Kasugamycin Resistant Mutants of *Bacillus stearothermophilus* Lacking the Enzyme for the Methylation of Two Adjacent Adenosines in 16S Ribosomal RNA

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**Summary.** Several mutants of *B. stearothermophilus* have been isolated that are resistant to the antibiotic kasugamycin. One of these is shown to lack dimethylation of two adjacent adenosines in the 16S ribosomal RNA. All mutants that were analyzed biochemically lack the enzyme that is able to methylate this site. Ribosomal sensitivity and resistance to kasugamycin in *B. stearothermophilus* is therefore, like in *E. coli*, closely connected with dimethylation of the adenosines.

**Introduction**

In *Escherichia coli* dimethylation of two adjacent adenosines (m6Am6A) near the 3' end of 16S ribosomal RNA determines ribosomal sensitivity to the antibiotic kasugamycin (Helser et al. 1971), an inhibitor of polypeptide chain initiation (Gale et al. 1981). Resistance is conferred by a mutation in the *ksgA* gene, coding for a methyl-transferase that specifically methylates these adenosines using SAM as methyl donor (Helser et al. 1972). This has enabled us to undertake a study of the functional and structural implications of this modification. The methylated A's are present in a hairpin loop (Van Charldorp et al. 1981). Functionally they appear to play a role in initiation of protein synthesis (Poldermans et al. 1979a) and in subunit interaction (Poldermans et al. 1980) and structurally they destabilize the hairpin stem (Van Charldorp et al. 1981b).

The characteristic sequence m6Am6A is present in every small ribosomal subunit RNA (Van Charldorp and Van Knippenberg 1982) except in yeast mitochondria (Klootwijk et al. 1975; and personal communication N. Martin, University of Texas, U.S.A.). To further our understanding of this part of the ribosome we are studying the 3' end of the 16S RNA of *Bacillus stearothermophilus* (Van Charldorp et al. 1981c). Here we describe the isolation of KsgA mutants of this organism that lack the specific methylase for these adenosines.

**Materials and Methods**

**Materials.** The following were commercial materials: kasugamycin sulphate (Boehringer Mannheim, FRG); S-adenosyl-l-methionine (Sigma, St. Louis, USA); [3H-methyl]-S-adenosyl-l-methionine (15 Ci/mmol; Amersham, England); [p-32P]-ATP (New England Nuclear, USA); and T4-polynucleotide kinase (Boehringer Mannheim). Wild-type *Bacillus stearothermophilus* strain NCA1503 was obtained from the PHLS Centre of Applied Microbiology and Research (England). The bacteriocin cloacin DF13 was a gift of Dr. F.K. De Graaf (Free University, Amsterdam, The Netherlands).

**Bacterial Growth and Isolation of Mutants.** Bacterial growth in liquid culture as well as on agar plates was at 60°C. Ksg-resistant mutants were isolated as follows. *B. stearothermophilus* NCA1503 was incubated in LC medium (per l: 10 g bacto-trypton, 5 g yeast extract, 8 g NaCl, 0.12 g Tris, 2 g MgSO4, 7 H2O and 0.025 g thymine; pH 7.2). Subsequently 0.1 ml samples of the exponentially growing culture (5 x 10⁸ cells/ml) were spread on LC agar (1.7 Difco agar) containing 0.25, 0.5, 1.0, 1.5, 2.0, and 2.5 mg/ml ksg and left overnight in a stove. The concentration of 1.5 mg/ml appeared appropriate to allow growth of resistant mutants and to suppress the sensitive wild-type on the selection plates. Visible colonies from these plates were purified by streaking three times in succession for single colonies on agar supplemented with 1.5 mg/ml of the drug. The minimal inhibitory concentrations (MIC) were determined by spreading appropriate dilutions of each culture on a series of LC agar plates containing increasing amounts of ksg, so that the lowest concentration preventing visible growth of about 500 single colonies on a plate after one night incubation was within the concentration range.

Large scale preparations of *B. stearothermophilus* NCA1503 and its KsgA descendants were performed under aeration in the same medium as above. Bacteria were harvested in the mid-exponential phase (about 7 x 10⁸ cells/ml), washed with buffer (10 mM Tris-HCl pH 7.5, 20 mM Mg(OAc)₂, 60 mM NH₄Cl, 6 mM 2-mercaptoethanol) and stored frozen.

**Preparation of S30 Extracts, Ribosomes and Cloacin Fragments.** Cells were lysed by sonicaton. Cell-debris was removed by centrifugation at 30,000 x g yielding an S30 extract. Ribosomes were prepared from S30 extracts by ultracentrifugation. 30S subunits were isolated by sucrose-gradient centrifugation after dissociation of 1 M NH₄Cl washed ribosomes at 1 M MgCl₂.

Cleavage of the 16S RNA in situ was achieved by treatment of 70S ribosomes with the bacteriocin cloacin DF13.
The ensuing 3' fragment of 52 nucleotides, called the 'cloacin' fragment, was isolated according to Baan et al. (1976).

**In vitro Methylation of 30S Subunits.** The methylation reaction was carried out in a volume of 0.05 ml containing 100 mM Tris-Cl pH 7.8, 5 mM Mg(OAc)$_2$, 60 mM NH$_4$Cl, 6 mM 2-mercaptoethanol, 0.15 mM of a mixture of [H-methyl]-SAM and unlabeled SAM (2064 dpm/ pmol), 0.1 A$_{260}$ units 30S ribosomes and 0.05 units of methylase (as defined by Poldermans et al. (1979b); the purification of this enzyme from *B. stearothermophilus* will be described elsewhere). When screening for the presence of the methyltransferase in a strain, 0.2 A$_{260}$ units of an S30 extract were used instead. After incubation at 60°C 1 ml 10% trichloroacetic acid was added to the mixture and the radioactivity of the precipitate was determined using nitrocellulose filters and a liquid scintillation counter. The percentage methylation was calculated from the manufacturer's data on the specific activity of [H-methyl]-SAM and with the assumption that 1 A$_{260}$ unit 30S ribosomes equals 70 pmol particles.

**Detection of Adenosines in the Cloacin Fragments.** To localize (unmodified) A residues in the cloacin fragment, use was made of the chemical sequence method developed by Peattie (1979). Compare Results for further details. Labelling of the cloacin fragments with $^{32}$P at their 5' ends and gel electrophoresis were performed as outlined by Van Charlardorp et al. (1981a).

**Results**

*B. stearothermophilus* strain NCA1503 is sensitive to kasugamycin with an MIC value (compare Materials and Methods) of 0.5-0.6 mg/ml. Spontaneous mutants resistant to the drug and having MIC values of 2.9-3.0 mg/ml were obtained with a frequency of $1 \times 10^{-5}$.

Ten mutants (designated PK101-PK110) were chosen for further study. These strains were grown in liquid culture. The bacteria were lysed by sonication and an S30 extract was prepared from each strain. From the extract of mutant PK101 the ribosomes were isolated and treated with the bacteriocin cloacin DF13. This bacteriocin cleaves the 16S RNA in situ at 52 nucleotides from the 3' end (Van Charlardorp et al. 1981c). The 'cloacin' fragment was isolated according to Baan et al. (1976) in order to elucidate the presence or absence of the methyl groups of the adenosines. As a control the corresponding fragment was also isolated from the wild-type strain. The fragments were labeled at their 5' ends with $^{32}$P using [$\gamma$-$^{32}$P]-ATP and polynucleotide kinase. Subsequently they were treated with diethylpyrocarbonate and cleaved as in the chemical sequence method of Peattie (1979). This treatment results in strand scission at purines, preferentially at adenosines, as is shown in Fig. 1. The known sequence of wild-type *B. stearothermophilus* is shown alongside the autoradiogram of a gel electrophoresis slab obtained with wild-type and mutant 'cloacin' fragments. Thick bands correspond to the position of A residues, except at positions 25 and 26 in the wild-type fragment. This is due to the dimethylation of the N$^6$ atoms of the adenosines (Van Charlardorp 1982). With the mutant fragment two prominent bands at these places indicate the presence of the unmethylated As here.

The results shown in Fig. 2 also demonstrate the absence of the methyl groups. 30S ribosomal subunits isolated from strain PK101 can be fully methylated by a purified methyl-transferase from wild-type *B. stearothermophilus* while the homologous wild-type 30S subunits do not function as a substrate.

To analyze for the presence of the methyl-transferase in the other mutants, their S30 extracts were incubated with

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**Fig. 1.** A Sequence of the cloacin fragment of *B. stearothermophilus* drawn in a secondary structure model (Van Charlardorp et al. 1981c). B Autoradiogram of (5'-32P) labeled wild-type (WT) and mutant (M) cloacin fragments after applying the A>G direct RNA sequence method of Peattie (1979). The numbers correspond to the positions of the A residues (compare A).