Review Article

BASIC TECHNIQUES FOR DNA CLONING AND CONDITIONS REQUIRED FOR STREPTOMYCETES AS A HOST

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To develop a host-vector system in streptomycetes for DNA cloning, we examined the technical problems encountered and the conditions required for use of *Streptomyces kasugaensis* MB273 as the host. Basic techniques, such as plasmid DNA isolation, regeneration of mycelia from protoplasts and elimination of plasmids from cells were investigated. These techniques were found to be useful for many streptomycetes. Strain M518, a derivative of *S. kasugaensis* MB273, was found to have the following useful characteristics as a host. The plasmids of MB273 were easily cured by regeneration of mycelia from protoplasts. The protoplasts prepared from M518 regenerated mycelia at high frequency using an improved method and were efficiently transformed by plasmid DNA. The extra and intra cellular DNase activities were very weak, and no restriction endonuclease activity was detected. The sensitivity to various antibiotics was determined. This strain did not show any pathogenicity in mice nor suvival in the digestive organs of rats. MB273 and its derivatives died rather quickly in natural soil. M518 still forms aerial mycelial conidia. These results indicate that *S. kasugaensis* M518, derived from MB273, has useful characteristics as a host for DNA cloning. The techniques thus developed were found to be useful in other streptomycetes.

Streptomycetes are prokaryotes forming mycelia and having distinct morphological differentiation, and the mole percentage of G+C in their DNA is around 71%. They are important as producers of secondary metabolites, including many antibiotics of medical and agricultural use, and other biologically active substances. The study of the application of DNA cloning technology to these organisms should be useful in establishing general methods to increase the fermentation yield of secondary metabolites and to produce useful new compounds. Secondary metabolites, such as antibiotics, are produced through many steps including special biosynthetic enzymes which exist in specific strains. Such biosynthetic pathways are not normally found in *Escherichia coli*. It will be very difficult to introduce all genes necessary for antibiotic production into *E. coli* by gene cloning. Moreover even if genes for antibiotic synthesis could be introduced into *E. coli*, components of the biosynthetic pathway may be degraded or otherwise altered by *E. coli* functions. Furthermore, the *E. coli* cell may have no resistance to the antibiotic, except for the involvement of inactivating enzymes. Therefore, a DNA cloning system needs to be established in a *Streptomyces* species.

When we apply to streptomycetes the recombinant DNA techniques that are widely used in *E. coli*, most of the techniques have to be modified. On the other hand, in the case of the production of peptides or proteins, *E. coli* strain K12 and its derivatives are generally useful as the host, but in the case of the production of secondary metabolites, the host strains should be prepared from many species of strepto-

mycetes. For that purpose, the following basic techniques and characteristics are required for the streptomycete to act as a host for DNA cloning.

1. A simple method for the extraction of plasmids.
2. A method for curing the plasmids carried in the potential host.
3. Transformation
   1) A method for regenerating mycelia from protoplasts.
   2) Selection of a host strain which has no or very weak extracellular DNase activity.
   3) Preparation of protoplasts which are not easily broken and from which chromosomal DNA is not released.
   4) A procedure for transformation of protoplasts by plasmid DNA.
4. Selection of a host strain which has little or no restriction endonuclease activity.
5. Selection of a host strain which has no extracellular protease activity.
6. Determination of sensitivity and resistance to various antibiotics.
7. No pathogenicity of the host strain.
8. No growth of the host strain in the digestive organs of animals.
9. The formation of aerial mycelial conidia by the host strain.
10. Rec$^-$ is preferable.

Among streptomycetes, *Streptomyces coelicolor*[1,2,3] and *Streptomyces lividans*[3], whose properties suggest that they belong to the same species, have already been used as hosts in DNA cloning experiments. This paper deals with the technical problems encountered and the selection of a host system for DNA cloning into *Streptomyces kasugaensis*.

Streptomyces kasugaensis Strains

Only two strains, M338 and MB273, have been identified as *Streptomyces kasugaensis* during 20 years of antibiotic screening at the Institute of Microbial Chemistry[4]. The former was isolated from soil collected at Kasuga Shrine in Nara Prefecture and the latter at Mt. Yoshino, located in the same prefecture. Both strains produce kasugamycin and aureothricin. The MB273 series was used in our study because this strain has better sporulation properties than M338, and its plasmid DNA is easier to isolate. The characteristics of various mutants derived from MB273 are shown in Table 1.

Table 1. Characteristics of *Streptomyces kasugaensis* MB273 and its derivatives.

Strains	Ksp	Atp (in liquid)	Atp (on agar)	Amy	Nutritional requirement				Cm
					Arg	Ilv	Cys	Met	
MB273	+	+	+	+	−	−	−	−	R
18a	+	−	−	−	+	−	−	−	R
M518	+	−	+	+	−	−	−	−	R
G1	+	−	+	+	−	+	−	−	R
G3	+	−	+	±	−	+	+	+	R
C401	+	−	+	±	−	+	+	+	S

Ksp: Kasugamycin productivity. Atp: Aureothricin productivity. Amy: Aerial mycelium formation. Arg: Arginine requirement. Ilv: Isoleucine and valine requirement. Met: Methionine requirement. Cys: Cysteine requirement. Cm: Chloramphenicol resistance.

Isolation of Plasmid DNA

Some streptomycetes are not lysed by egg white lysozyme. Our past studies have shown that the medium composition and cultural conditions used for growing streptomycetes significantly influence the response of cells to lysozyme. A suitable medium was obtained by adding glycine at sub-inhibitory con-

centrations and by reducing the concentration of the carbon source. A phosphate buffer was used to stimulate growth and repress DNase formation in the culture. Streptomycetes were incubated for 5~6 hours after reaching the stationary phase. These improvements to the medium composition and cultural conditions were effective with either defined or complex media. A typical defined medium consisted of 0.3~0.6% glycerol, 0.2% L-asparagine (or 0.4% Casamino Acids), 0.1~1.0% glycine, 0.05% $MgSO_4 \cdot 7H_2O$, 0.5% trace element solution[5], 0.2% KH_2PO_4 and 0.8% $Na_2HPO_4 \cdot 12H_2O$. An organic medium, GPYG, containing 0.3~0.6% glycerol, 0.2% peptone, 0.4% yeast extract, 0.1~1.5% glycine, 0.05% $MgSO_4 \cdot 7H_2O$, 0.2% KH_2PO_4 and 0.8% $Na_2HPO_4 \cdot 12H_2O$ was also used.

To extract plasmid DNA, it would be important to lyse mycelia under conditions inhibiting DNase activity and to avoid binding of plasmid DNA to cellular components. The procedure for isolation of plasmid DNA recently established in our laboratory is shown in Fig. 1. Reproducible yields of plasmid DNA were achieved by adding the following three procedures to the previous method[5]. First, treatment of the mycelia with RNase together with lysozyme increased the plasmid yield. Second, SDS was added at a concentration of 1.5%, and the mixture was then incubated at 50°C for 20 minutes. These procedures at this high temperature solubilized most of the protein and lipid. Third, to remove most of the chromosomal DNA, the viscous lysate was filtered by mild suction through a layer of cellulose powder. This procedure significantly increased the yield of clear lysate. A similar filtration was initially developed for yeast cells by FUKAZAWA (personal communication). We were successful in the application of this technique to streptomycetes by using cellulose powder instead of the Hyflo Super Cell used for yeasts. Compared to the previous method[5], the improved procedure increased the plasmid yield by about 1.5 fold in the case of *S. kasugaensis* and other species (*S. ribosidificus* KCCS-0923, *S. niveus* KCCS-0599, *S. omiyaensis* NIHJ At-95, *S. fradiae* ATCC 10745 and *Streptoverticillium mashuense* KCCS-0059).

Fig. 1. Isolation of cccDNA.

Culture (100 ml)
- centrifuge
- wash in 1×TES
- centrifuge

Washed mycelium (2 g wet weight)
- suspend in 40 ml 2×TES
- add 1 ml lysozyme (40 mg/ml) and 1 ml RNase (5 mg/ml) incubate at 37°C 20~90 minutes
- add 6 ml SDS (10%)
- incubate at 50°C for 20 minutes

Viscous lysate
- filter by mild suction through a layer of cellulose powder on a Büchner funnel

Filtrate
- add 5 M NaCl (37°C) to 1 M final concentration
- leave at 0°C, 2.5 hours
- centrifuge (8,000 rpm, 20 minutes, 0°C)

Cleared lysate
- add Pronase (5 mg/ml) to 100 μg/ml final concentration
- incubate at 37°C for 20 minutes

Digested lysate
- add PEG 6000 (40%) to 10% final concentration
- leave at 4°C overnight

Refregerated sample
- centrifuge (5,000 rpm, 15 minutes, 0°C)

PPT
- dissolve in 4.7 ml DSB
- dialyze against DSB

Concentrated sample

1×TES: 25 mM Trizma base, 25 mM trisodium ethylenediaminetetraacetate and 25 mM NaCl, pH 7.4 with HCl. SDS: sodium dodecylsulfate. PEG: polyethylene glycol. DSB: 10 mM Trizma base, 1 mM trisodium ethylenediaminetetraacetate, and 10 mM NaCl, pH 7.6 with HCl.

Elimination of Plasmids from Cells

Plasmids, especially those incompatible to the potential vector, must be cured from the host strain. However, it can be difficult to eliminate plasmids from an organism carrying a high copy number of plasmids in its cells. Moreover, most plasmids found in streptomycetes are cryptic, and the phenotype of

plasmid-cured cells can not be distinguished from that of cells carrying plasmids. Therefore, an effective new method for high frequency elimination of plasmid was needed. Acridine dyes, ethidium bromide, mitomycin C and novobiocin[6] have generally been used for curing plasmids. Plasmids pSK1, pSK2 and pSK3 have been found in *S. kasugaensis* MB273[7]. The pSK1 and 2, which show a high copy number, were not cured by treatment with the above agents.

A spontaneous mutant, 18a, was isolated from strain MB273. It was found to have a pleiotropic mutation (Amy$^-$, Arg$^-$, Atp$^-$), and it contained very small amounts of pSK1 and pSK2 but a large amount of pSK3. Protoplasts of 18a were prepared and incubated on R3 medium[8]. Regeneration from the protoplasts occurred at a frequency of more than 90%. Unexpectedly, more than 40% of the regenerated colonies had lost the pleiotropic mutation found in strain 18a, and moreover, about half of them had lost all three kinds of plasmids. No integration of plasmids pSK1 and pSK2 into the chromosome could be detected[9] by Southern blotting experiments. The protoplast regeneration thus caused not only plasmid curing but also variations in the copy number of plasmids[10,11]. Curing by protoplast regeneration has been reported independently by HOPWOOD[12] using *S. coelicolor* A3(2). According to a personal communication from VINING, the plasmid found in *Streptomyces* sp. 13a, a chloramphenicol producer, was also cured by using this regeneration procedure; this curing method may be of general use in the genus *Streptomyces*.

On Transformation Ability

Regeneration of Mycelia from Protoplasts

Genetic transformation in streptomycetes requires establishing a method for regenerating mycelia from protoplasts. We recently reported an improved regeneration method[8]. This improved method was applied to six species of actinomycetes which did not regenerate well by a method reported in 1976[13]. As shown in Table 2, five of the 6 species tested showed a regeneration frequency higher than 30%, with *Streptomyces humidus* ISP 5263 being the only exception. To achieve high frequency of regeneration, as a function of the strain used, suitable modification is needed of the concentration of an appropriate osmotic stabilizer and the concentrations of divalent cations in R3 medium.

Table 2. Regeneration frequency in two methods.

Protoplasts prepared	Spreading method medium R2	Double layer method medium R3
S. kasugaensis MB273-18a-M518	<2%	80~90%
S. fradiae KCC S-0579	<2	>90
S. niveus ISP 5088	<2	>90
S. kanamyceticus At-533	<2	30
S. humidus ISP 5263	<2	10
Streptoverticillium mashuense ISP 5221	<2	70

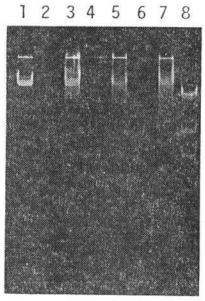
DNase and Restriction Endonuclease Activity

At first, the extracellular release of DNase was observed by incubating streptomycetes on a *Staphylococcus*-DNase-test agar supplemented with 0.2% glucose. The excreted DNase of strains MB273 M518 and G3 was detected; the amount was much smaller compared with *Staphylococci*. The presence of mycelial DNases, including restriction endonucleases were also examined. Sonicated samples were prepared from mycelia cultured for 10 hours, 18 hours and 26 hours and incubated with λDNA as the substrate; analysis was done by gel electrophoresis. As Fig. 2 shows, all samples showed a slight smear, indicating slight DNase activity against λDNA. Restriction endonuclease activity was not detected under the conditions we tested.

As another approach, to investigate the presence or absence of restriction and modification systems

Fig. 2. Detection of DNase and restriction endonuclease.

The mycelia of *S. kasugaensis* G3 were harvested from cultures incubated for 10 hours, 18 hours and 26 hours in GPYG medium. *Ca.* 1.2 g wet weight of mycelia was suspended in 6 ml of buffer (50 mM tris-HCl, 5 mM $MgCl_2$, 0.2 mM EDTA and 5 mM 2-mercaptoethanol), and sonicated 10 times at 20 KHz for 30 seconds in the cold. After centrifugation at 80,000×*g* for 60 minutes, 5% streptomycin sulfate solution was added to the supernatant solution to give a final concentration of 1%. This was centrifuged at 9,000×*g* for 20 minutes, followed by dialysis against the buffer mentioned above. Protein content of specimens was adjusted to give 4.2 mg/ml with the buffer. The reaction mixture to detect DNase and restriction endonuclease consisted of 10 μl of cell-free extract, 2 μl of restriction buffer (×10), 2 μl of λDNA solution (OD_{260}=5.0) and 6 μl of distilled water. After incubation at 37°C for 1 hour, 5 μl of halting solution was added and heated at 65°C for 7 minutes. The mixture was submitted to agarose gel electrophoresis.



1. λDNA
2. Extract from 10 hour-cultured cells
3. λDNA+Extract from 10 hour-cultured cells
4. Extract from 18 hour-cultured cells
5. λDNA+Extract from 18 hour-cultured cells
6. Extract from 26 hour-cultured cells
7. λDNA+Extract from 26 hour-cultured cells
8. λDNA+*Hin*d III

Table 3. Relative plaque-forming efficiency of φK1.

Host of φK1	Indicator strain						
	MB273	18a	M518	G1	G3	M338	MA350
S. kasugaensis MB273 and its derivatives							
MB273	1	5.4×10^{-1}	1.2×10^{-1}	3.1×10^{-1}	2.6×10^{-1}	7.7×10^{-1}	1
18a	5.5	1	9.8×10^{-1}	1.4	8.3×10^{-1}	2.9	5.0
M518	4.0	1.4	1	2.8	2.1	9.2×10^{-1}	6.4
G1	3.1	1.5×10^{-1}	5.4×10^{-1}	1	4.0×10^{-1}	6.5×10^{-1}	1.9
G3	4.2	9.2×10^{-1}	2.6×10^{-1}	1.4	1	1.9	3.4
S. kasugaensis M338	9.0×10^{-1}	4.7×10^{-2}	7.0×10^{-2}	1.0×10^{-1}	7.4×10^{-2}	1	3.5×10^{-1}
S. kasugaspinus MA350	1.5	7.4×10^{-2}	9.7×10^{-2}	2.8×10^{-1}	1.1×10^{-1}	1.4	1

An appropriate amount of φK1-containing filtrate and 2 ml of a host culture were inoculated into 100 ml of AC medium (1.0% glucose, 1.0% yeast extract and 0.73% $CaCl_2\cdot2H_2O$, pH 7.0) in a shaking flask and incubated at 27°C for 24 hours on a reciprocating shaker. The lysate obtained was centrifuged at 7,500×*g* for 10 minutes to remove the cell debris and then the supernatant solution was filtered through a membrane filter having a 0.2 μm pore size. The same strain as the host used for propagation of the phage. The relative plaque-forming efficiency was assayed against three species as well as the mutants derived from *S. kasugaensis* MB273.

in *S. kasugaensis*, we measured the efficiency of plating (EOP) actinophage grown in different strains. An actinophage, φK1, capable of infecting *S. kasugaensis* MB273 was isolated after testing 130 soil samples. As shown in Table 3, φK1 (MB273) grown in strain MB273 as the host strain showed EOPs of 1.0, 7.7×10^{-1} and 1.0 against *S. kasugaensis* MB273, M338 and MA350, respectively. φK1 (M338) grown in strain M338 showed EOPs of 1.0, 9.0×10^{-1} and 3.5×10^{-1} against M338, MB273 and MA350, respectively. φK1 (MA350) grown in MA350 showed EOPs of 1.0, 1.5 and 1.4 against MA350, MB273 and M338, respectively. A slight variation in the EOP was also observed among the derivatives (18a, M518, G1 and G3) of MB273. The adsorption rate of the phage to the host cells was the same for MB

273 and G3 (data not shown). These findings indicate that restriction and modification systems to ϕK1 do not exist in *S. kasugaensis*.

Leakage of Chromosomal DNA from Protoplasts

We have found that the presence of viscous chromosomal DNA in a protoplast suspension can interfere with the transformation of protoplasts by plasmid DNA. Therefore, we measured the free chromosomal DNA in a suspension of G3 protoplasts prepared for transformation experiments. DNase I was added at a concentration of 100 units/ml to a protoplast suspension (2×10^9/ml) in P medium, and this was incubated at 28°C for 10 minutes. After centrifugation, the ultraviolet absorption spectrum of the supernatant was measured using P medium as a reference. The values of OD_{260} and OD_{280} were 0.36 and 0.255, respectively, indicating the free nucleic acid at 18 μg/ml, and the free protein at 130 μg/ml, at most. This concentration of nucleic acid did not interfere with plasmid transformation.

Transformation of Protoplasts

The transformation system reported by BIBB *et al.*[14], combined KOMANO's transformation method[15] using polyethyleneglycol for *E. coli* and our regeneration method[13] for streptomycete protoplasts. This system has been successfully used to transform protoplasts prepared from *S. coelicolor*[1,2,14], *S. lividans* 66[3,16], and *S. parvulus*[14]. We confirmed that it was also useful for *S. kasugaensis*, but that polyethyleneglycol 4000 was always more effective than polyethyleneglycol 1000.

Extracellular Protease

When we attempt to produce a protein significantly different from those of the host by recombinant DNA technology, protease activity in the host may prevent the accumulation of the foreign protein. Extracellular protease activity in strain M518 was very low or non-existent; liquefaction of gelatin and coagulation or peptonization of milk casein were not detected.

Sensitivity to Various Antibiotics

The sensitivity of a host to various antibiotics should be determined for the selection of an appropriate vector or for convenient identification of the host strain. The sensitivity test was carried out using the sensitivity discs widely employed in hospitals. As shown in Table 4, the derivatives, M518 and G3, showed a similar pattern of sensitivity, but C401, selected as a mutant highly sensitive to chloramphenicol, was somewhat more sensitive to various antibiotics. Generally, *S. kasugaensis* M518 and its derivatives were resistant to the penicillin group, the cephem group, paromomycin, neomycin, ribostamycin, colistin and polymyxin, sensitive to streptomycin, kanamycin A, kanamycin B, gentamicin, dibekacin, tobramycin, the tetracycline group, the macrolide group, lincomycin, novobiocin and mikamycin, and very sensitive to josamycin and clindamycin. Mutations conferring antibiotic resistance or nutritional requirements and their back mutations often accompanied regeneration from the protoplasts. However, these antibiotic resistance and nutritional requirements were stable in the normal intact mycelia and the strains already regenerated from protoplasts. Since these phenomena produce ambiguous problems, it is best to avoid using such unstable characteristics as markers in DNA cloning experiments.

Pathogenicity

S. kasugaensis M338 has been used to produce about 100 tons of kasugamycin every year in several factories. In the last 12 years, the workers coming into contact with this strain never reported health problems that could be associated with *S. kasugaensis*.

The potential virulence of strain M518 was examined by injection intravenously into mice (Table 5).

Table 4. Sensitivity of *S. kasugaensis* mutants to various antibiotics.

Antibiotic	M518 & G3	C401	Antibiotic	M518 & G3	C401
Penicillin group			Doxycycline 200 μg	S	S
Penicillin 20U	R	R	Minocycline 200 μg	S	S
Propicillin 30 μg	R	R	Macrolide group		
Cloxacillin 30 μg	R	R	Erythromycin 50 μg	S	SS
Ampicillin 30 μg	R	R	Oleandomycin 30 μg	S	SS
Hetacillin 30 μg	R	R	Leucomycin 30 μg	S	SS
Carbenicillin 30 μg	R	R	Josamycin 30 μg	VS	SS
Sulbenicillin 30 μg	R	R	Spiramycin 30 μg	S	VS
Cephem group			Midecamycin 30 μg	S	SS
Cephaloridine 30 μg	R	R	Chloramphenicol 100 μg	S	SS
Cephalexin 30 μg	R	R	Sulfa drugs		
Cefazolin 30 μg	R	R	Sulfisoxazole 400 μg	S	SS
Aminoglycoside group			Sulfisomezole 400 μg	S	VS
Streptomycin 50 μg	S	S	Sulfamonomethoxin 400 μg	R	S
Kanamycin 50 μg	S	S	Sulfamethizole 400 μg	R	S
Kanamycin B 50 μg	S	R	Others		
Paromomycin 30 μg	R	R	Colistin 150U	R	R
Neomycin 20 μg	R	R	Polymyxin B 100U	R	R
Gentamicin 30 μg	S	S	Lincomycin 30 μg	S	VS
Dibekacin 30 μg	S	S	Clindamycin 30 μg	VS	SS
Ribostamycin 50 μg	R	R	Novobiocin 20 μg	S	SS
Tobramycin 30 μg	S	S	Bacitracin 2U	R	S
Tetracycline group			Nalidixic acid 50 μg	R	R
Tetracycline 200 μg	S	VS	Mikamycin 30 μg	S	S

R: <1.0 mm, S: 1.5~4.0 mm, VS: 4.5~8.0 mm, SS: >8.0 mm (inhibition zone).

Fig. 3. Change in body weight of mice with time; with or without inoculation of *S. kasugaensis* M518.

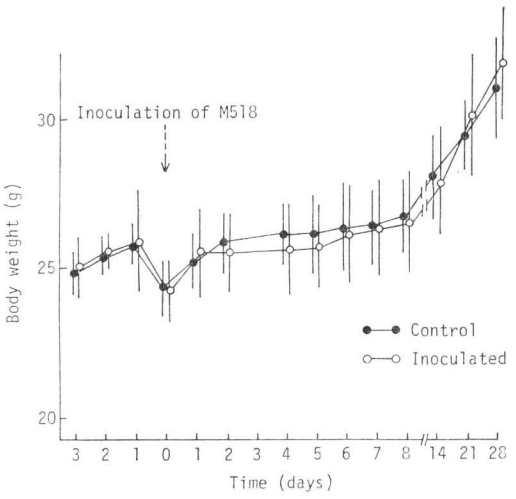


Fig 4. Change in body weight with time cortisone-treated mice with or without inoculation of *S. kasugaensis* M518.
C (arrow): Administration of cortisone

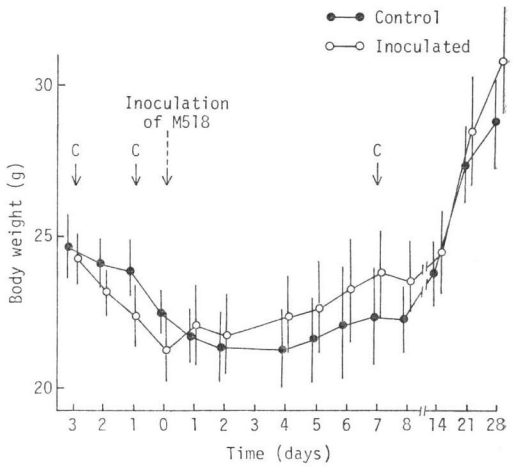


Table 5. Pathogenicity of *S. kasugaensis* MB273-18a-M518 in mice after intravenous inoculation.

Method	Mice: ICR(SPF), female, 4 weeks old Experimental groups: Normal group: 20 mice Cortisone-treated group: 20 mice; Cortisone was administrated intramuscularly on Days 3 (2.5 mg/mouse) and 1 (1.5 mg/mouse) before inoculation and on Day 7 (1.5 mg/mouse) after inoculation Inoculation of M518: 1×10^7 CFU/mouse intravascularly
Results	1. Normal group (No cortisone group) Survival: Control; 10/10 after 28 days Inoculated; 10/10 after 28 days Body weight: No significant difference was detected between control and inoculated groups 2. Cortisone-treated group Survival: Control; 10/10 after 28 days Inoculated; 10/10 after 28 days Body weight: No significant difference was detected between the control and inoculated groups

Table 6. Survival of *S. kasugaensis* M518 in the digestive tract of rats.

Method	Rats: Wister (SPF), female, 6 weeks old Administration of M518: 1×10^9 CFU/rat, orally, 6 rats Medium for counting viable cells: Maltose 10 g Yeast extract 3 g Agar 16 g Ampicillin 0.2 g Tetracycline 0.005 g Deionized water 1 liter (pH 7.2) Viable count: 0.1 ml of the suspension, which contained 1.0 g of fecal specimen in MS solution (10 ml), was spread on the agar plate and incubated at 28°C for 3 days, followed by counting the colonies grown.
Results	No colonies of M518 were detected from all of the fecal specimens on Days 1, 3, 7 and 14 after administration.

Forty mice were employed and separated into 2 groups: one was used without injection of cortisone and the other was treated with cortisone in advance. 1×10^7 CFU of a fresh culture of M518 was injected to half of the mice in each group. Both with and without the cortisone treatment, all mice survived on the 28th day without any toxic signs after injection of M518. The body weights between the control and the M518-injected mice were not significantly different, with or without the cortisone treatment (Figs. 3 and 4).

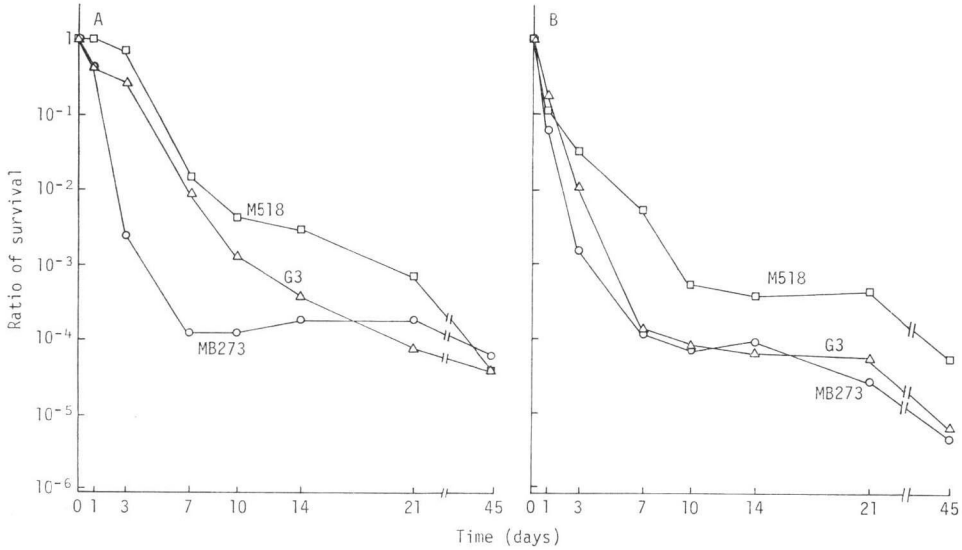
Survival in Digestive Organs

The survival of M518 in the digestive organs of rats was examined (Table 6). Freshly grown 1×10^9 CFU of strain M518 were administrated orally to each of 6 rats. The culture medium used for counting viable cells contained ampicillin and tetracycline to select for strain M518. The viable count in the feces was made as described in Table 6. No colonies of M518 were detected from any of the fecal specimens. These results indicate that M518 is rapidly eradicated from the digestive organs of rats.

Survival in Soil

The habitat of most streptomycetes is the soil. Strain M518 is non-pathogenic but it is also highly desirable that the organism should not remain viable in soil for a long period of time. Therefore, the survival of 3 strains (MB273, M518 and G3) in natural farm soil and in barren soil was examined. To 1 g of soil sample, 10^7 CFU of mycelia were inoculated and incubated at 20°C at 100% humidity. In the nutritionally rich farm soil, the survival of strain MB273, carrying plasmids showed a rapid decline, but the plasmid-free strain M518 and its nutritional mutant, G3, showed a slow decline (Fig. 5A). In the barren soil, MB273 showed a rapid decline; the decline for M518 was slow, as in the farm soil, but

Fig. 5. Survival of *S. kasugaensis* in nutritionally rich farm soil (A) and in nutritionally poor barren soil (B).



mutant G3 displayed a rapid decline different from that in the farm soil (Fig. 5B). Nevertheless, after 3 weeks, even strain M518 decreased to less than 10^{-3}.

Conclusion

In Table 7, the experimental results on the characteristics of *S. kasugaensis* M518 as a DNA cloning host are summarized. From these results, it can be concluded that *S. kasugaensis* strain M518, derived

Table 7. Characteristics of *S. kasugaensis* M518 as a candidate of the host for DNA cloning.

1	Isolation of plasmid DNA:	Easily from original strain MB273
2	Carrying plasmids:	Eliminated (pSK1$^-$, pSK2$^-$, pSK3$^-$)
3	Transformation ability	
	1) Regeneration from protoplast:	>90% by the improved regeneration method
	2) Extracellular DNase:	Detectable but little
	3) Release of chromosomal DNA from protoplasts:	Little
	4) Transformation method:	Protoplasts-PEG4000 system
4	Restriction endonuclease:	Not detected
5	Extracellular protease:	Almost none
6	Sensitivity to antibiotics:	
	a) Resistant to penicillin group, cephem group, paromomycin, neomycin, ribostamycin, colistin and polymyxin.	
	b) Sensitive to streptomycin, kanamycin, kanamycin B, gentamicin, dibekacin, tobramycin, tetracycline group, macrolide group, lincomycin, novobiocin and mikamycin.	
	c) Very sensitive to josamycin and clindamycin.	
7	Pathogenicity in mice:	None
8	Survival in the digestive organs of rats:	None
9	Survival in natural soil:	Low (10^{-3} after 3-week incubation)
10	Formation of aerial mycelium and its conidia:	Positive
11	Others:	Maximum temperature for growth: At 34°C Growth form in liquid culture: Fragments

from MB273, is useful as a host for DNA cloning. Moreover, it may be necessary to describe here the following additional characteristics of *S. kasugaensis*. The maximum temperature for its growth is 34°C. Vegetative mycelia grown in liquid medium consist of fragmented hyphae, like bacilli. Streptomycete conidia make up the only generation consisting of single cells in the life cycle of *Streptomyces*. Accordingly, the formation of conidia of M518 is useful for genetic analysis. Biological containment would be compromised if the host's conidia possessed heat resistance. However, the conidia of mutant M518 died rapidly, decreasing by 10^{-5} at 60°C for 10 minutes and less than 10^{-8} at 60°C for 20 minutes. Unlike spores of *Bacillus subtilis*, the conidia of M518 are, therefore, heat susceptible.

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