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ORIGINAL PAPER

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Lagging strand replication of rolling-circle plasmids in *Streptomyces lividans*: an RNA polymerase-independent primer synthesis

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Abstract The rolling circle (RC) mechanism of DNA replication generating single-stranded DNA (ssDNA) intermediates is common in various high-copy circular plasmids in *Streptomyces*, and the ssDNA released after leading strand synthesis is converted to its double-stranded form (dsDNA) by the host proteins. The in vivo and in vitro lagging strand syntheses from ssDNA replicative intermediates of RC plasmid pSN22 in *Streptomyces lividans* was characterized. The presence or absence of the single-strand origin (*sso*), the replication initiation site of lagging strand synthesis, did not significantly affect the copy numbers of pSN22 derivatives. In vivo lagging strand synthesis was not affected by the rifampicin inhibition of *S. lividans* RNA polymerase. Likewise, in vitro lagging strand synthesis using cell-free extracts revealed*sso*-independent, rifampicin-resistant lagging strand synthesis in *S. lividans*. Although all four dNTPs are usually required for the initiation of such synthesis, the presence of only one NTP was sufficient to carry outlagging strand synthesis in vitro. Interestingly, the cell-free extract of exponential-phase cells required less ATP than that of stationary-phase cells. These results reveal a predominant RNA polymerase-independent priming system in *S. lividans* that may be a result of the stabilization of RC plasmids lacking *sso* in *S. lividans*.

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Introduction

Rolling circle (RC) replication is the replication mechanism of a large number of small circular plasmids isolated from gram-positive bacteria (Novick 1989; Gruss and Ehrlich 1989; Jannière et al. 1993; Khan 1997; Leenhouts et al. 1991; del Solar et al. 1993). RC replication yields strand-specific single-stranded circular plasmid DNA (ssDNA) replication intermediates (te Riele et al. 1986a,b), whose conversion from ssDNA to a double-stranded (ds) plasmid molecule through lagging strand synthesis is initiated at the single strand origin (*sso*), also called the minus origin (M-O). The replication mechanisms of small circular plasmids in the genus *Streptomyces* have been investigated in order to develop vectors useful in the genetic manipulation of this industrially important microorganism. Studies showed that most small circular plasmids replicate via the RC mechanism (Deng et al. 1988; Hagège et al. 1993; Kataoka et al. 1994b; Muth et al. 1995; Pigac et al. 1988; Servín-González et al. 1995; Yokoyama et al. 1996), with *Streptomyces lividans* being the major host strain used.

sso-initiated lagging strand replication is a problem in the initiation of RC replication. As for other RC plasmids from gram-positive bacteria, the lack of *sso* decreases the efficiency of initiating lagging strand replication in *S. lividans*, resulting in the accumulation of a large number of ssDNAs in host cells (Deng et al. 1988; Pigac et al. 1988). *S. lividans* plasmid pIJ101 (Kieser et al. 1982) lacking *sso* leads to a decrease in plasmid copy number without causing instability by plasmid segregation (Deng et al. 1988). However, the copy number of its derivative, pIJ702 (Katz et al. 1983), is more than 100 molecules per chromosomal DNA (Zaman et al. 1993) and the plasmids are stable in *S. lividans* cells. In contrast, the decrease in RC plasmid copy number to less than ten molecules per cell in *Staphylococcus aureus* (Gruss et al. 1987) due to the lack of the

sso sequence, which causes plasmid segregation, indicates the suppression of the initiation of complementary strand synthesis at random nucleotide sequences of the ssDNA replication intermediate in *S. aureus* (Boe et al. 1989). However, the lack of the *sso* sequence does not affect the copy number of RC plasmids in *Bacillus subtilis* although there is an accumulation of ssDNA replication intermediates in *B. subtilis* cells (Gruss et al. 1987). *S. lividans* and *B. subtilis* thus appear to be efficient in terms of initiation mechanisms at random DNA sequences on ssDNA replication intermediates even without *sso* sequences.

In this report, the in vivo and in vitro *sso*-independent lagging strand replications of a pSN22-derived RC plasmid in *S. lividans* were studied. The 11-kbp pSN22 is a high-copy-number, broad-host-range, and self-transmissible plasmid isolated from *Streptomyces nigrifaciens* SN22 (Kataoka et al. 1991). pSN22 is stable in *S. lividans* and replicates by the RC mechanism (Kataoka et al. 1994b). The effect of *sso* sequence on the copy number of pSN22 derivatives was examined. In vitro conversion of ssDNA to dsDNA using cell-free extract of *S. lividans* TK21, a plasmid-free strain, revealed that *S. lividans* has a rifampicin-resistant *sso*-independent lagging strand synthesis activity. The *S. lividans* extract was characterized in order to determine the optimal conditions for random *sso*-independent lagging strand synthesis.

Materials and methods

Bacterial strains, plasmids and media. *Escherichia coli* strain JM109 was used as host for pUC19 (Yanisch-Perron et al. 1985) and pBluescript II SK + (Stratagene), *E. coli* JM105 as a host for M13mp18 and M13mp19 (Yanisch-Perron et al. 1985), and *E. coli* BL21 (Studier and Moffatt 1986) for cell-free extract (fraction II) preparation. *S. lividans* TK21 (Kieser et al. 1982) was the host strain for *Streptomyces* plasmids and was used for cell-free extract preparation. *E. coli* cultures were grown in 2xYT medium (Sambrook et al. 1989). For the in vivo experiments of plasmid replication, *S. lividans* cultures were grown in liquid YEME medium I (Hopwood et al. 1985). When required, thiostrepton was added at 5 μg/ml. To determine the role of RNA polymerase in the conversion of ssDNA to dsDNA, protein synthesis was blocked by adding 100 μg erythromycin/ml to the medium and RNA synthesis was blocked by adding 100 μg rifampicin/ml. For preparation of the cell-free extract, *S. lividans* was cultured in liquid YEME medium II (Thompson et al. 1984) containing 0.5% (w/v) polyethyleneglycol (PEG) 6000 instead of sucrose. The plasmids used in this study are listed in Fig. 1.

DNA manipulations. Plasmid and bacteriophage DNA isolation, in vitro DNA manipulations, and *E. coli* transformations were done as described by Sambrook et al. (1989). *S. lividans* TK21 protoplasts were prepared and transformed as described by Hopwood et al. (1985). The enzymes used for DNA manipulations were purchased from TAKARA (Kyoto, Japan).

Detection of ssDNA accumulation. Total DNA was isolated from *S. lividans* transformants as described by Hopwood et al. (1985). To detect both ds and ss plasmid molecules, 1.6 μg total DNA was digested with *Eco*RI, applied to a 1% agarose gel, and fractionated by electrophoresis. The denatured DNA was transferred onto nylon membranes (Hybond-N, Amersham) and the plasmid molecules were detected by Southern hybridization (Southern 1975) using a DIG luminescent detection kit (Boehringer Mannheim) with DIG-labeled pBluescript II SK + as probe. To detect ssDNA molecules only, 1.6 μg total DNA was electrophoresed and then transferred onto the nylon membrane without prior denaturation (te Riele et al. 1986a).

Preparation of cell-free extract. Cell-free extracts of *S. lividans* were prepared from the plasmid-free strain *S. lividans* TK21. Cells were grown at 30°C in 6 l of YEME medium II using a 10-l jar fer-

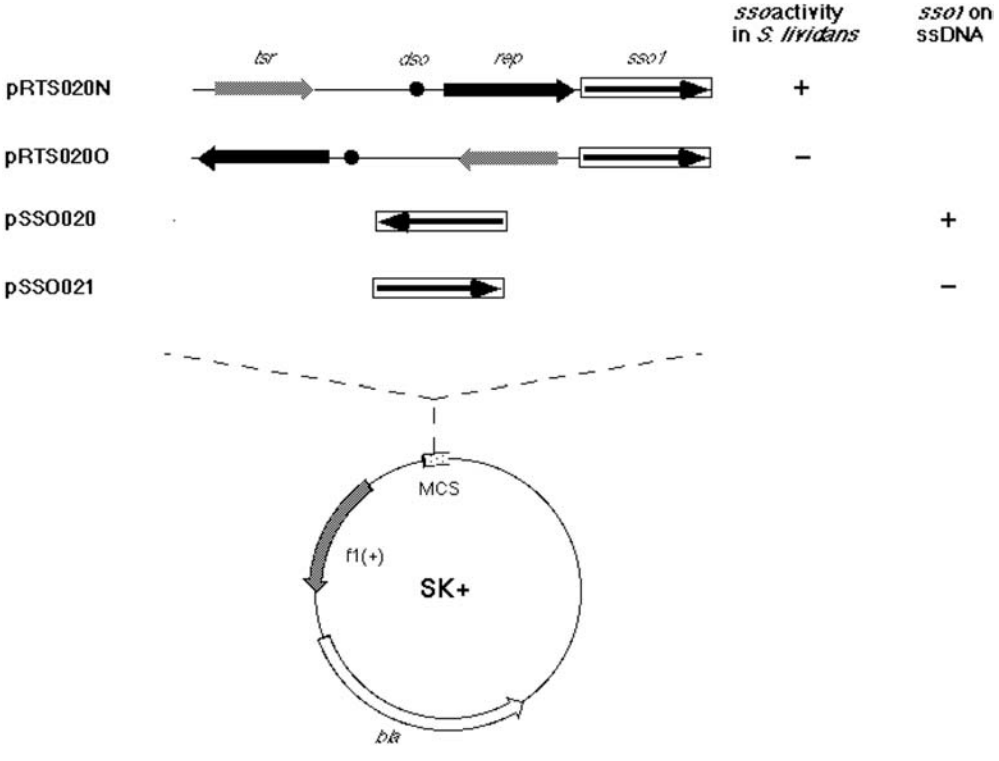

Fig. 1 Plasmids used in this study. *sso* activity indicates the in vivo activity of pRTS020N and pRTS020O (Suzuki et al. 1997a). pRTS020N and pRTS020O harbor the *PstI–Bgl*II fragment of pSN22-containing *sso1* inserted in the normal (5′ to 3′) and opposite (3′ to 5′) orientations, respectively, in accordance with the double-strand origin (*dso*)-rep of pSN22 (Kataoka et al. 1994a,b; Suzuki et al. 1997b). Thiostrepton-resistance gene (*tsr*) from pIJ6 (Thompson et al. 1982) served as a selection marker. pSS020 and pSS021 harbor the *Pst*I–*Bgl*II fragment with or without *sso1*, respectively, on a single-stranded phagemid

mentor stirred at 200 rpm and 1 vvm. Mycelia from the mid-exponential phase (12 h) or stationary phase (36 h) were harvested by filtration (Thompson and Cundliffe 1981) at room temperature and then stored at –80°C. The cells were resuspended in S30 buffer (50 mM HEPES–KOH pH 7.5, 60 mM NH_4OAc, 10 mM $Mg(OAc)_2$, 5 mM 2-mercaptoethanol, and 10% (v/v) glycerol) based on 2.5 ml buffer per gram wet weight. The cell suspension was subjected to a French press at 70–80 MPa, and the lysate obtained was centrifuged for 30 min at 39,000×*g* at 4°C. Endogenous DNA in the lysate was removed by streptomycin sulfate precipitation as described by Conrad and Campbell (1979). To 1.0 ml of the supernatant, 0.1 ml of a 33% (w/v) streptomycin sulfate solution was added, and the mixture was stirred for 30 min at 0°C. The collected supernatant after 30-min centrifugation at 39,000×*g* at 4°C was subjected to ammonium sulfate precipitation at 70% saturation and stirred for 30 min at 0°C. The precipitate obtained was suspended in S30 buffer and the suspension was dialyzed against the same buffer for 3 h at 4°C, after which the volume of the dialyzed material was adjusted to the initial lysate volume. The protein concentration of the prepared cell-free extract, determined using Protein Assay Kit I (Bio-Rad) with IgG as standard, was 6.4 mg/ml. The cell-free extract was subdivided into small aliquots and stored at –80°C.

ssDNA preparation. M13mp18 ssDNA was isolated as described by Sambrook et al. (1989). Recombinant pBluescript II SK + ssDNA was prepared according to the protocol provided by Stratagene using the helper phage VCSM13. The ssDNAs were further purified by alkaline agarose gel electrophoresis through electroelution. The presence of residual small oligonucleotide fragments in the purified ssDNA solution was tested by measuring the incorporation of [5-^{3}H]dCTP using the Klenow fragment. The incorporation reaction mixture, carried out at 37°C for 30 min in a total volume of 12.5 µl, contained 20 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 50 µM of each dATP, dGTP, and dTTP, 1 µl (4 µM, 25 Ci/mmol) [5-^{3}H]dCTP, 0.5 µg ssDNA and 0.16 U Klenow fragment/ml. Termination of the reaction and measurement of [5-^{3}H]dCTP incorporation were carried out as described below. After alkaline agarose gel electrophoresis, the incorporation of [5-^{3}H]dCTP was reduced 55-fold to 0.06 pmol per 0.5 µg M13mp18 ssDNA.

RNase treatment of cell-free extract and template ssDNA. To eliminate possible contaminating endogenous RNA that might prime lagging strand synthesis, the cell-free extracts and template ssDNAs were treated with RNase A and RNase T1 (90 u/ml each) for 1 h at 30°C and then passed through a NAP-10 column (Pharmacia) to remove the digested ribonucleotides.

Assay for in vitro ssDNA replication. The standard reaction mixtures (12.5 µl) contained 50 mM HEPES–KOH pH 7.5, 60 mM NH_4OAc, 7.5 mM $Mg(OAc)_2$, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, 5 µg rifampicin/ml, 2 mM ATP, 50 µM each of dATP, dGTP, and dTTP, 1.0 µl (4 µM, 25 Ci/mmol)[5-^{3}H]dCTP or 0.5 µl (0.132 µM, 3,000 Ci/mmol) of [α-^{32}P]dCTP, 0.5 µg ssDNA, and 2.0 µl cell-free extract. When using 0.5 µg poly(dT) (Pharmacia) as a template, 0.5 µl (0.132 µM, 3, 000 Ci/mmol) [α-^{32}P]dATP was added and the three dNTPs were omitted. Incubation was carried out at 30°C. Reactions using [5-^{3}H]dCTP were terminated by adding 500 µl of cold 10% (w/v) trichloroacetic acid–0.1 M sodium diphosphate (TCA–NaPPi) solution. The precipitates were collected on glass-fiber filters as described by Inuzuka and Helinski (1978), and radioactivity was determined using a liquid scintillation counter. Reactions involving [α-^{32}P]dATP and poly(dT) were terminated and analyzed as described above. Reactions using [α-^{32}P]dCTP were terminated by adding 1.25 µl 500 mM EDTA. The samples were fractionated by 0.7% agarose gel electrophoresis. The reaction products were identified by autoradiography of dried gels.

Preparation of E. coli cell-free extracts. *E. coli* cell-free extracts (fraction II) were prepared from the plasmid-free strain BL21 as described by Fuller et al. (1981). The in vitro lagging strand synthesis reaction mixtures (12.5 µl) contained 50 mM HEPES–KOH (pH 7.5), 60 mM NH_4OAc, 7.5 mM $Mg(OAc)_2$, 5 mM 2-mercaptoethanol, 10% (v/v) glycerol, 50 µM each of dATP, dGTP, and dTTP, 1.0 µl[5-^{3}H]dCTP, 0.5 µg M13mp18 ssDNA, and 12.8 µg cell-free extract proteins. The mixture were incubated for 30 min at 37°C.

Results

Lack of sso has no effect on copy number of pSN22 derivatives in S. lividans. The lack of the *sso* sequence does not cause significant segregational instability of the pSN22 derivatives, but leads to the accumulation of ssDNA molecules in *S. lividans* cells (Kataoka et al. 1994a). Hence, either *sso* deletion does not influencethe copy number of the plasmid or pSN22 has a specific segregation mechanism. To assess the former possibility, the copy numbers of the two pSN22 derivatives (Suzuki et al. 1997a), pRTS020N (carrying *sso1* in active orientation) and pRTS020O (carrying *sso1* in inactive orientation), with and without the *sso* sequence, respectively, were determined. As shown in Fig. 2, the intensity ratios of both plasmids compared to the chromosome showed no significant differences, indicating that the lack of *sso1* does not affect the plasmid copy number of the derivatives in *S. lividans*. Alternatively, the *sso*-independent lagging strand synthesis of pSN22 derivatives is effective in preserving the homeostasis of plasmid copy number.

Effect of rifampicin on lagging strand synthesis of pSN22 derivatives in S. lividans. It has been postulated that lag-

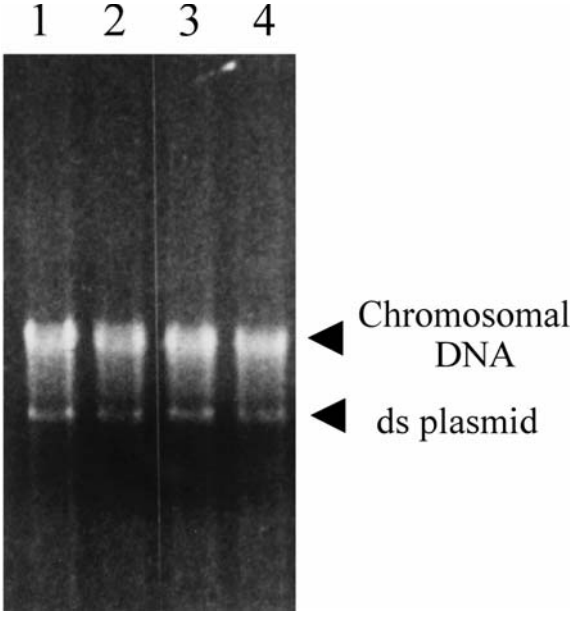

Fig. 2 Copy number of pSN22 derivatives in *Streptomyces lividans*. *Eco*RI-digested total DNA (1.6 µg) was fractionated by 1% agarose gel electrophoresis and visualized by ethidium bromide staining. *Lanes 1*,, *2* pRTS020N (carrying *sso1* in the active orientation); *lanes 3*', *4* pRTS020O (carrying *sso1* in the inactive orientation)

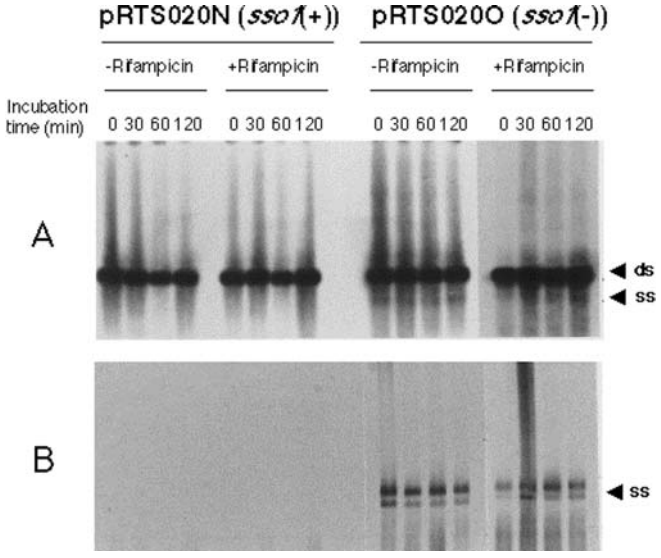

Fig. 3a,b Effect of rifampicin on lagging strand synthesis of pSN22 derivatives in *S. lividans*. After adding 100 µg rifampicin/ml, total DNA was isolated from *S. lividans* transformants harboring pRTS020N (active *sso1*) or pRTS020O (inactive *sso1*) at the indicated times. Erythromycin (100 µg/ml) was added simultaneously to all the samples to further inhibit protein synthesis and thus another cycle of replication. The electrophoresed total DNA (1.6 µg) was detected by Southern hybridization as described in "Materials and methods". *Arrows* indicate the positions of *Eco*RI-linearized ds plasmid and ss plasmid (circular or linear) molecules as marked. *Rif* rifampicin. **a** Southern blotting of denatured DNA. **b** Southern blotting without prior denaturation. The ss plasmid molecules are observed as two signals, probably indicating circular and linear forms, since the circular ss plasmid could be linearized during the DNA extraction process

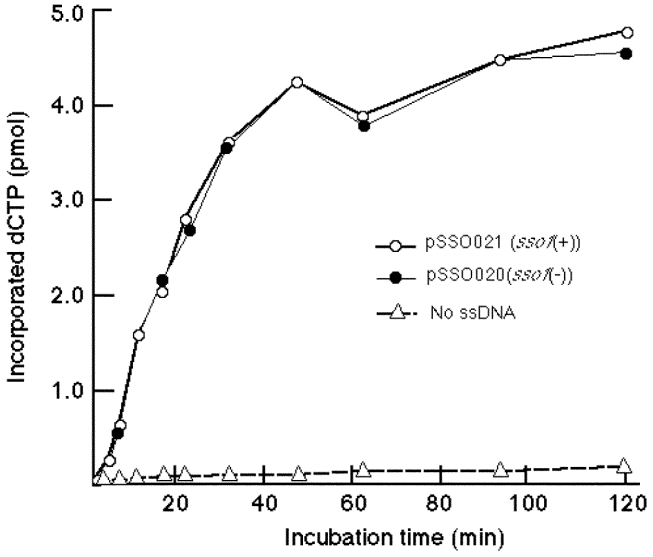

Fig. 4 Time course of lagging strand synthesis using cell-free extract from exponential-phase cells of *S. lividans*TK21. The reaction mixture (300 µl) described in "Materials and methods" was incubated under standard assay conditions with [5-^{3}H]dCTP, and 12.5-µl aliquots were sampled, acid-precipitated, and assayed at different time intervals. Twelve µg ssDNA, pSSO021 (active *sso1*, *open circles*), or pSSO020 (inactive *sso1*, *closed circles*) were used as templates. *Open triangle*s No ssDNA template was added to the reaction mixture

ging strand synthesis is initiated by a primer RNA synthesized from *sso* by RNA polymerase (RNAP) (Birch and Khan 1992; Dempsey et al. 1995; Kramer et al. 1997, 1998). To determine the involvement of RNAP in the lagging strand synthesis of pSN22 derivatives in *S. lividans*, the effect of the RNAP inhibitor rifampicin on ssDNA conversion was examined. *S. lividans* TK21 did not grow on the agar plate containing 50 µg rifampicin/ml (data not shown). Two strains harboring pRTS020N or pRTS020O were examined. Cultures were grown to the mid-exponential phase, and 100 µg rifampicin/ml (final) was added to half of the cultures. Simultaneously, 100 µg erythromycin/ml (final)was added to all of the cultures to inhibit the initiation of plasmid replication. The cells were incubated at 30°C for 30–120 min after which DNA was extracted, and plasmid DNA molecules were determined by Southern hybridization using DIG-labeled pBluescript II SK + as a probe. Interestingly, in contrast with the lagging strand synthesis of RC plasmids in *S. aureus* cells, in which the amount of ssDNA molecules increases (Boe et al. 1989), rifampicin had no effect on ssDNA and dsDNA with either of the plasmids (Fig. 3), indicating that replication from a ss plasmid in *S. lividans* is independent of RNAP. However, both first and lagging strand syntheses might have been inhibited simultaneously by rifampicin.

sso-independent lagging strand synthesis using cell-free extracts of S. lividans. As shown above, *sso1* exerts no effect on the copy number of pSN22 derivative in *S. lividans*, implying that lagging strand synthesis in *S. lividans* can be initiated without the *sso* sequence. The in vitro lagging strand synthesis using the cell-free extract of the plasmid-free strain *S. lividans* TK21 confirmed likewise that the rates and total amounts of incorporated [5-^{3}H]dCTP on the ssDNA template were similar in the presence or absence of *sso1* (Fig. 4). In addition, a similar [5-^{3}H]dCTP incorporation rate was obtained for M13 mp18 ssDNA, which harbors no DNA fragment from pSN22 (data not shown). These results show that lagging strand synthesis is initiated on a random nucleotide sequence, but they do not explain the in vivo result that plasmid without*sso1* accumulates in its ss form. The factor necessary for efficient lagging strand synthesis of the *sso1* sequence is still unknown.

sso-independent lagging strand synthesis analyzed from cell-free extracts prepared from exponential-phase *S. lividans* cells showed relatively sharp bands consistent with DNA synthesis on ssDNA templates (Fig. 5a). To exclude any influence of *sso*, M13mp18 ssDNA was used as the template. Similar to in vivo experiments, the addition of 5 µg rifampicin/ml had no effect on the amount of ssDNA (Fig. 5a, lanes 2, 4), suggesting that initiation of lagging strand synthesis of the RC plasmid is independent of RNAP in *S. lividans*. Furthermore, the presence of all four NTPs is not a prerequisite for *sso*-independent lagging strand synthesis, as the process proceeded well even with

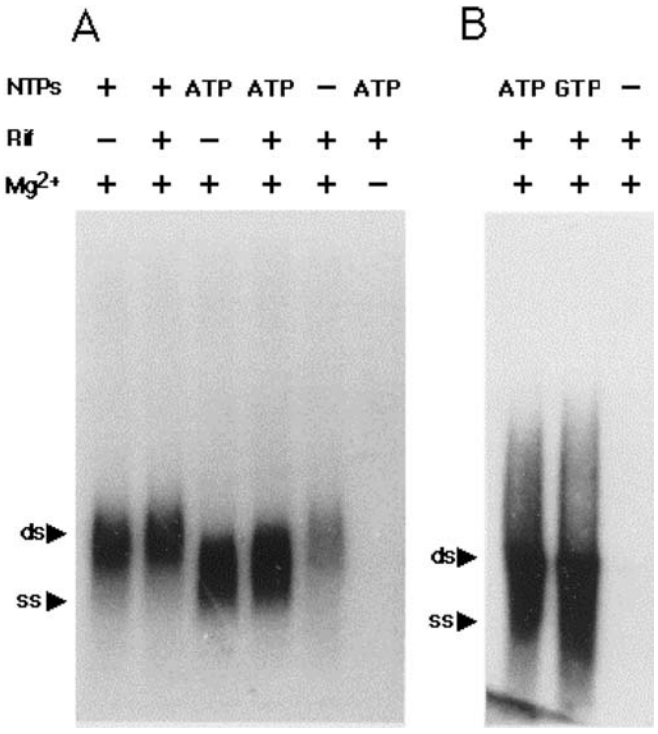

Fig. 5 Effects of rNTPs, rifampicin, and Mg^{2+} on *sso*-independent lagging strand synthesis using cell-free extracts obtained from different growth phases of *S. lividans*. Cell-free extract was prepared from **a** exponential-phase or **b** stationary-phase mycelium. Products obtained from standard reaction mixtures (12.5 µl), containing alkaline-gel-purified M13mp18 ssDNA as template and [α-^{32}P]dCTP, and incubated at 30°C for 30 min, were separated by agarose gel electrophoresis. [α-^{32}P]dCTP incorporation was detected by autoradiography. The presence or absence of NTPs, rifampicin, and Mg^{2+} in the reactions is indicated. The components of the reaction mixture are as follows: *NTPs* 2 mM ATP and 500 µM each of CTP, GTP, and UTP; *ATP* 2 mM ATP only; *GTP* 2 mM GTP only; *Rif* 5 µg rifampicin/ml ; *Mg*$^{2+}$ 7.5 mM $Mg(OAc)_2$. *Arrows* indicate the positions of circular ds and ss plasmid molecules as marked

Table 1 Requirements for *sso*-independent lagging strand synthesis using *Streptomyces lividans* TK21 extracts. The complete reaction was carried out using standard reaction mixture (12.5 µl) with 0.5 µg M13mp18 ssDNA, at 30°C for 30 min. In the complete system, 1.84 and 0.286 pmol [5-^{3}H]dCTP were incorporated into the reactions with exponential-phase cell extract and stationary-phase cell extract, respectively

Reaction mixture	Exponential phase (%)	Stationary phase (%)
Complete	100	100
M13mp18 ssDNA	1	14
Cell-free extract	0	0
dATP, dGTP, dTTP	1	17
ATP dependency		
ATP	76	28
+2 mM CTP	100	100
+2 mM GTP	100	93
+2 mM UTP	100	100
+2 mM ADP	100	93
Mg^{2+} dependency		
$Mg(OAc)_2$	3	9

Table 2 *sso*-independent lagging strand synthesis using *S. lividans* TK21 extracts and poly(dT) as template. The complete reaction was carried out using the standard reaction mixture (12.5 µl) with 0.5 µg poly(dT) and 5 µg rifampicin/mlat 30°C for 30 min. Cell-free extract from exponential-phase mycelium was used. In addition, the incorporation of [α-^{32}P]dATP on M13mp18 ss DNA was 0.112 pmol

Reaction mixture	[α-^{32}P]dATP incorporation ($\times 10^{2-}$ pmol)
Complete	1.08
ATP	0.84
$Mg(OAc)_2$	0.42
poly(dT)	0.37

ATP alone (Fig. 5a, lanes 3, 4). Moreover, it should be noted that the incorporation of labeled dCTP was observed in reactions without NTPs but with Mg^{2+} (Fig. 5a, lanes 5, 6, and Table 1). No incorporation of [α-^{32}P]ATP was observed even with addition of all four NTPs when the cell-free extract from exponential-phase cells was used (data not shown). Likewise, rifampicin-resistant lagging strand synthesis was observed in the extract from stationary-phase cells (Fig. 5b, lane 1). However, in contrast to the extract prepapred from exponential-phase cells, the absence of ATP impeded lagging strand synthesis even when Mg^{2+} was added to the reaction mixture (Fig. 5b, lane 3, and Table 1). GTP could substitute for ATP (Fig. 5b, lane 2) or any other NTP (Table 1). These results indicate that lagging strand synthesis in cell-free extracts prepared from stationary-phase mycelium is dependent on at least one NTP. Lagging strand synthesis on a random nucleotide sequence was also confirmed using poly (dT) as a template, and Mg^{2+}-dependent incorporation of [α-^{32}P] dATP was observed (Table 2).

The incorporation of dNTPs in exponential-phase cells was absolutely dependent on the presence of Mg^{2+} (Fig. 5, lane 6, and Table 1) at an optimal concentration of 7.5 mM (Fig. 6a). The incorporation of [5-^{3}H]dCTP was optimal at pH 7.5 (Fig. 6b) and the maximum incorporation was at 0.5 µg template ssDNA (Fig. 6c) and 12.8 µg cell-free-extract proteins (2 µl; Fig. 6d).

Comparison of in vitro lagging strand synthesis between S. lividans and E. coli extracts. Results of the in vitro study suggested the similarity of *sso*-independent lagging strand synthesis in *S. lividans* cell-free extract to the "general priming" reaction of *E. coli*. The general priming reaction is well characterized in *E. coli* ssDNA phages such as ϕX174 (Kornberg and Baker 1992), which are rifampicin-resistant, *sso*-independent, and require ATP to assemble the protein complex for primer synthesis ("primosome")(Arai and Kornberg 1981b; Arai et al. 1981). In the absence of three NTPs but in the presence of ATP and four dNTPs, the primosome synthesizes deoxyribonucleotide primers under the condition of the general prim-

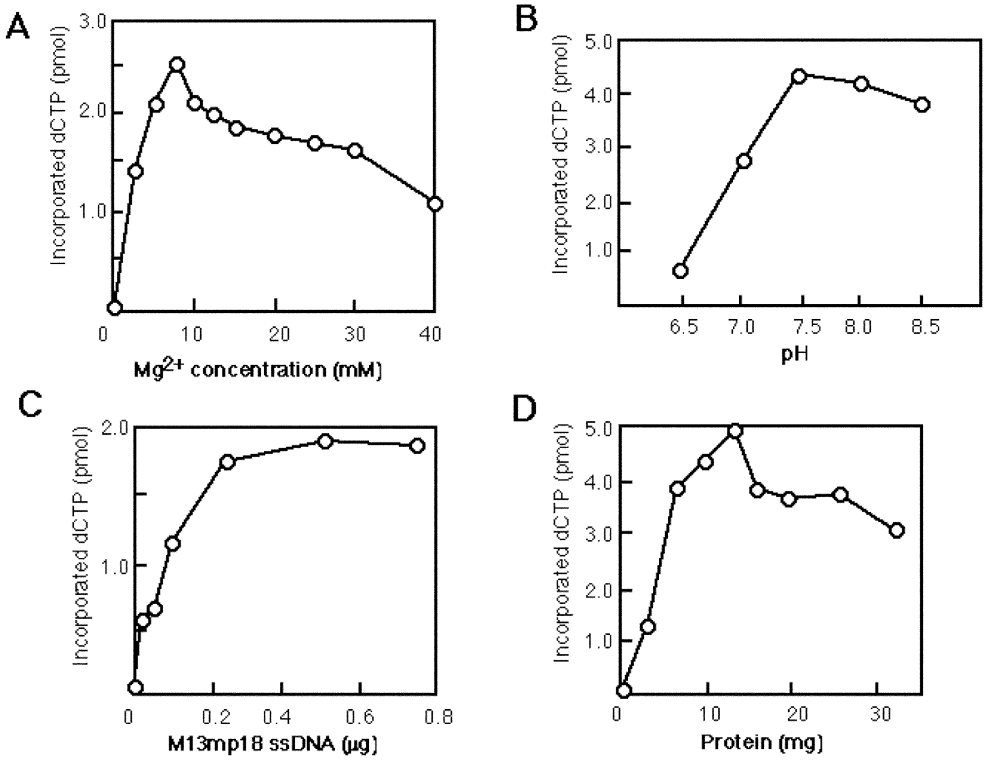

Fig. 6 Effects of **a** Mg^{2+}, **b** pH, **c** amount of ssDNA template, and **d** protein concentration on in vitro lagging strand synthesis using *S. lividans* extracts. Standard mixtures (12.5 µl) contain $Mg(OAc)_2$ at various concentrations, or HEPES–KOH buffer at various pH, or M13mp18 ssDNA or various amounts of cell-free extract prepared from exponential-phase cells. The mixtures were incubated at 30°C for 30 min

Table 3 Effects of NTPs and rifampicin on *sso*-independent lagging strand synthesis in *S. lividans* (from exponential-phase mycelia) and *E. coli* extracts. Reactions were carried out for 30 min at 30°C with *S. lividans* extract and at 37°C using *E. coli* extract.. *NTPs* 2 mM ATP, 500 µM each CTP, GTP and UTP was added. *ATP* 2 mM ATP was added only. *Rifampicin* 5 µg rifampicin/ml was added. In the complete system; 3.72 and 1.84 pmol of [5-^{3}H]dCTP were incorporated in the reactions using *S. lividans* and *E. coli* extract, respectively.

NTPs	Rifampicin	*sso*-independent lagging strand synthesis	
		S. lividans cell-free extract (%)	*E. coli* fraction II (%)
NTPs	–	100	100
NTPs	+	79	37
ATP	+	79	21
None	+	58	8

ing in vitro (Arai et al. 1981). The *sso*-independent lagging strand synthesis in *S. lividans* was compared with that in *E. coli* cell-free extract using alkaline-gel-purified M13mp18 ssDNA as a template. The *sso* of M13mp18 (minus-strand origin) is recognized by host-encoded RNAP in *E. coli* (Kaguni and Kornberg 1982). Table 3 shows that rifampicin decreased the incorporation of [5-^{3}H]dCTP in the *E. coli* extract to 37%, which further decreased to 21% upon the elimination of GTP, UTP, and CTP. Conversely, the withdrawal of three NTPs did not change the level of [5-^{3}H]dCTP incorporation by *S. lividans* extract from exponential-phase mycelium. The residual rifampicin-resistant and NTP-independent (ATP-dependent) activity of the *E. coli* extract, which was relatively lower than that of the *S. lividans* extract, corresponds to general priming. These results suggest that *sso*-independent lagging strand synthesis in *S. lividans* differs from general priming in terms of the NTP requirement for primer synthesis.

Discussion

Copy number control of pSN22. The maintained plasmid copy number could have induced segregational stability of pSN22 derivatives in *S. lividans* even without the *sso*, although there was an accumulation of ssDNA. Lagging strand synthesis of the RC plasmid is initiated by the host-encoding factor (Gruss and Ehrlich 1989), and it was shown that *sso*-independent primer synthesis in *S. lividans* is sufficiently efficient to maintain plasmid copy number. In RC replication, the ssDNA intermediate is produced from a ds plasmid molecule by Rep. In *sso*-dependent lagging strand synthesis, ssDNA is immediately converted to dsDNA; thus, there is no accumulation of ssDNA. If the plasmid lacks *sso*, ssDNAs accumulate since *sso*-independent primer synthesis is less efficient than *sso*-dependent primer synthesis. In contrast, the difference in the efficiency of primer synthesis in the presence or absence of *sso* did not affect the copy number of pSN22 derivatives, suggesting that the amount of ds plasmid molecules is limited whether the plasmid carries *sso* or not. Different from what was observed in pSN22, the deletion of *sso* was previously shown to decrease the plasmid copy number of pIJ101 derivatives to 50–100 copies from the expected 40–300 copies in *S. lividans* (Deng et al. 1988). It should be noted that the copy number of pSN22 is approximately 60 (unpublished data), which is lower than that of the pIJ101 derivatives without *sso*. These observations

suggest that *sso*-independent primer synthesis in *S. lividans* is able to convert 50–100 copies of ssDNA to dsDNA, which is sufficient for maintaining the copy number of pSN22 derivatives, but not that of pIJ101 derivatives.

Rifampicin effect. Different from lagging strand synthesis in *Staphylococcus aureus* (Birch and Khan 1992; Boe et al. 1989; Dempsey et al. 1995) rifampicin failed to inhibit both *sso*-dependent and *sso*-independent lagging strand replications in *S. lividans*, though the strain was found to be rifampicin-sensitive and most probably have a rifampicin-sensitive RNAP. The only known rifampicin-resistant lagging strand replication in gram-positive bacteria occurs in pWVO1 in *Lactococcus lactis*, in which the activation of the *sso* of the plasmid has been suggested to involve two different routes; RNAP-dependent and RNAP-independent (Leenhouts et al. 1991; Seegers et al. 1995) routes. If RNAP is required for the preferential lagging strand replication at *sso*, rifampicin should have inhibited the lagging strand replication of the pSN22 derivative containing *sso* leading to ssDNA accumulation. However, our results suggest otherwise, as RNAP is not required for both in vivo *sso*-dependent and *sso*-independent lagging strand syntheses, suggesting the plausible existence of a dominant RNAP independent priming system in *S. lividans*.

Lagging strand replication in vitro. While *sso* hampered the accumulation of ss plasmid molecules in the cells during in vivo lagging strand synthesis (Fig. 3), there was no difference in complementary strand synthesis in the presence or absence of *sso* during in viitro synthesis (Fig. 4). These results indicate that the cell-free extract might have been deficient in factors that drive in vivo *sso*-dependent lagging strand synthesis. In *E. coli*, a single-stranded DNA binding protein (SSB) is involved in recognition of the signal sequence for primer synthesis of the RC-replicating bacteriophage (Arai and Kornberg 1979). *E. coli* SSB (Stratagene) was added to the reaction mixture of in vitro lagging strand synthesis using *S. lividans* extract, but there was no significant effect on *sso* recognition (data not shown). Further studies are required to characterize the factors initiating lagging strand synthesis at the *sso* of RC plasmids in *S. lividans*.

Figure 5 shows the in vitro replication product of lagging strand synthesis in the exponential and stationary phases of *S. lividans*. Although M13mp18 RF has two *Cla*I sites, digestion by the restriction enzyme failed to produce the expected fragments (data not shown), indicating that the products were neither initiated at a specific site nor completely replicated. It should be noted that M13mp18 cloned into pSN22 derivatives in different orientations all showed ssDNA accumulation, and consequently, no sso activity in *S. lividans* in vivo (unpublished data). Lagging strand synthesis on a random nucleotide sequence was also confirmed using poly(dT) as template (Table 2). In addition, eliminating other dNTPs in the reaction induced no dCTP incorporation (Table 1), implying that dCTP incorporation is independent of nonspecific dNTP incorporation into ssDNA such as mediated by terminal transferase activity. Thus, the molecular structure of the products is partly assumed to be that of dsDNA consisting of short DNA fragments (primers) on template ssDNA. Further studies are required to determine the precise molecular structure of the in vitro replication products.

As priming activity in the extract was resistant to rifampicin and all four dNTPs, and only one NTP was sufficient to carry out lagging strand synthesis in vitro, RNAP is not involved in lagging strand initiation of RC plasmids in *S. lividans*. It is therefore possible that in vitro priming synthesis in *S. lividans* is similar to that in *E. coli* primase. *E. coli* primase and DnaB require ATP and all four dNTPs for the general priming system in vitro (Arai and Kornberg 1981b), and ATP is required for the assembly of the primase complex onto template ssDNA (Arai and Kornberg 1981a). Similarly, ATP or any one of the NTPs, but not rNTPs, was required for efficient, Mg^{2+}-dependent lagging strand synthesis in cell-free extract from stationary-phase cells. However, lagging strand synthesis differs from the "general priming" synthesis in cell-free extract from exponential-phase cells, where ATP was not an absolute requirement. If the primase complex present in *E. coli* is involved in lagging strand synthesis in *S. lividans*, the complex could have been activated during exponential growth. In addition, a similar RNAP-independent lagging strand replication was reported in *B. subtilis*, where synthesis was attributed to the presence of primosome assembly involving DnaE (Bruand et al. 1995). It appears then that the *sso*-independent, rifampicin-resistant lagging strand replication mechanism in *S. lividans* is not unique but is found in a wide variety of gram-positive bacteria.

The specific requirement of ATP or any one of the NTPs for efficient lagging strand synthesis in *S. lividans*, particularly in cell-free extracts prepared from stationary-phase mycelium, suggests a role for ATP as an activator during such synthesis. The difference in NTP requirement between exponential-phase and stationary-phase extracts was not due to contamination by endogenous NTPs because lagging strand synthesis was also observed without NTPs in further purified fractions of the exponential-phase extract using three steps of liquid chromatography, i.e. gel filtration, Q-Sepharose, and heparin (data not shown). The difference in the efficacy of ATP stimulation between exponential-phase and stationary-phase mycelia is indicative of the difference in the components of the replicating pool at each phase, that is, ATP stimulation is dependent on the growth phase of *S. lividans*. Signal transduction, including the protein phosphorylation system, regulating aerial mycelial development and secondary metabolism in *Streptomyces* (Beppu 1995) may contribute to DNA replication in *Streptomyces*.

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