A RNA polymerase with transcriptional activity at 0°C from the Antarctic bacterium *Pseudomonas syringae*

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Abstract A DNA-dependent RNA polymerase was purified from the Antarctic psychrotrophic bacterium *Pseudomonas syringae*. The RNA polymerase showed a typical eubacterial subunit composition with β, β′, α_2 and σ subunits. The subunits cross-reacted with antibodies raised against holoenzyme and the individual subunits of the RNA polymerase of *Escherichia coli*. However, the enzyme was considered unique, since unlike the RNA polymerase of mesophilic *E. coli* it exhibited significant and consistent transcriptional activity (10–15%) even at 0°C. But, similar to the enzyme from the mesophilic bacterium, the RNA polymerase from *P. syringae* exhibited optimum activity at 37°C. The study also demonstrates that the RNA polymerase of *P. syringae* could preferentially transcribe the cold-inducible gene *cspA* of *E. coli* only at lower temperatures (0–22°C). The polymerase was also observed to be relatively more rifampicin-resistant during transcription at lower temperature.

Key words: Antarctic bacterium; Psychrotroph; Transcription at low temperature; RNA polymerase

1. Introduction

Psychrotrophic bacteria and yeasts from Antarctica have evolved various strategies to survive and grow at low temperatures (0–30°C) [1–3]. In an attempt to understand the mechanism by which these microorganisms transcribe and regulate gene expression at the extremely cold temperatures of Antarctica [4], we attempted to characterise the transcription process and gene regulation in the Antarctic psychrotrophic bacteria. In eubacteria, the enzyme RNA polymerase plays a crucial role in the control of gene expression [5]. This enzyme has a fairly constant subunit composition consisting of the β′, β and α_2 units which constitute the core RNA polymerase and several σ factors which are the key regulators for promoter selection during various growth conditions and developmental stages [6–9]. However, the bacterial RNA polymerases which have been characterised so far are from mesophiles [7,8,10] which do not exhibit any transcriptional activity at low temperature such as 0°C. Therefore, there is hardly any information available as to how RNA polymerase functions at 0°C, which is important for organisms growing at low temperatures. Therefore, it would be important to isolate, purify and characterise the DNA-dependent RNA polymerase from psychrotrophic bacteria. In this paper, we describe the isolation, purification and some of the characteristics of the major RNA polymerase from the psychrotrophic bacterium *Pseudomonas syringae*, which has been used as a model system in our laboratory to study various aspects of cold adaptation [3].

2. Materials and methods

2.1. Bacterial strain and growth

The psychrotrophic bacterium *P. syringae* Lz4W was isolated from a soil sample of Schirmacher Oasis, Antarctica [11]. The strain was maintained on ABM agar (0.5% peptone, 0.2% yeast extract and 1.5% agar) at 4°C and was routinely grown in ABM broth at room temperature (~22°C) as described earlier [11].

2.2. Purification of RNA polymerase

P. syringae RNA polymerase was purified by a procedure based on Burgess and Jendrisak [12] followed by a final purification step on a heparin-Sepharose CL-6B column [13]. Briefly, crude RNA polymerase was prepared from cell lysate by Polymin P (polyethyleneimine, PEI) precipitation and extraction of the precipitate (PEI pellet) with 0.5–1.0 M NaCl in TGED buffer (10 mM Tris, pH 8.0, 5% glycerol, 0.1 mM EDTA and 0.1 mM DTT). The extract of the precipitate (PEI pellet eluant) was further purified by Sephacryl S-300 chromatography. The active fraction from the gel filtration step was subsequently purified by heparin-Sepharose CL-6B chromatography as described by Kumar and Chatterji [13]. The purified RNA polymerase was dialysed either against TGED to remove salts, or in TGED containing 50% glycerol for long-term storage at −20°C.

2.3. Assay of RNA polymerase activity

RNA polymerase activity was measured with calf thymus DNA or *P. syringae* DNA as the templates following the method of Lowe et al. [14]. The typical assay mixture (40 μl) contained 40 mM Tris-HCl (pH 7.9), 0.2 M NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA (pH 8.0), 0.2 mM each of ATP, GTP, CTP, 0.05 mM UTP, 0.5 μCi of [^{3}H]UTP (specific activity 10.9 Ci/mmol) and 35 μg/ml DNA template. The assay was generally carried out for 30 min with RNA polymerase from *P. syringae* or for 15 min with *Escherichia coli* RNA polymerase. The temperature of the reaction was varied depending on the requirement of the experiment.

2.4. Assay of thermal stability

The thermal stability of RNA polymerase was studied by preincubating the purified enzymes (protein concentration 1 mg/ml) at desired temperatures and then assaying the enzyme activity at 30°C (for *P. syringae*) or at 37°C (for *E. coli*) with calf thymus DNA as template. The activity of the unincubated enzyme was taken as 100%.

2.5. Protein determination and SDS-PAGE analysis

Protein concentration was estimated by the method of Lowry et al. with bovine serum albumin (BSA) as the standard [15] and the proteins were analysed by electrophoresis in polyacrylamide gels, containing sodium dodecyl sulphate (SDS-PAGE) following the method of Laemmli [16].

2.6. Western blot analysis

The immunogenic similarities between *P. syringae* RNA polymerase and *E. coli* RNA polymerase were studied by Western blot analysis [17] using rabbit antisera raised against *E. coli* RNA polymerase hol-

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oenzyme, core enzyme and σ^{70} subunit. The antibodies (Abs) were raised in rabbits in the laboratory following standard methods [17]. Antibodies obtained from other laboratories, such as Ab against core RNA polymerase (Dr M. Chamberlin, University of California, Berkeley, CA, USA), Abs against β, β′ and σ subunits (Dr H. Heumann, Max Planck Institute for Biochemie, Martinsried, Germany and Dr A. Ishihama, National Institute of Genetics, Japan) were also used in the study. All the antibodies were found to be specific for the respective subunits. The detection of the immunogenic reaction was carried out either with alkaline phosphatase-conjugated anti-rabbit immunoglobulin G or with ^{125}I-labelled protein A.

2.7. Assay for promoter specificity

The promoter specificity of *P. syringae* Lz4W RNA polymerase was monitored by in vitro production of transcripts from pLz4W (an indigenous plasmid of *P. syringae*), T7 (A_2) promoter, the *rrnB* (P1) promoter, and the *csp*A promoter of the cold shock gene of *E. coli*. The T7 (A_2) promoter was present on plasmid pAR1539 [18,19]. The *rrnB* (P1) promoter was on plasmid pRP1 which was constructed by cloning a 349 bp DNA fragment of pKK3535 [20] into the *Sma*I site of pUC18 [19,21]. The *E. coli* cold shock gene, *cspA*, was on plasmid pJJG02, a derivative of pJJG01 [22] from which the whole of *cspA* could be retrieved as a 1.0 kb *Eco*RI-*Hin*dIII DNA fragment. Transcription analyses of *cspA* were made both on the 1.0 kb linear DNA and on the supercoiled plasmid pJJG02 as templates. The plasmid template had the replication-inhibitor-promoter P4 of *oriV* which produces a 108 bases long transcript of RNA I [23]. Transcription assay was carried out in a reaction mixture similar to the standard conditions as mentioned earlier except that 10 μCi of [α-^{32}P]UTP was used instead of [^{3}H]UTP. The RNase inhibitor RNasin (2 units) was also included in the reaction mixture. After 30 min of incubation the ^{32}P-labelled transcripts were precipitated with 2.5 M ammonium acetate and 2.5 volumes of absolute ethanol. The transcripts were separated by electrophoresis either on a 1.5% agarose gel containing formaldehyde, or on an 8% polyacrylamide-8 M urea gel, and subsequently detected by autoradiography [17]. The intensity of signals from transcripts was quantified with a phosphorimager (Molecular Dynamics). The size of the run-off transcripts was determined by using the mobility of the ϕX174RF *Hae*III digest and 23S, 16S and 4S RNAs as standards on the denaturing gel.

The susceptibility of the RNA polymerase-DNA binary complex to rifampicin was studied by incubating the plasmid DNA (pJJG02) (0.6 pmol) with the enzyme (4 pmol) at low (10°C) and high (30°C) temperatures, and analysing the transcripts produced at various time points by adding the ribonucleotides, [α-^{32}P]UTP and rifampicin (4.8 pmol), simultaneously, following the method described above.

2.8. Amino acid sequencing

The NH_2-terminal amino acid sequence of the α subunit was determined by the Edman degradation method on an automated protein sequencer (Applied Biosystem, Model 473 A), following transfer of the protein from SDS-polyacrylamide gel onto a PVDF membrane.

3. Results and discussion

3.1. Purification of RNA polymerase from P. syringae

The DNA-dependent RNA polymerase from the psychrotroph *P. syringae* was purified by a three-step procedure involving PEI precipitation, gel filtration (Sephacryl S-300) and heparin-Sepharose affinity chromatography. The specific activity of the pure enzyme was enhanced about 170-fold (Table 1). The polymerase so purified was about 90–95% pure as judged by SDS-PAGE analysis.

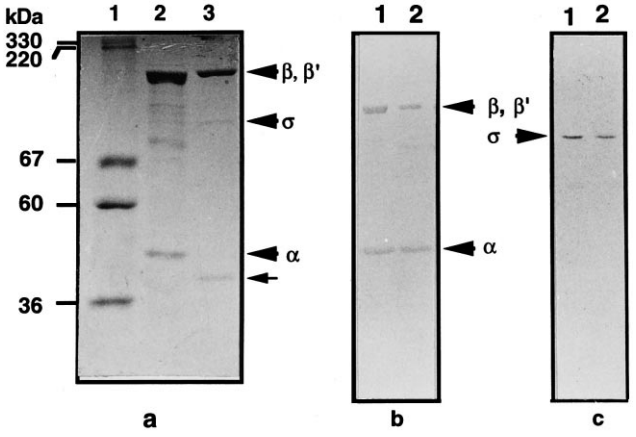

Fig. 1. Analysis of *P. syringae* RNA polymerase. a: 7.5% SDS-PAGE. Lane 1, high molecular weight marker proteins showing thyroglobulin (330 kDa), ferritin (220 kDa), albumin (67 kDa), catalase (60 kDa) and lactate dehydrogenase (36 kDa); lane 2, purified RNA polymerase of *P. syringae*; lane 3, purified RNA polymerase of *E. coli*. The arrow indicates the α subunit of *E. coli* RNA polymerase. b,c: Western blot analysis. Hybond C (Amersham) membrane filters containing *P. syringae* RNA polymerase were probed with polyclonal antibodies raised against *E. coli* core RNA polymerase (b) and against *E. coli* σ^{70} (c). The immunogenic reaction was detected by alkaline phosphatase-conjugated secondary antibody. Both lanes of b and c contain *P. syringae* RNA polymerase with approximately 20 and 12 μg of protein, respectively.

3.2. Subunit composition of the RNA polymerase

Gel filtration on Sephacryl S-300 column indicated that the molecular mass of the RNA polymerase from *P. syringae* was similar to that of the RNA polymerase from *E. coli* which had a molecular mass of about 470 kDa (data not shown). The enzyme had a typical eubacterial subunit composition with β′, β, α_2 and σ subunits (Fig. 1a). The tentatively identified β, β′ subunits (based on electrophoretic mobility on SDS-PAGE) had a molecular mass similar to the corresponding subunits of *E. coli* RNA polymerase. However, the α subunit from *P. syringae* was larger (apparent molecular mass 45 kDa) than that of *E. coli* α subunit (37 kDa), thus confirming an earlier observation that the α subunits from *Pseudomonas* species are generally larger than that of *E. coli* [24]. However, the NH_2-terminal 16 amino acids of the α subunit of *P. syringae* (MQISVNEFLTPRHIDV) were found to be similar to those of *E. coli* (MQGSVTEFLKPRLVDI) [25] and *P. putida* (MQFXVNEFLTPRXIDQ; X represents an unidentified amino acid residue)[26].

Antibodies to the core RNA polymerase of *E. coli* cross-reacted with all the core subunits (β′, β and α subunits) of *P.*

Table 1
Purification of RNA polymerase from the Antarctic bacterium *P. syringae*[a]

Purification step	Volume (ml)	Protein (mg)	Total activity (U)	Specific activity[b] (U/mg of protein)	Purification (fold)
Crude lysate	300	4530	ND[c]	ND[c]	–
PEI pellet eluant	90	756	510	0.67	1
Sephacryl S-300	22.5	160	1404	8.8	13.1
Heparin-Sepharose	17.2	19.1	2185	114	170

[a]From 40 g of wet cells of *P. syringae*.
[b]Activity (U) expressed as nmol of [^{3}H]UMP per mg of protein at 22°C under the assay conditions.
[c]Because of the presence of nucleases in the crude lysate, these values were not determined (ND).

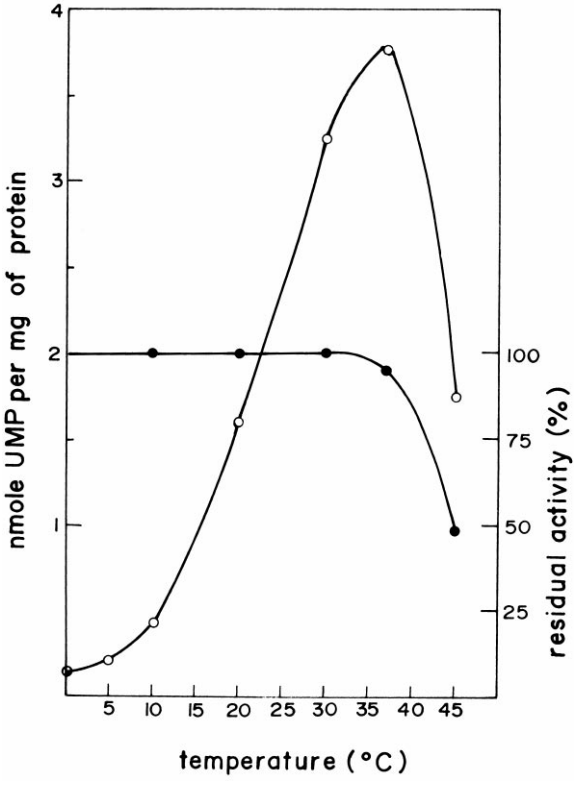

Fig. 2. Activity (○) and thermal stability (●) of the *P. syringae* RNA polymerase. Polymerase activity in the presence of calf thymus DNA and thermal stability were measured as described in Section 2. Stability is expressed as percentage of residual activity of the RNA polymerase at the respective temperatures.

syringae RNA polymerase (Fig. 1b) thus indicating that the subunits of RNA polymerase from these two eubacteria are probably structurally homologous. The specific antibodies against individual subunits, such as β and β′, also cross-reacted with the corresponding subunits of *P. syringae* (data not shown). The antibody against the σ^{70} subunit of *E. coli* also cross-reacted with the σ subunit of *P. syringae* (Fig. 1c) which had a similar electrophoretic mobility corresponding to 87 kDa protein, as observed for *E. coli* σ^{70} on SDS-PAGE. It was also noted, from the densitometer scanning data of Coomassie blue-stained protein bands, that the purified RNA polymerase from *P. syringae* was about 25–30% saturated with the σ subunit.

3.3. General properties of the RNA polymerase from *P. syringae*

Transcription by RNA polymerase of *P. syringae* was dependent on a DNA template and exhibited optimum activity in the presence of 10 mM Mg^{2+}, 0.2 M NaCl and at a pH of 7.9 similar to *E. coli* RNA polymerase. Maximum activity was observed between 30 and 37°C (Fig. 2) and this was unexpected since the psychrotroph grows optimally around 22°C. The transcription efficiency at 22°C was about 60–65% of its maximal activity when *P. syringae* DNA was used as template, but was about 45% when calf thymus DNA was used as a template (data not shown). At 0°C, the efficiency of transcription was about 10–15% with *P. syringae* DNA as the template, whereas with the calf thymus DNA as the template it was only about 4%. The RNA polymerase of *E. coli* under similar conditions did not exhibit any transcriptional activity. An Arrhenius plot of the data from Fig. 2 indicated that below 5°C and above 25°C the rate of transcription is slightly affected (data not shown).

3.4. Heat-labile nature of the RNA polymerase

The RNA polymerase from *P. syringae* was heat-labile compared to the RNA polymerase from the mesophilic *E. coli*. It lost more than 50% of its activity at 45°C within 30 min whereas the *E. coli* enzyme under identical conditions retained almost 100% of its activity. Further, the enzyme was totally inactivated at 56°C (Fig. 2). A heat-labile RNA polymerase with subunit composition similar to the *E. coli* polymerase was reported earlier from a marine *Pseudomonas* BAL-31 [27]. This enzyme, unlike the *P. syringae* enzyme, exhibited optimal activity at 28°C (the optimal growth temperature of the bacterium) and was more heat-labile, losing 50% of the activity on exposure to 41°C for 10 min. It is, however, important to note that the heat stability of the enzyme may be influenced by several factors, including the concentration of the enzyme used during thermal preincubation. Therefore a direct comparison between the two enzymes was not possible in the present study. However, the enzymes of psychrotrophic and psychrophilic bacteria and yeasts are generally known to be heat-labile [2,3].

3.5. In vitro specificity of transcription by RNA polymerase from *P. syringae*

It was observed that the RNA polymerases of *P. syringae* and *E. coli* differ in their promoter specificity of transcription in vitro. For example, the polymerase of *P. syringae* was capable of transcribing the cryptic plasmid pLz4W of *P. syringae* [28] to produce two major transcripts of 1.4 kb and 0.6 kb

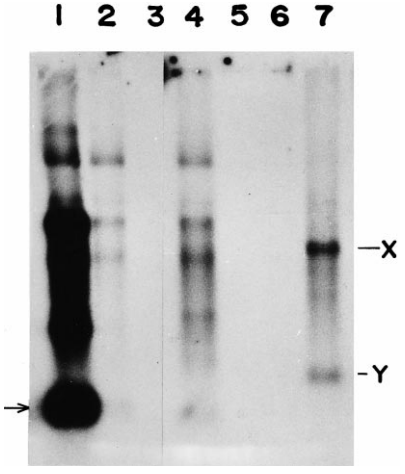

Fig. 3. In vitro transcription products of *P. syringae* and *E. coli* RNA polymerases from T7 (A_2) (lanes 1–5) and pLz4W promoters (lanes 6 and 7). Transcription was carried out in the presence of [α-^{32}P]UTP and the transcripts were analysed on 1.5% formamide agarose gel. No heparin was used in these experiments and therefore the transcripts were the products of multiple initiation. Lanes 1 and 3, transcription with *E. coli* holo-RNA polymerase at 37°C and 0°C, respectively; lane 2, transcription with *E. coli* core RNA polymerase at 37°C; lanes 4 and 5, transcription with *P. syringae* RNA polymerase at 30°C and 0°C, respectively; lane 6, transcription by *E. coli* holo-RNA polymerase at 37°C; lane 7, transcription by *P. syringae* RNA polymerase at 30°C. The arrow indicates the expected 310 nt run-off transcript from T7 (A_2) promoter, and X and Y indicate the two transcripts produced from plasmid pLz4W.

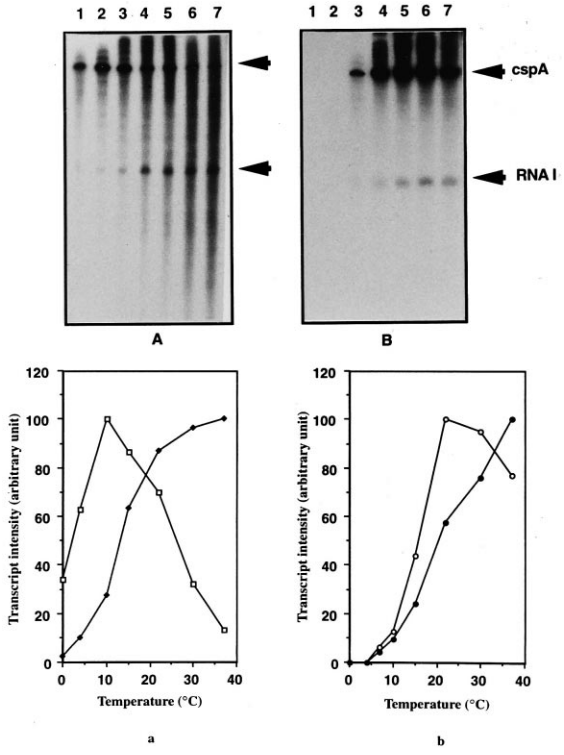

Fig. 4. Analysis of in vitro transcription products from the plasmid pJJG02 containing the gene for *cspA*. The transcription was carried out in the presence of [α-^{32}P]UTP and the transcripts were analysed on 8% polyacrylamide-8 M urea gel. No heparin was used as in Fig. 3. The transcripts of *cspA* and RNA I are marked only by arrows. Lanes 1–7 contain the transcripts produced at 0, 4, 10, 15, 22, 30 and 37°C by RNA polymerase of *P. syringae* (A) and of *E. coli* (B). For quantitation, the densitometer scanning data of the transcripts of *cspA* (□, ○) and RNA I (■, ●) were plotted for *P. syringae* (a) and *E. coli* (b), respectively. The values are the average of three separate experiments. The highest intensity of the transcripts was taken as 100 in all the cases.

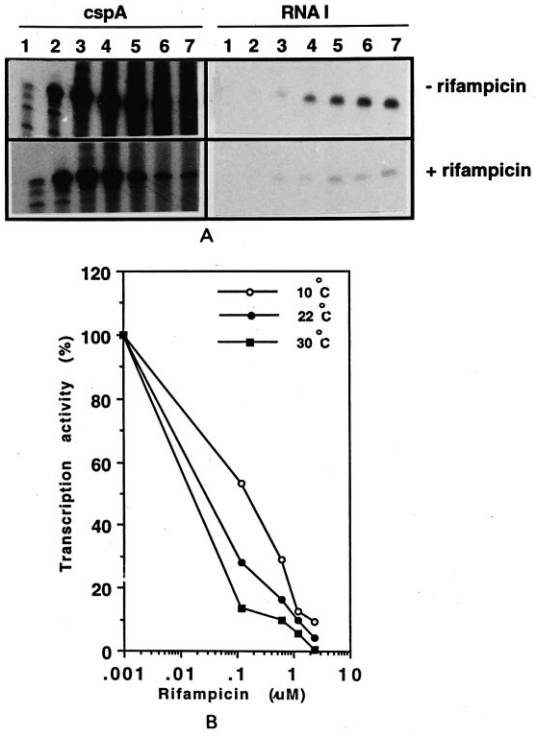

Fig. 5. A: Autoradiogram showing the amount of transcripts of *cspA* and RNA I produced by *P. syringae* RNA polymerase at 0, 4, 10, 15, 22, 30 and 37°C (lanes 1–7, respectively) in the absence or presence of rifampicin (0.12 μM). For the sake of clarity *cspA* and RNA I transcripts are shown from two different autoradiograms. B: Effect of various concentrations of rifampicin (0.12, 0.6, 1.2 and 2.4 μM) on transcription of *cspA* at 10°C (○), 22°C (●) and 30°C (■). The amount of transcripts produced in the absence of rifampicin has been taken as 100%, at the respective temperatures.

(Fig. 3), but the polymerase of *E. coli* failed to do so. Similarly, the RNA polymerase from *E. coli* could transcribe from the *rrnB* P1 promoter but the polymerase from *P. syringae* could not (data not shown). In contrast, the phage promoter T7 (A_2) was recognised by both polymerases in the in vitro transcription assay (Fig. 3). However, this promoter was transcribed weakly by the *P. syringae* RNA polymerase but strongly by the *E. coli* RNA polymerase (Fig. 3, lanes 4 and 1). Neither of the two polymerases could transcribe from the promoter at 0°C (Fig. 3, lanes 3 and 5).

The in vitro transcription from the promoter of *cspA*, the cold shock gene of *E. coli* [22], was more interesting. When a 1.0 kb linear DNA fragment containing *cspA* was used as a template, both the *P. syringae* and the *E. coli* RNA polymerases produced a major transcript of the expected length of about 400 bases [29,30] only between 20°C and 37°C (data not shown). At 0°C neither of the enzymes produced this transcript from the linear DNA fragment. However, when the supercoiled plasmid pJJG02 containing the *cspA* gene was directly used for transcription analysis, the RNA polymerase of *P. syringae* produced more transcript of *cspA* at lower temperatures (0–22°C) (Fig. 4). In contrast, the transcript of RNA I originating from the replication inhibitor promoter P4 (*oriV*) was produced more at higher temperatures (22–37°C) only. Interestingly, *E. coli* RNA polymerase did not transcribe the *cspA* between 0 and 5°C but could transcribe both *cspA* and RNA I with increased efficiency at higher temperatures (22–37°C) from the same supercoiled plasmid (Fig. 4). The fact that *E. coli* RNA polymerase can transcribe *cspA* in vitro between 22 and 37°C with equal efficiency supports the earlier observation that the upregulation of *cspA* at low temperature in vivo may depend on some other cellular factors [29].

The results indicate that the RNA polymerase of the psy-

Table 2
Rifampicin sensitivity of *P. syringae* RNA polymerase

Temperature	Residual activity of RNA polymerase (%)[a]			
	0.1 μg/ml rifampicin		1 μg/ml rifampicin	
	Calf thymus DNA	*P. syringae* DNA	Calf thymus DNA	*P. syringae* DNA
0°C	37	35	22	20
22°C	10	7	5	3

[a]Data are the average of two independent experiments.

chrotrophic *P. syringae* could discriminate between the promoters of *cspA* and RNA I at low and high temperatures (0–37°C) (Fig. 4). Although there are differences in the promoter sequence of the *cspA* (−35 and −10 sequences being TTGCAT and CTTAAT, respectively) and RNA I (−35 and −10 sequences being TTGAAG and TACACT, respectively), these sequences are no more divergent than observed in others, such as T7 (A_2) and *rrnB* P_1 promoters. The *cspA* promoter, however, contains a Y-box or CCAAT sequence motif [29]. There is also a possibility, however small, that certain co-purified factor(s), present in minute quantity in the RNA polymerase fraction of *P. syringae*, might be responsible for the promoter discrimination. However, an important additional finding of the study is that the supercoiled structure of template DNA has a crucial role in transcription of *cspA* at lower temperature.

3.6. Rifampicin sensitivity of the RNA polymerase of P. syringae

During the course of our study on general properties of the RNA polymerase of *P. syringae* it was observed that the purified polymerase was sensitive to rifampicin (0.1 μg/ml inhibited 90–95% activity) at 22°C (Table 2) but interestingly, it was relatively less sensitive to rifampicin at 0° and 5°C. At 0°C, the RNA polymerase retained about 35–37% and 20–22% of its activity in the presence of 0.1 and 1 μg/ml of rifampicin, respectively (Table 2). The lower rifampicin sensitivity at lower temperatures was also observed during transcription from the specific promoters, such as for those of *cspA* and RNA I of *E. coli* (Fig. 5). For example, 50% inhibition (I_{50}) of transcription of *cspA* at 10, 22 and 30°C was observed at concentrations of 165 nM, 75 nM and 60 nM of rifampicin, respectively (Fig. 5b). It was also noted that a binary complex of the plasmid pJJG02 (containing *cspA* and RNA I promoters) and the *P. syringae* RNA polymerase formed at higher temperature (30°C) was more susceptible to attack by rifampicin than the binary complex formed at lower temperature (10°C) (data not shown). Whether this reflects the differential stability of the enzyme-promoter complex at different temperatures and the K_a (association constant) of rifampicin to such a complex requires more detail study. It was postulated earlier that the more stable a complex, the lower is the K_a [31].

4. Concluding remarks

In conclusion, the RNA polymerase from the psychrotrophic *P. syringae* was found to be similar to other eubacterial RNA polymerases, such as that of *E. coli*, in terms of subunit composition and immunogenic cross-reactivity. The enzyme exhibited optimum activity at 37°C in vitro, similar to mesophilic enzymes. However, the purified enzyme had transcription ability at lower temperatures (0–4°C), unlike mesophilic *E. coli* RNA polymerase. The activity at lower temperatures could be due to the intrinsic property of the enzyme. Additionally, the cold-active RNA polymerase of *P. syringae* could discriminate between the promoters of *cspA* and RNA I of *E. coli* during in vitro transcription at low and high temperatures. The relatively increased rifampicin resistance of the *P. syringae* RNA polymerase during transcription at low temperatures may reflect a change in conformation of the enzyme. Proof for such an assumption would require further study of the enzyme.

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