosomal stringency and ambiguity in vivo can be determined by the expression of nonsense and frameshift mutations in a suppressor-minus genetic background [13,22–24]. We made use of well-characterized nonsense mutants in *lacIZ* gene fusions [12,13,25] and of a frameshift mutant in *lacZ* [14,15]. Kasugamycin- and streptomycin-resistant mutants of these strains were selected after spontaneous mutation. The *ksgA* genotype was established by determining the lack of the specific methyltransferase and the presence of ribosomes lacking dimethylation of 16 S RNA [16]. The β-galactosidase activities in the *ksgA* mutants were compared to those of the isogenic *ksgA*$^+$ type (table 2). The *lacIZ* nonsense mutants and the *lacZ* frameshift mutant became more leaky in the *ksgA* background. The increase varied from 1.2 to 1.9, depending on the nature of the mutation and on the 'context'. In comparable studies on the effect of *ram* (*rpsD* coding for ribosomal protein S4) using the same *lacIZ* fusion mutants, the increase in leakiness varied 2- to 50-fold [13]. Other *ram* mutants were found to 'suppress' nonsense in the ornithine transcarbamoylase gene by a factor of 2–6 [24].

The effect of mutation to streptomycin resistance (table 2) is as expected and in good agreement with previous work with these nonsense and frameshift mutants [23,25]. Since reinitiation of protein synthesis can take place subsequent to termination in the *lacI* gene [26] and in the *lacZ* gene [27] it was important to rule this out as the cause of leakiness. By determining the size of the β-galactosidase made in vivo in one of the *ksgA* mutants we could exclude reinitiation as a cause of leakiness (not shown).

Unlike with *strA* and *ram* mutants [9] we have been unable to detect ribosomal ambiguity of *ksgA* ribosomes in vitro (compare table 1, exp.3). Probably this system is not sensitive enough to detect a difference with wild-type ribosomes.

4. DISCUSSION

Our results show clearly that the antibiotic kasugamycin, in addition to its inhibitory effect on initiation of protein biosynthesis [2,29], increases the accuracy of translation in vitro. Both the misincorporation of histidine in MS2 coat protein and the read-through of the coat protein gene are counteracted by the antibiotic. These activities are exerted by the antibiotic at concentrations lower than those giving inhibition of synthesis of coat protein.

The lack of inhibition of coat protein synthesis in this system needs some explanation because previously rather strong inhibitions of phage RNA-directed protein synthesis at low concentrations ($<100\,\mu$g/ml) of kasugamycin have been reported [5,28]. We never attained these high levels of inhibition in this system (maximally 50% inhibition at $400\,\mu$g kasugamycin/ml, compare [29]). The studies reported by authors in [3] may afford an explanation. They found that kasugamycin inhibits initiation at the A protein cistron of phage f2 RNA more than it does at the coat protein cistron. In the above-mentioned studies [5,28] the intactness of the phage RNA and the nature of the products were not tested. However, with unfolded or degraded RNA the ribosomes select the A protein cistron for translation [30] while with intact RNA the predominant product is the coat protein [31] (cf. also fig.1). We believe, therefore, that inhibition of initiation at the coat protein cistron by kasugamycin, as recorded at high concentrations in a purified system [29], does not occur under the conditions used here.

Kasugamycin is an antibiotic widely used in Japan to control rice blast disease caused by the fungus *Piricularia oryzae* [32]. It is interesting that an increase in translational fidelity inhibits sporulation in another fungus, *Podospora anserina* [33]. It has been suggested that translational ambiguity may play a role during differentiation of other eukaryotic cells [34]. Our findings in *E. coli*, an organism that is relatively insensitive to the antibiotic (unpublished), suggest that the accuracy of translation is the target for antibiotic kasugamycin.

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