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DNase I Induced DNA Degradation is Inhibited by Neomycin

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Preparations of antimicrobials from biotechnological sources containing nucleic acids may serve as vector for the dissemination of resistance genes. An essential prerequisite for the acquisition of a new resistance phenotype in a transformational scenario is the availability of physically intact DNA molecules capable of transforming competent microorganisms. DNA is thought to be an easy target for catabolic processes when present in the natural habitat of bacteria (*e.g.* gastrointestinal tract, soil) due to the overall presence of nucleolytic enzymes. Aminoglycoside antibiotics are known to display a strong affinity to nucleic acids rendering these compounds to be primary candidates for exerting DNA protective functions in the gastrointestinal tract when applied orally during antibiotic chemotherapy. Using a DNase I protection assay it could be demonstrated that neomycin B at a concentration of 2 mM completely inhibited degradation of plasmid DNA *in vitro*. No inhibition of degradation was observed with streptomycin and kanamycin and the non-aminoglycoside antibiotics oxytetracycline and ampicillin under identical assay conditions. Thus, neomycin preparations may be able to promote structural integrity of contaminating DNA-fragments in DNase-rich environments.

Antibiotic preparations contaminated with nucleic acids from the producer strain organism may play a distinct role in horizontal gene transfer events as they may serve as a source for resistance genes[3,30,31]. One prerequisite of several others for an effective acquisition of a new trait in a transformational scenario would be the existence of physically intact DNA molecules capable of transforming a competent bacterial host in its natural habitat. Until recently it was a common doctrine that orally ingested DNA would be an easy target for degradation by nucleolytic enzymes and unfavourable ionic conditions of the mammalian gastrointestinal tract *in vivo*. Nevertheless evidence is accumulating that DNA is more resistant to degradation than previously thought[8,11,26]. DNA-fragments from ingested sources are not completely metabolized and can be amplified from feces of animal origin by PCR[7] and the survival of M13 phage DNA in the gastrointestinal tract, bloodstream and several tissues of mice has been demonstrated recently[18,19]. The detection of foreign DNA in these environments is transient. This observation raises the question whether there are factors which may be able to promote DNA survival in natural habitats. It could be shown that adsorption to clay minerals may protect nucleic acids against degradation by nucleases in soil[8]. Preparations of antimicrobial agents engulf DNA fragments tightly and therefore may serve as a physical barrier, but in the case of aminoglycoside antibiotics the active compounds themselves show a strong affinity to nucleic acids[4]. Direct interactions with biologically essential RNA-elements are well documented. Neomycin B, a 4,5 disubstituted 2-deoxystreptamin aminocyclitol, produced by *Streptomyces fradiae*, is known to be an effective inhibitor of bacterial protein synthesis[12] and group I intron splicing[27], interacts with the hammerhead ribozyme[20] and blocks binding of HIV Rev protein to the Rev-responsive element[32]. At high concentrations neomycin precipitates RNA[4]. Unspecific binding occurs with DNA inducing a transition of B- to A-DNA which renders the molecule once again very similar to RNA[16]. These observations led us to the question, whether neomycin is capable of exerting a protective function against nucleolytic degradation *via* direct interactions with the target DNA. An experimental

setup comparable to a DNase protection assay containing plasmid DNA, neomycin and bovine pancreatic deoxyribonuclease I (DNase I) allowed us to validate this hypothesis *in vitro*. Additionally several other aminoglycoside and non-aminoglycoside antibiotics were screened for DNA protective functions.

Material and Methods

Materials

Neomycin sulfate (85% neomycin B, remainder neomycin C), paromomycin sulfate, streptomycin sulfate, kanamycin A monosulfate, oxytetracycline-HCl, ampicillin sodium salt, Trisma base and EDTA were purchased from Sigma-Aldrich, Vienna, Austria. DNase I (bovine pancreas, grade II), bovine serum albumin (BSA) and DNA λ ladder III and VII were from Roche, Vienna, Austria. Acetic acid, $CaCl_2$ and $MgCl_2$ were from Merck, Darmstadt, Germany. The plasmid EGFP-C1 and *E. coli* host strain DH5α were supplied by Clontech, Palo Alto, USA. Agarose was from USB, Cleveland, Ohio; ethidium bromide from Oncor, Gaithersburg, MD, USA. Ion exchange columns were supplied by Qiagen, Hilden, Germany.

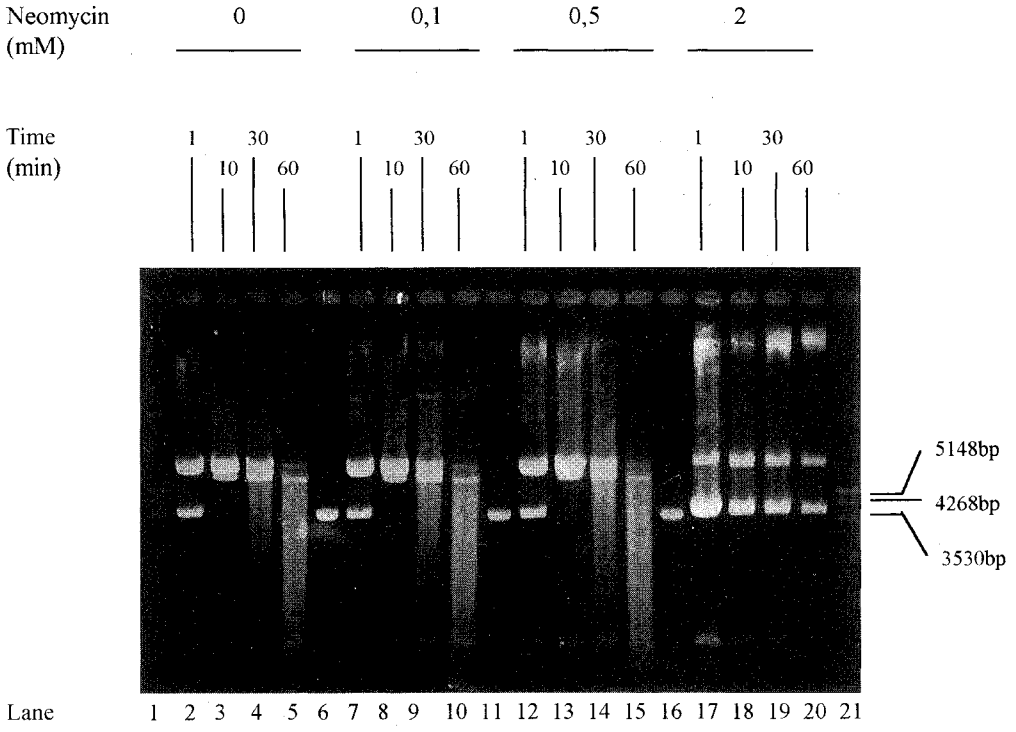
Plasmid Preparation

$CaCl_2$ competent *E. coli* DH5α was transformed with plasmid DNA as described[17]. DNA was isolated and purified using alkaline lysis and weak anion exchange chromatography from Qiagen according to the manufacturer's instructions[15].

DNase I Protection Assay

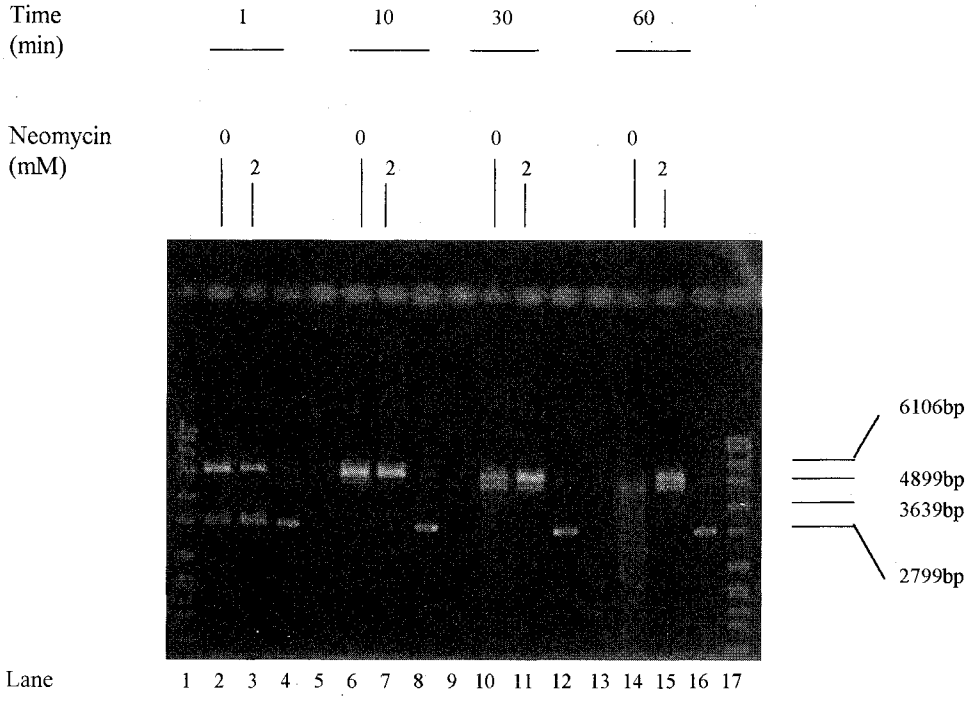
The standard assay buffer contained 50 mM Tris-HCl (pH 8), 10 mM $MgCl_2$ and 50 μg/ml BSA. 20 μg plasmid DNA was added and preincubated with various concentrations (*e.g.* 0.1 mM, 0.5 mM, 1 mM, 2 mM) of the respective antibiotic for 30 minutes on ice. The tubes were transfered to room temperature and 20 units of DNase I were added to all samples simultaneously. The reaction volume was a total

Fig. 1. Neomycin-DNase I protection assay.



DNA degradation appears after 30 minutes of incubation (lanes 4, 9, 14). Relaxation of the supercoiled (sc) plasmid conformation is displayed at neomycin concentrations up to 0.5 mM (lanes 3~5, 8~10, 13~15). Conservation of scDNA and degradation protection take place with 2 mM neomycin (lanes 17~20). DNA molecular weight marker: lanes 1 and 21; EGFP-plasmid DNA, untreated: lanes 6, 11, 16. Neomycin concentrations 0 mM: lanes 2~5; 0.1 mM: lanes 7~10; 0.5 mM: lanes 12~15; 2 mM: lanes 17~20. Incubation periods for each antibiotic concentration: 1 minute, 10 minutes, 30 minutes, 60 minutes.

Fig. 2. DNase I–neomycin incubation.



DNase I was incubated with 2 mM neomycin for 30 minutes at room temperature and then transfered into the DNase protection assay. Assays containing 2 mM neomycin show DNA degradation protection but no stabilisation of the supercoiled plasmid DNA conformation (lanes 7, 11, 15). In assays without neomycin DNA is degraded (lanes 10, 14).

Neomycin concentrations were: 0 mM (lanes 2, 6, 10, 14) and 2 mM (lanes 3, 7, 11, 15). Incubation periods: 1 minute (lanes 2~3); 10 minutes (lanes 6~7); 30 minutes (lanes 10~11); 60 minutes (lanes 14~15). DNA molecular weight marker: lanes 1, 17. EGFP-plasmid DNA, untreated: lanes 4, 8, 12, 16. Empty: lanes 5, 9, 13.

of 100 μl. To observe the progress of degradation 10 μl aliquots were taken from each sample after 1, 10, 30 and 60 minutes of incubation. The reaction was stopped with 5 μl 0.5 M EDTA on ice. Aliquots were analysed on an 0.8% agarose gel in 1×TAE buffer[17] stained with ethidium bromide after the gel run. Neomycin stock solutions (250 mM, 12.5 mM) were prepared in H_2O and stored at −20°C.

Results

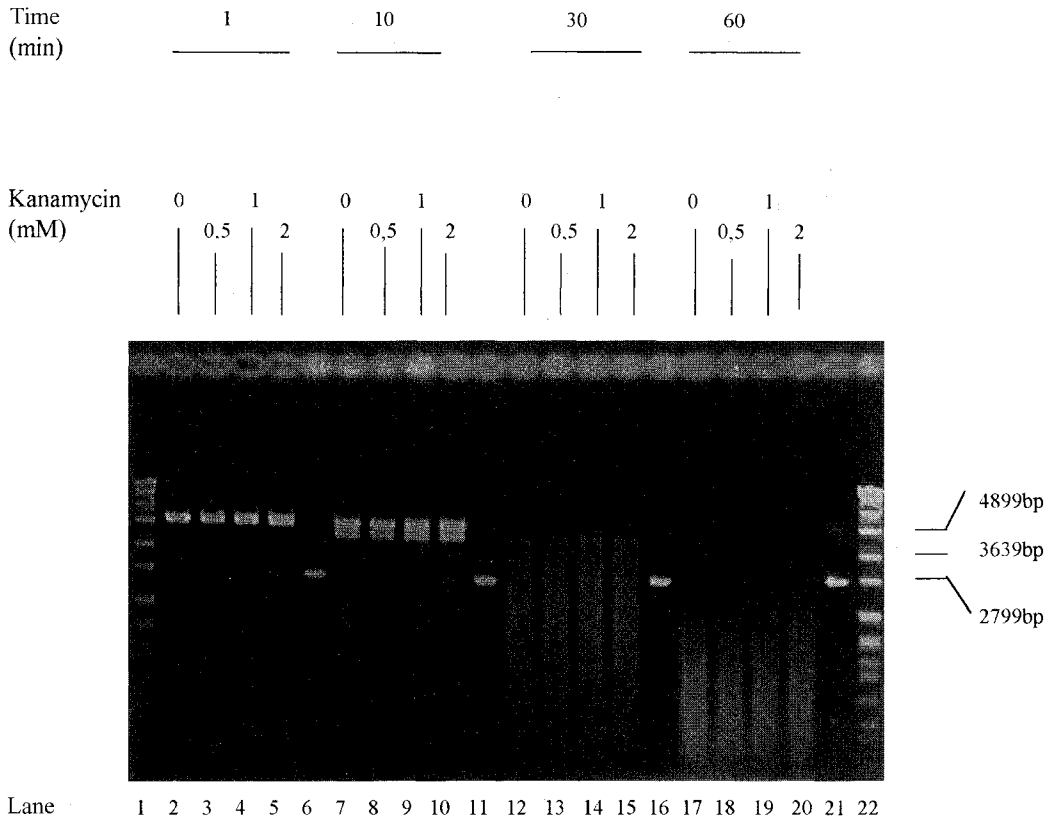
DNase Protection Assay

Incubation of the plasmid EGFP in DNase I assay buffer supplemented with 2 mM neomycin sulfate resulted in a complete degradation protection during the scanning period of up to 1 hour (Fig. 1). There was also no degradation detectable after 2 hours under the applied conditions (data not shown). Plasmid DNA incubated in neomycin concentrations ≥2 mM displayed a distinct bandshif the gel (Fig. 1). The effect was intensified by higher concentrations of the antibiotic (data not shown; compare Fig. 7).

Samples without neomycin showed a distinct alteration in the DNA banding pattern during prolonged incubation. The supercoiled form of the plasmid diminished after 10 minutes. An additional band appeared below the closed circular form of EGFP indicating a linearisation of the plasmid in the sample. After 30 minutes of incubation a degradation activity was displayed by the appearance of a DNA smear below the prominent two bands of the circular and linear form of EGFP. The high molecular weight target DNA was degraded completely after more than 60 minutes in samples lacking neomycin (Fig. 1).

Control lanes (EGFP without incubation) indicated a predomination of the supercoiled form of the plasmid in the assay as there were only faint DNA bands visible on the

Fig. 3. Kanamycin-DNase I protection assay.



There is no DNA degradation protection detectable (lanes 14, 15; 19, 20).
Kanamycin concentrations were: 0 mM (lanes 2, 7, 12, 17), 0.5 mM (lanes 3, 8, 13, 18), 1 mM (lanes 4, 9, 14, 19) and 2 mM (lanes 5, 10, 15, 20). Incubation periods: 1 minute (lanes 2~5); 10 minutes (lanes 7~10); 30 minutes (lanes 12~15); 60 minutes (lanes 17~20). DNA molecular weight marker: lanes 1, 22. EGFP-plasmid DNA, untreated: lanes 6, 11, 16, 21.

gel at the appropriate positions for the other plasmid conformations. This was in accordance with the DNA isolation and purification procedure which preferentially yielded supercoiled plasmid DNA species.

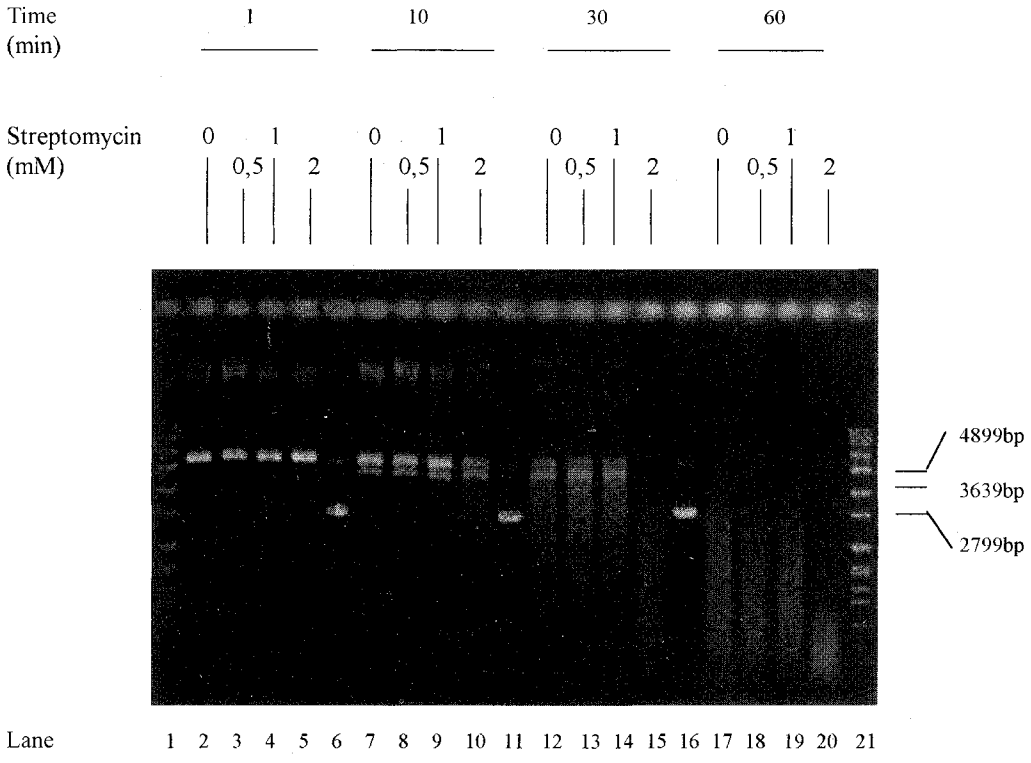
Direct Inhibition of DNase I

To test whether DNase I is directly inhibited by neomycin a DNase I stock solution was incubated with 2 mM neomycin for 30 minutes at room temperature. By transferring 2 μl of the treated DNase I stock solution the concentration of the aminoglycoside antibiotic was diluted 1 : 50 and thus, reached only levels far below of the putative inhibition threshold of 2 mM neomycin in the protection assay. Samples without neomycin preincubation were degraded by DNase I from the neomycin treated stock solution, whereas samples with neomycin preincubation once again showed a protective effect (Fig. 2).

A DNA protective effect and DNA bandshifts could not be detected under identical assay conditions with paromomycin (Fig. 7), kanamycin (Fig. 3), streptomycin (Fig. 4), oxytetracycline (Fig. 5) or ampicillin (Fig. 6).

Since paromomycin differs from neomycin only in a hydroxyl group instead of an amino moiety at C6′ this antibiotic was chosen for further investigation. A strong DNA degradation protection was detectable at 10 mM paromomycin. A transition from the supercoiled to the closed circular plasmid conformation was visible, but the circular conformation appeared to be strongly conserved over a wide range of antibiotic concentrations and for assay periods up to 6 hours (Fig. 7 and data not shown).

Fig. 4. Streptomycin-DNase I protection assay.



There is no DNA degradation protection detectable (lanes 14, 15; 19, 20). High streptomycin concentrations seem to accelerate DNA degradation (lanes 15, 20). Streptomycin concentrations were: 0 mM (lanes 2, 7, 12, 17), 0.5 mM (lanes 3, 8, 13, 18), 1 mM (lanes 4, 9, 14, 19) and 2 mM (lanes 5, 10, 15, 20). Incubation periods: 1 minute (lanes 2~5); 10 minutes (lanes 7~10); 30 minutes (lanes 12~15); 60 minutes (lanes 17~20). DNA molecular weight marker: lanes 1, 21. EGFP-plasmid DNA, untreated: lanes 6, 11, 16.

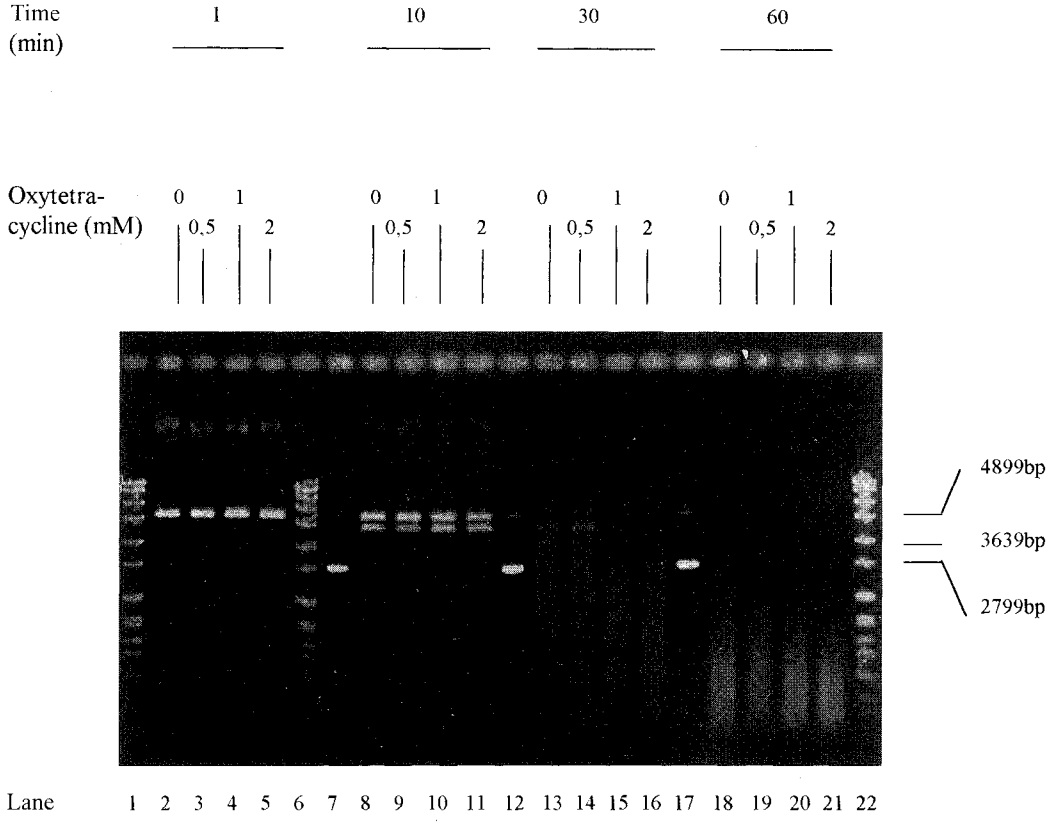
Discussion

Our results demonstrated inhibition of DNase I mediated DNA degradation by neomycin. This was in accordance with the previously reported observation that binding of neomycin to double-stranded (ds) DNA imposes a transition from B-DNA to the A-DNA conformation especially in GpG rich sequences[16]. This structural distortion leads to an inaccessibility of the minor groove, the target binding area of DNase I[24,29]. Neomycin binding to DNA additionally results in a global decrease of flexibility of the helix, once again a prerequisite for effective DNA cutting by DNase I[23]. Both findings may be the reason why enzyme activity on its substrate is abolished. Neomycin present in commercially available antibiotic preparations therefore will enhance stability of engulfed DNA fragments in a DNase I containing environment. There are indications that DNase I is not inhibited by direct interaction with neomycin. DNase I mediated DNA degradation is blocked most likely by neomycin molecules docking to target DNA, either inhibiting enzyme binding or cutting. This hypothesis is supported by the observation that all aminoglycoside compounds mediating DNA degradation protection at lower concentrations ($\leq$10 mM) induce distinct DNA bandshift patterns on the gel. No bandshift is detectable with antibiotics, which do not inhibit DNase I function in the assay.

Paromomycin essentially shows the same DNA protective effects but only at increased antibiotic concentrations. This observation may be due to a lower affinity to the phosphate backbone mediated by unfavourable electrostatic interactions induced by an additional hydroxyl moiety instead of a positivley charged amino group, which is present in neomycin.

Neomycin B, a secondary metabolite of *Streptomyces*

Fig. 5. Oxytetracycline-DNase I protection assay.



There is no DNA degradation protection detectable (lanes 15, 16; 20, 21). Oxytetracycline concentrations were: 0 mM (lanes 2, 8, 13, 18), 0.5 mM (lanes 3, 9, 14, 19), 1 mM (lanes 4, 10, 15, 20) and 2 mM (lanes 5, 11, 16, 21). Incubation periods: 1minute (lanes 2~5); 10 minutes (lanes 8~11); 30 minutes (lanes 13~16); 60 minutes (lanes 18~21). DNA molecular weight marker: lanes 1, 6, 22. EGFP-plasmid DNA, untreated: lanes 7, 12, 17.

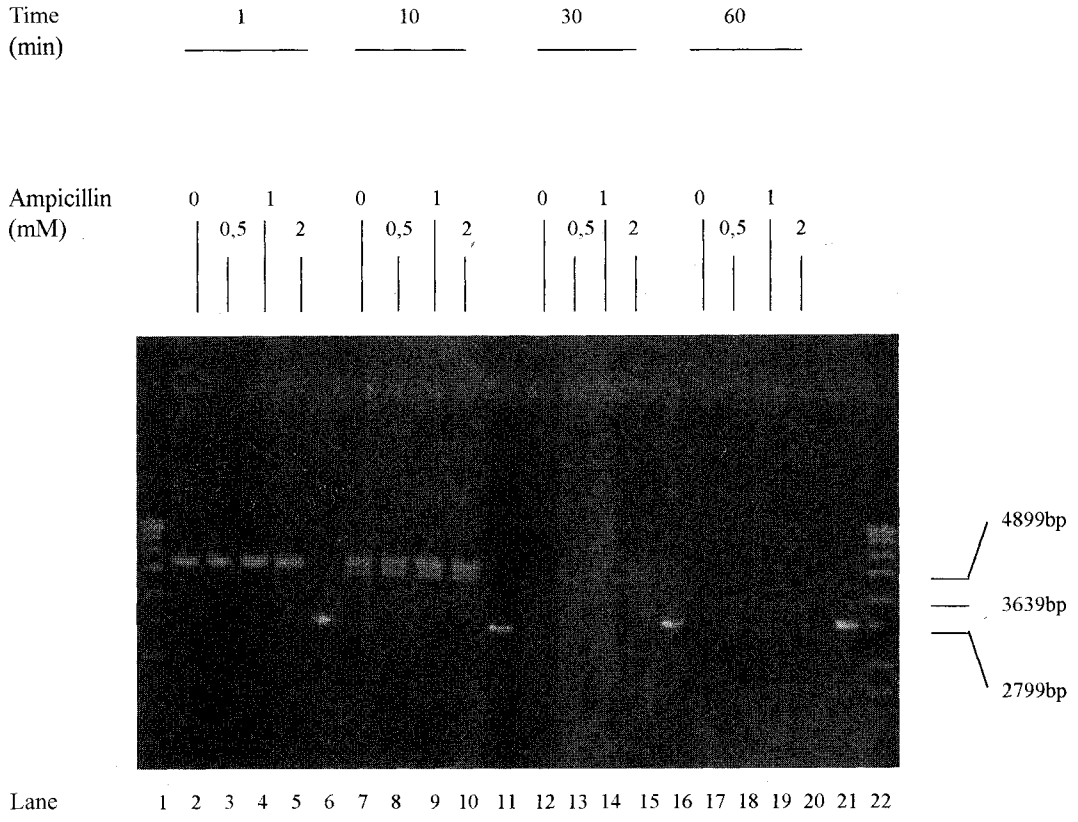
fradiae, provides six primary amino groups on a rigid molecular frame building a stereochemically well defined array of hydrogen bond donors and acceptors[6,16]. Under physiologic conditions neomycin is densely positively charged and hence prone to interact with the negatively charged phosphate backbone of nucleic acids[16,22].

DNase I recognizes sequence dependent variations in DNA-helix topology for cutting but does not act as sequence specific endonuclease[23]. For hydrolysis divalent cations are required. Mg^{2+} preferentially induces single strand nicks, whereas Mn^{2+} leads to double strand breaks. At a physiologic pH-optimum (7.5~8.5) the enzyme electrostatically interacts with phosphate moieties from both strands opposing each other in the minor groove and imposing a partial widening of the groove[23]. Thus, A- and Z-DNA are proposed to be resistant to DNase degradation[24].

Transformation of competent bacteria may be considered as the genuine way in evolution of horizontal gene transfer. Due to its simple nature gene uptake by transformation has to meet only two preconditions: the temporal and spatial presence of physically intact DNA molecules and bacteria ready for uptake and genomic integration[8].

Orally applied preparations of antimicrobial agents contaminated with DNA support both requirements as they physically protect nucleic acids, releasing their contents at locations with the highest probability of getting into contact with dense bacterial populations and interfere profoundly with the prokaryotic metabolism. Antibiotics at subinhibitory concentrations may stimulate DNA uptake[5]. Thus antibiotics from biotechnological sources may be one piece in the puzzle of reconstructing the story of the emergence and dissemination of resistance genes[3,30,31]. But until now it has been impossible to demonstrate a gene transfer event

Fig. 6. Ampicillin-DNase I protection assay.



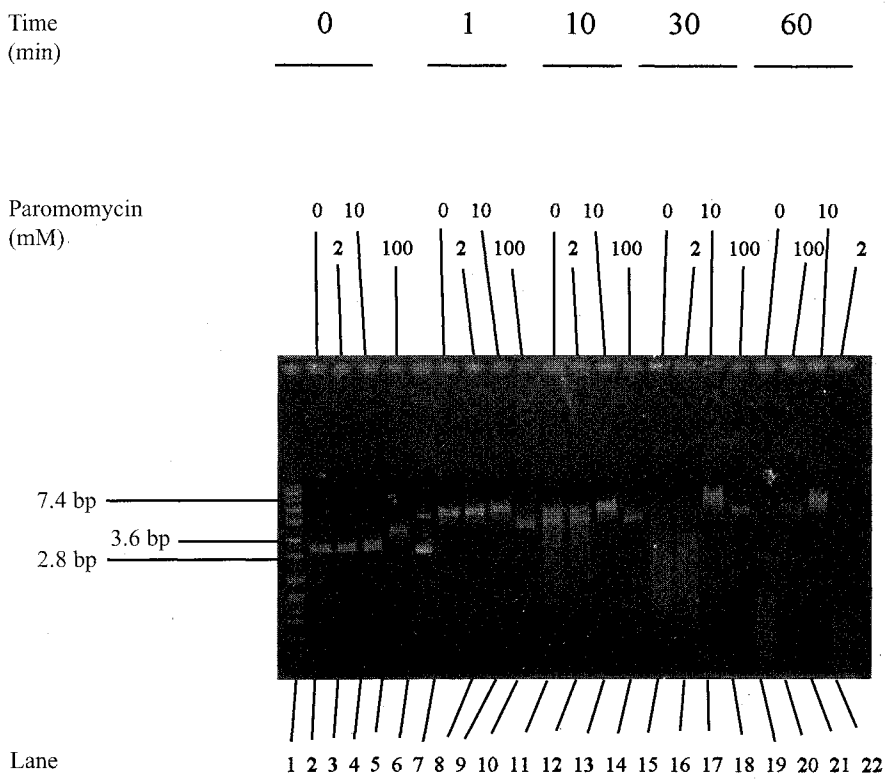
There is no DNA degradation protection detectable (lanes 14, 15; 19, 20). Ampicillin concentrations were: 0 mM (lanes 2, 7, 12, 17), 0.5 mM (lanes 3, 8, 13, 18), 1 mM (lanes 4, 9, 14, 19) and 2 mM (lanes 5, 10, 15, 20). Incubation periods: 1minute (lanes 2~5); 10 minutes (lanes 7~10); 30 minutes (lanes 12~15); 60 minutes (lanes 17~20). DNA molecular weight marker: lanes 1, 22. EGFP-plasmid DNA, untreated: lanes 6, 11, 16, 21.

based upon passing over a resistance determinant present in an antibiotic preparation. This is probably due the inability to identifiy the appropriate initial DNA acceptor strain in the human or animal ecosystem because the invading pathogen (isolated in the diagnostic laboratory and treated by the physician) which displays a resistant phenotype may not be the primary candidate for gene transfer mediated by contaminated antibiotics[5].

Nevertheless there are strong indications that this mechanism indeed plays a role. Already twentyfive years ago it was pointed out that several resistance genes isolated in nosocomial pathogens closely resembled those found active in the antibiotic producer strain[2,28]. Concomitant coevolution of a resistance gene in the antibiotic producer and a pathogenic microbe is rather uncommon. Horizontal transfer of the determinant from the producer organism is more likely. Whether the gene exchange took place by direct transformation of the pathogenic strain or *via* a combination of different gene transfer events (transformation, conjugation, transduction) including several genera remains unknown[5].

Microbial producers of antibiotics are not the only source for resistance genes. Soil bacteria, regardless whether they synthesize antimicrobials or not, constitute a large pool of known, cryptic or other yet uncharacterized resistance determinants[5]. On the other hand it could be demonstrated that "housekeeping" genes harboured in each bacterial cell and performing essential tasks in bacterial metabolism have a potential to modify intruding antibiotics and, thus, mediate resistance. In this respect aminoglycoside phosphotransferases show striking similarities in the mode of action and sequence homologies to bacterial protein kinases[5]. Aminoglycoside acetyltransferases found in actinomycetes, which do not produce any aminoglycosides,

Fig. 7. Paromomycin-DNase I protection assay.



DNA degradation protection is detectable at 10 mM paromomycin (lanes 13, 17, 21). A weak bandshift is visible with 2 mM (lanes 3, 8, 12) and 10 mM (lanes 4, 9, 13, 17, 21), a strong shift with 100 mM (supercoiled plasmid DNA; lanes 5, 10, 14, 18, 20). Paromomycin concentrations were: 0 mM (lanes 2, 7, 11, 15, 19); 2 mM (lanes 3, 8, 12, 16, 22), 10 mM (lanes 4, 9, 13, 17, 21), 100 mM (lanes 5, 10, 14, 18, 20). Incubation periods: before addition of DNase I (lanes 2~5); 1 minute (lanes 7~10); 10 minutes (lanes 11~14); 30 minutes (lanes 15~18); 60 minutes (lanes 19~22). DNA molecular weight marker: lane 1. EGFP-plasmid DNA, untreated (2 μg): lane 6.

are asumed to have other functions than inactivation of antimicrobials[25] and there are indications that aminoglycoside acetyltransferases found in mycobacteria[1] and *Providencia stuartii*[13] participate in the synthesis of peptidoglycan. Resistant pathogenic bacteria may also serve as donor for resistance genes especially in clinical settings. After cell death and lysis their DNA may become accessible to bacteria residing in their close surrounding. A concomitant presence of neomycin enhances DNA stability. It is thus possible for all these non-antibiotic producers to provide aminoglycoside resistance determinants for horizontal gene transfer events *via* natural bacterial transformation.

Antibiotics exert an enormous selective pressure for resistance in bacterial populations leading to a profound change in prokaryotic metabolism and at several occasions to the activation and transfer of silent resistance determinants[5,21]. All these events are to be seen in close connection with alterations in membane structures and uptake properties of the target cells eventually promoting the uptake of exogenously presented DNA molecules. Moreover discussion should not be restricted to orally applied antibiotic preparations, since DNA contact by contaminated antibiotics of microbial origin may occur at several other locations like skin, tissue and bloodstream.

Evidence is accumulating that DNA is much more stable in natural environments than previously thought. SCHUBBERT *et al.*[18] could demonstrate the survival of orally ingested DNA in mice by amplification of DNA fragments in feces up to 1500 bp long and even showed M13 phage DNA to appear transiently in the bloodstream and in several tissues[19].

Thus, the search for factors promoting DNA survival in natural human or animal environments (*e.g.* gastrointestinal

tract, skin) is legitimate. Aminoglycoside antibiotics are primary candidates for evaluation because these compounds are known to bind tightly to RNA and DNA[4]. Besides well documented specific interactions with several RNA-subspecies (16S rRNA[12,14], group I introns[27], hammerhead ribozyme[20], HIV rev responsive element[32]) neomycin inhibits initiation of DNA replication[9] and anecdotal reports link this compound to ssDNA templates for protein synthesis[10].

Ingested DNA fragments surviving the passage through the stomach probably will get into contact with DNases secreted from the pancreas in the duodenum but one should bear in mind that bacteria themselves are capable of excreting large amounts of deoxyribonuclease especially under imbalanced growth conditions leading to cell death in the population[8]. Enzymes with nucleolytic functions are present on the skin and mucosa as well. Thus, compounds capable of inhibiting DNase functions may be of significant relevance concerning the transfer of resistance genes *via* transformation.

Kanamycin and oxytetracyline do not interfere with DNA-DNase I interactions although both compounds interact with ribosomal RNA[12]. Streptomycin was shown to be incapable of inducing a B- to A-DNA transition[16] and ampicillin per se should not display any interactions with nucleic acids. This was in accordance with our results as in both cases no DNase degradation protection was observed.

The data presented in this study indicate that 4,5 disubstituted 2-deoxystreptamin aminocyclitols, with neomycin B being the most effective exponent, may promote structural integrity of DNA molecules under DNase-rich environmental conditions.

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