Depression of streptomycin production by *Streptomyces griseus* at elevated growth temperature: studies using gene fusions

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***Streptomyces griseus* ATCC 12475 fails to produce streptomycin when grown at 34 °C or above, although growth is appreciable up to at least 37 °C. This depression of streptomycin production at elevated growth temperature is manifest equally in liquid and on solid, and with complex and minimal, media. We report studies with gene fusions of the reporter genes *aph* or *xylE* to restriction fragments containing the streptomycin biosynthesis promoter *PstrB1*. *aph* constructs were in high, and *xylE* constructs in low, copy number vectors. Two *strB1* promoter fragments were used, one requiring activation by the pathway-specific activator StrR of *S. griseus*, the other reportedly activator independent. *PstrB1* expression in the *aph* constructs in *S. griseus* and in *S. lividans* was significantly reduced at 37 °C compared to 30 °C. Some of this reduction could be explained by lower plasmid copy number at the higher temperature, but *strR*-dependent expression was clearly temperature controlled. Using the *xylE* reporter system, the temperature dependence of *PstrB1* expression was confirmed but, surprisingly, the *strR* dependence of the two promoter fragments differed from that observed in the multicopy *aph* constructs. These data identify a temperature-dependent promoter which may contribute to the depressive effect of elevated growth temperature on streptomycin production.**

Keywords: antibiotic production, antibiotics, regulation of secondary metabolism, *Streptomyces*, streptomycin

INTRODUCTION

Secondary metabolite production in streptomycetes is subject to complex physiological and environmental controls (Martín & Demain, 1980). A frequent finding is that growth at elevated temperature leads to a marked depression in production or none at all (Votruba & Vanek, 1989). Three hypotheses accounting for this effect postulate at the elevated growth temperature: (i) lowered expression – which may involve reduction in transcription, translation initiation, or mRNA stability, or any combination of these – of one or more genes whose products are needed for secondary metabolite synthesis; (ii) *in vivo* heat lability of one or more proteins involved in secondary metabolite production; and/or (iii) diminished

availability of precursors due, in some way, to the altered physiological state of the culture. In principle, hypothesis (i) can be distinguished from (ii)/(iii) by its testable prediction of diminished gene expression at the elevated temperature.

One of the best-characterized secondary metabolite systems in terms of regulation is streptomycin biosynthesis and resistance in *Streptomyces griseus* (Retzlaff *et al.*, 1993; Piepersberg, 1994). *str* genes, encoding enzymes of streptomycin synthesis, are activated by the presumably pathway-wide activator StrR (Distler *et al.*, 1987b, 1992). Current models suggest that physiological and environmental parameters determine, through a regulatory cascade, the expression of *strR* and thus the internal level of StrR, which in turn controls the extent of activation of the biosynthetic genes (Vujaklija *et al.*, 1993; Piepersberg, 1994). We have studied regulation at the promoter *PstrB1*, which directs expression of the *strB1* gene; the gene order

Abbreviations: APH, aminoglycoside 3'-phosphotransferase; CO, catechol oxygenase.

in this region of the *S. griseus* chromosome is *strD-strR-aphD* (= *strA*)-*PstrB1-strB1*, where *aphD* encodes the major streptomycin resistance enzyme, the 6-phosphotransferase (Retzlaff *et al.*, 1993). We report here the use of fusions of *PstrB1* to the reporter genes *aph* and *xylE* in distinguishing between the above hypotheses (i) and (ii)/(iii) as to the nature of the effect of elevated growth temperature on streptomycin production.

METHODS

Bacterial strains and plasmids. The bacterial strains used were *S. griseus* ATCC 12475 and *S. lividans* TK54 (Hopwood *et al.*, 1983), a *his leu* *Spc*^r derivative of 1326. The plasmids used are listed in Table 1, and the structures of the gene fusions employed are shown in Fig. 1.

Media and growth conditions. Complex media R5 and YEME, and minimal media MM and NMMP, were as in Hopwood *et al.* (1985); modified YEME was as in Fazeli *et al.* (1995). Spore suspensions were prepared according to Hopwood *et al.* (1985).

The following growth regimes were used with liquid media. For the estimation of streptomycin production in liquid media, *S. griseus* spores were inoculated either directly into the final modified YEME or MM medium, or used to provide a vegetative preculture as follows. Spores were inoculated into modified YEME and grown at 30 °C to provide a first preculture, which was inoculated either into the final modified YEME or into MM to provide a second preculture, the latter being inoculated into the final MM. The modified YEME or MM cultures were incubated at the appropriate temperature, the mycelium being sampled at intervals for estimation of dry weight and the supernatant medium assayed for streptomycin. (Further details are in Fazeli *et al.*, 1995.) For the estimation of streptomycin yield at different temperatures, incubation was continued for up to 8 d; the streptomycin level recorded was

either the maximum or that attained at the end of incubation. For the growth of cultures to be assayed for APH (amino-glycoside 3'-phosphotransferase, encoded by *aph* from Tn5), inocula consisted of frozen aliquots of YEME precultures. These were inoculated into NMMP medium containing glucose and thiostrepton. Cultures were incubated at 30 °C or 37 °C for 72 h (*S. lividans*) or 48 h (*S. griseus*); preliminary experiments showed that these time points gave the maximum enzyme activities, up to 30 % above those of samples 24 h on either side. Mycelia were harvested, washed with phosphate buffer, resuspended in Tris/Mg²⁺/NH₄⁺ buffer, and disrupted ultrasonically, cell debris being removed by centrifugation to provide protein extracts. (Further details are in Lindley *et al.*, 1995.) All liquid cultures in flasks were grown with coiled stainless steel springs.

The following growth regimes were used with solid media. For the estimation of streptomycin production on solid media, 10⁷ *S. griseus* spores were inoculated on to 9 cm diameter sterile dialysis membranes (Gallenkamp) on the surface of modified YEME or MM agar plates. The membranes were removed at appropriate times, the mycelium being collected for estimation of cell dry weight (carried out as for liquid cultures) and the underlying agar assayed for streptomycin (see below). For cultures to be assayed for CO (catechol oxygenase, the product of the *xylE* gene), spores were inoculated on to dialysis membranes on the surface of R5 plates supplemented with appropriate antibiotics. In this case, one membrane for each combination of plasmid pairing and temperature was removed daily; the mycelium was resuspended, disrupted ultrasonically, and freed of cell debris by centrifugation to provide protein extracts. (Further details are in Lindley *et al.*, 1995.)

Estimation of streptomycin in culture media. For liquid cultures, this was performed by bioassay as in Vallins &

Table 1. Plasmids used in this work

For pIJ702, pIJ486 and pIJ2839, see Hopwood *et al.* (1985), Ward *et al.* (1986), and Ingram *et al.* (1989), respectively. All the other plasmids are described in Lindley *et al.* (1995), and their construction is depicted in Fig. 1.

Plasmid		Plasmid vector/replicon in <i>Streptomyces</i>	Plasmid vector/replicon in <i>E. coli</i>	Inserted fragment bearing the <i>PstrB1</i> promoter	Reporter gene	Inserted fragment bearing an <i>strR</i> allele
pHL623	} Fig. 1(b)	pIJ486	pBR322	None	<i>aph</i>	None
pHL708		pIJ486	pBR322	'Short' fragment	<i>aph</i>	None
pHL614		pIJ486	pBR322	'Long' fragment	<i>aph</i>	None
pHL723	} Fig. 1(c)	pIJ702	None	None	<i>aph</i>	None
pHL728		pIJ702	None	'Short' fragment	<i>aph</i>	None
pHL714		pIJ702	None	'Long' fragment	<i>aph</i>	None
pIJ2839	} Fig. 1(d)	SCP2*	pBR322	None	<i>xylE</i>	None
pHL308		SCP2*	pBR322	'Short' fragment	<i>xylE</i>	None
pHL314		SCP2*	pBR322	'Long' fragment	<i>xylE</i>	None
pES2	} Fig. 1(e)	pIJ702	pACYC184	None	None	None
pES2 <i>strR</i>		pIJ702	pACYC184	None	None	<i>strR</i>
pES2 <i>strRA</i> 7		pIJ702	pACYC184	None	None	<i>strRA</i> 7

Fig. 1. Gene fusions used in this work. (a) Region of the *S. griseus* chromosome employed. Restriction sites: B, *Bam*HI; E, *Eco*RI; N, *Nru*I. Transcription startsites are denoted by arrows. The *strΔ7* fragment is described in O'Neill (1992). (b) pH623, 708 and 614. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; H, *Hind*III; X, *Xho*I. — and — represent DNA derived from pIJ486 and pBR322 respectively (Lindley *et al.*, 1995). (c) pHL723, 728 and 714. Restriction sites: Bg, *Bgl*II; P, *Pst*I; X, *Xho*I. Pi, Pii and Piii are three of the *Pst*I sites within pH623, 708 and 614 (the *Bam*HI site in pH623 into which the 'short' and 'long' fragments are cloned lies between Pi and the 5' end of *aph*). (d) pIJ2839, 308 and 314. Restriction sites: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III. — here represents DNA of *Streptomyces* origin (including the replication/maintenance region of SCP2*); — represents DNA of Gram-negative origin (including the *xyIE* reporter gene and the replication region of pBR322). (e) pES2, pES2strR and pES2strΔ7. Restriction sites: B, *Bam*HI; Bc, *Bcl*I; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; Sp, *Sph*I. —, — and — represent DNA derived from pIJ486, pACYC184 and pGM160, respectively.

Baumberg (1985) with the modifications of Fazeli *et al.* (1995). For cultures on solid media, 7 mm plugs of agar were removed from the medium underlying a mycelial mat on a dialysis

membrane, and transferred to similarly sized wells in bioassay plates. The bioassay was carried out as in Fazeli *et al.* (1995).

Enzyme assays. APH, CO and protein were assayed as in Lindley *et al.* (1995). As described in that reference, CO activity was barely detectable in liquid-grown cultures, and for its estimation mycelia had to be grown by inoculation of spores on to a set of dialysis membranes placed on R5 agar appropriately supplemented with antibiotic(s), as indicated above. Activities shown in Table 3 were the maximum recorded with a particular set of membranes, usually in samples harvested at 3–4 d. Experiments (results not shown) showed that neither APH nor CO activities were reduced by holding protein extracts at 37 °C for up to 2 h, far longer than the length of any incubation employed in enzyme assays in this work. A further control was performed to check whether CO activity fell *in vivo* on incubation of mycelia at 37 °C. Cultures known to show high levels of CO (*S. lividans* carrying pHL308 and pH314 together with pES2strR – see below) were incubated on solid media, in the usual way for CO assay, for 3 d at 30 °C and then shifted to 37 °C. No consistent decrease in CO activities was found. Statistical tests on enzyme specific activities (standard deviation of means, *t*-test) were carried out according to Parker (1973).

RESULTS

Streptomycin production by *S. griseus* at elevated growth temperature in liquid media

S. griseus ATCC 12475 was grown in liquid complex or minimal media at temperatures from 20 °C to 37 °C. A typical time course demonstrating the kinetics of growth and streptomycin production is shown in Fig. 2(a); in complex medium growth was rapid but streptomycin only began to appear as growth slowed, while in minimal medium growth was much slower but was accompanied by streptomycin formation. A plot of the highest streptomycin levels reached at each temperature is shown in Fig. 2(b). Whatever the medium, streptomycin production dropped to virtually zero at 34 °C, although at this temperature growth was still about 50 % of that at 30 °C. This strain of *S. griseus* thus clearly shows depression of streptomycin production at elevated growth temperatures in liquid media.

Streptomycin production by *S. griseus* at standard and elevated growth temperature on solid media

Streptomycetes in their natural environment, the soil, grow on the surfaces of particles; growth in liquid suspension is therefore in a sense unnatural. We therefore wished to examine whether the kinetics of streptomycin production, and its variation with growth temperature, differ as between cultures on solid and in liquid media. Cultures were grown (see Methods) on dialysis membranes placed on solid complex or minimal media. Growth was estimated by removing the membranes, washing, and estimating dry weight, while the streptomycin accumulated in the agar was determined by bioassay. The properties of the mycelia grown on solid media, such as heterogeneity due to diffusion limitation, partial anoxia, and degree of branching, might well be quite different as between solid- and liquid-grown cultures on media of the same composition. The kinetics of growth and strep-

Fig. 2. (a) Growth and streptomycin yield in liquid cultures (see Methods) as a function of length of incubation at 30 °C. △, ▲, Cell dry weight in minimal medium (△) and complex medium (modified YEME) (▲); ○, ●, streptomycin yield in minimal medium (○) and complex medium (modified YEME) (●). (b) Growth and streptomycin yield in liquid cultures (see Methods) at different temperatures. The values plotted are the maximum values detected and are the means of duplicate experiments. Symbols as for (a).

tomycin yield on complex and minimal agar at 30 °C as a function of time are shown in Fig. 3(a). On complex medium, streptomycin production lagged behind growth, whereas on minimal medium, they accompanied each other. These results are qualitatively very similar to those for liquid-grown cultures in the corresponding media shown in Fig. 2(a). Fig. 3(b) shows the variation of maximum streptomycin level with growth temperature on complex and minimal media. Though growth at 34 °C was appreciable, streptomycin production was undetectable at this temperature. Again, behaviour on solid media is strikingly similar to that in the corresponding liquid media.

Expression at 37 °C of the *aph* reporter gene under the direction of *PstrB1* in high copy number plasmid constructs

A 200 bp leader region separates *PstrB1* from the beginning of *strB1*. Because this shows a complex pattern of repeats and for other reasons, it was initially proposed

Fig. 3. (a) Growth and streptomycin level in the underlying agar medium as a function of length of incubation in cultures grown on solid media (see Methods) at 30 °C. Symbols as for Fig. 2(a). (b) Growth and streptomycin yield in cultures grown on solid media (see Methods) at different temperatures. Symbols as for Fig. 2(a).

that StrR might act as an anti-terminator to antagonize transcription termination within this region. This now seems unlikely, and StrR probably functions by binding to DNA in the vicinity of *PstrB1* and stimulating the initiation of transcription there (Retzlaff *et al.*, 1993). As shown in Fig. 1(a), two *PstrB1*-containing restriction fragments were used to construct fusions to reporter genes: a 'short' fragment which lacks most of the inverted repeats within the *PstrB1*-*strB1* leader region, and a 'long' fragment which includes the entire leader region and the beginning of *strB1*. The DNA sequence of these fragments is given in Distler *et al.* (1987a, b). These fragments were cloned (Lindley *et al.*, 1995) upstream of *aph* first in the pBR322-pIJ486 hybrid pHL623 to give plasmids pHL708 and 614 (Fig. 1b), carrying the 'short' and 'long' fragments respectively. Two contiguous *Pst*I fragments carrying the promoterless *aph* alone or *aph* fused to the 'short' or 'long' fragments were then cloned into pIJ702 to give pHL723, 728 and 714 respectively (Fig. 1c). We showed (Lindley *et al.*, 1995) that the 'short' fragment fused to *aph* in pHL708 and pHL728 directs StrR-independent APH expression. Results are shown in Table 2(a) for pHL723, 728 and 714 in *S. lividans* grown at

Table 2. APH specific activities in *S. lividans* and *S. griseus* carrying *aph* gene fusions in high copy number

Cultures of *S. lividans* or *S. griseus* carrying the plasmids indicated were grown (see Methods) at the temperature shown, mycelium harvested, and its protein extracted and assayed for APH, as described in Methods. For the definitions of 'short' and 'long' promoter-bearing fragments, see Fig. 1. The number in parentheses following the mean is the number of determinations. Specific activities are in (units mg⁻¹) × 10³.

Plasmid	Promoter-bearing fragment	Mean APH activity ± SD at 30 °C†	Mean APH activity ± SD at 37 °C	Ratio of mean at 30 °C to mean at 37 °C
(a) Host: <i>S. lividans</i>				
pHL723	None	4.1 (4) ± 1.0	5.0 (3) ± 1.6	0.8
pHL728	'Short'	110 (5) ± 12	37 (4) ± 6.9	3.0
pHL714	'Long'	8.3 (3) ± 1.9	1.3 (3) ± 0.22	6.2
(b) Host: <i>S. griseus</i>				
pHL723	None	3.5 (4) ± 1.1	4.3 (4) ± 0.71	0.8
pHL728	'Short'	110 (6) ± 6.5	51 (5) ± 2.7	2.2
pHL714	'Long'	47 (7) ± 3.7	6.9 (6) ± 1.1	6.8

† Data from Lindley *et al.* (1995).

30 °C or 37 °C. APH activity in extracts of cultures carrying pHL723, lacking the *PstrB1* promoter, was low and almost unchanged as between 30 °C and 37 °C. Where the cultures carried pHL728, with the 'short' promoter fragment capable of directing *aph* expression in the absence of StrR, APH activity at 30 °C was 27-fold higher than with pHL723, and fell by a factor of about 3 at 37 °C. With pHL714, with the 'long' fragment unable to direct *aph* expression in the absence of StrR, APH activity at 30 °C was not significantly different (by *t*-test) from that with pHL723. However, it fell at 37 °C by an even larger factor, about 6, than with pHL728; the activity at 37 °C was not significantly different from that with pHL723 (0.05 < *P* < 0.1 by *t*-test). As we did not possess a plasmid compatible with pHL708 that carried *strR*, the effect of growth at 37 °C on StrR-activated expression of *PstrB1* had to be carried out in *S. griseus*. Table 2(b) shows the APH activities in *S. griseus* at 30 °C and 37 °C directed by the same three plasmids. Activities with pHL723 were much as in *S. lividans*. Activity with pHL728 at 30 °C was identical to that in *S. lividans*, and at 37 °C was not significantly different (again by *t*-test). With pHL714, at 30 °C the APH activity in *S. griseus* was only 42 % of that with pHL728, implying that insufficient StrR was present for the high copy number plasmid. At 37 °C, APH activity was not significantly different from that with pHL723 (0.05 < *P* < 0.1 by *t*-test).

Changes in copy number between 30 °C and 37 °C may affect the expression of cloned genes, especially for high copy number vectors. We have estimated pIJ702 copy number in *S. griseus* by subjecting mycelial total DNA extracts to agarose gel electrophoresis, the photographic trace after ethidium bromide staining then being scanned densitometrically (H. K. Lindley, unpublished results). The results suggest that at 37 °C plasmid copy number is

one-third to one-half that at 30 °C (mean copy number approximately 100 and 250 respectively).

Expression at 37 °C of the *xylE* reporter gene under the direction of *PstrB1* in low copy number plasmid constructs

The availability of low copy number promoter-probe vectors such as pIJ2839 (Ingram *et al.*, 1989; Clayton & Bibb, 1990; Fig. 1d) based on SCP2*, and of *strR* cloned in the pACYC184-pIJ702 hybrid pES2 (Fig. 1e; Lindley *et al.*, 1995) permitted testing in *S. lividans* of the effect of growth at 37 °C on *PstrB1*-directed expression of *xylE* in the presence of *strR*. The effect of the cloned *strR* could be compared with that of the deletion variant *strRA7* (O'Neill, 1992; Lindley *et al.*, 1995). Results for the nine possible combinations of promoter-bearing fragment (none, 'short' in pHL308, or 'long' in pHL314; Fig. 1D) and *strR* allele cloned in pES2 (none, wild-type *strR*, or *strRA7*) are shown in Table 3. At 30 °C, as reported by Lindley *et al.* (1995), CO activities showed no increase with the 'short' fragment in the absence of StrR (pHL308/pES2) over those where no promoter-bearing fragment was present (pIJ2839 with any of the three pIJ702 derivatives). The presence of the cloned *strR* in the companion plasmid gave an approximately 20-fold increase in CO activity with both the 'short' and 'long' promoter-bearing fragments. *strRA7* unexpectedly appeared to give appreciable stimulation of *xylE* expression with pHL308 – yielding a CO activity about 25 % of that with *strR* – but not with pHL314 (the difference between mean activities with pHL314 and pIJ2839 is not significant by *t*-test). At 37 °C, no combination of promoter and *strR* allele gave activity significantly above background, implying a lowering in *xylE* expression from that at 30 °C by a factor of at least ~ 30.

Table 3. CO specific activities in *S. lividans* carrying *xyIE* gene fusions in low copy number

Cultures of *S. lividans* carrying the plasmids indicated were grown (see Methods) at the temperature shown, mycelium harvested, and its protein extracted and assayed for CO, as described in Methods. For the definitions of 'short' and 'long' promoter-bearing fragments, see Fig. 1. The number in parentheses following the mean is the number of determinations. Specific activities are in milliunits mg⁻¹.

SCP2*-derived plasmid carrying <i>xyIE</i> (Fig. 1d)	Promoter-bearing fragment	pIJ702 derivatives with <i>strR</i> or <i>strRA7</i> (Fig. 1e)	Mean CO activity $\pm$ SD at 30 °C†	Mean CO activity $\pm$ SD at 37 °C	Ratio of mean at 30 °C to mean at 37 °C
pIJ2839	None	pES2	4.6 (6) $\pm$ 1.1	3.3 (6) $\pm$ 0.39	1.4
pIJ2839	None	pES2 <i>strR</i>	2.4 (5) $\pm$ 0.49	4.3 (6) $\pm$ 1.3	0.6
pIJ2839	None	pES2 <i>strRA7</i>	4.3 (6) $\pm$ 0.46	4.0 (6) $\pm$ 0.90	1.1
pHL308	'Short'	pES2	6.7 (6) $\pm$ 1.1	4.7 (6) $\pm$ 0.66	1.4
pHL308	'Short'	pES2 <i>strR</i>	140 (6) $\pm$ 66	4.7 (6) $\pm$ 0.77	30
pHL308	'Short'	pES2 <i>strRA7</i>	36 (6) $\pm$ 7.0	4.9 (6) $\pm$ 0.65	7.3
pHL314	'Long'	pES2	6.4 (6) $\pm$ 0.75	6.1 (6) $\pm$ 0.95	1.1
pHL314	'Long'	pES2 <i>strR</i>	190 (6) $\pm$ 75	6.1 (6) $\pm$ 0.70	32
pHL314	'Long'	pES2 <i>strRA7</i>	9.2 (6) $\pm$ 2.3	6.5 (6) $\pm$ 0.82	1.4

† Data from Lindley *et al.* (1995), expressed in the units defined in this paper.

DISCUSSION

We have shown above that in *S. griseus* ATCC 12475, streptomycin production is depressed at elevated growth temperatures. This applies in both complex and minimal media, and both in liquid and on solid media.

Lindley *et al.* (1995) and others (Distler *et al.*, 1987b, 1992; Vujaklija *et al.*, 1991) have shown that the 'short' *Bam*HI fragment, when fused to *aph* in high copy number pIJ101-based constructs, directs (at 30 °C) substantial levels of APH in *S. lividans* in the absence of StrR; on the other hand, as reported by Lindley *et al.* (1995), this fragment when fused to *xyIE*, whether in low copy number SCP2*-based constructs or in high copy number pIJ101-based constructs, failed in the same situation to direct levels of CO above background with no promoter-bearing fragment present. The reason for this reporter gene dependent difference is unknown. The promoter activity of the 'short' fragment in *S. lividans* permitted the estimation of the effect of growth at 37 °C on APH in the absence of StrR. *aph* expression directed by pHL728, carrying the 'short' *PstrB1*-bearing fragment fused to *aph* within pIJ702, fell threefold in *S. lividans* and twofold in *S. griseus*. It was pertinent to consider whether this reduction might be accounted for by a change in plasmid copy number. If the level of gene product were proportional to gene dosage – which is not always the case – the fall in StrR-independent APH activity directed by the 'short' fragment at 37 °C in both *S. lividans* and *S. griseus* could be due solely to change in plasmid copy number. However, the activities directed by pHL714, with the 'long' fragment plus *aph* within pIJ702, determined solely in *S. griseus* due to the requirement for StrR activation, showed a considerably greater reduction, the activity at 37 °C not being significantly different from the pHL723 control lacking a promoter fragment. This could be due to: (i) a greater drop in copy number for pHL714 than for pIJ702 and

presumably for pHL728, which seems unlikely (because the replicon and most of the insert are the same in all cases) but cannot be ruled out; or (ii) a genuine reduction in StrR-activated *PstrB1* expression. Trivial explanations based on enzyme instability for the reduction in APH specific activity in extracts from cultures grown at 37 °C seem unlikely: as noted in Methods, no *in vitro* instability could be detected, while the similarity in activities conferred by the control plasmid pHL723 as between 30 °C and 37 °C in both *S. lividans* and *S. griseus* makes it unlikely that the higher growth temperature causes the appearance of an APH-inactivating proteolytic activity.

With the SCP2*-based low copy number constructs with *xyIE* as reporter gene, there is virtually no detectable increase in expression of the reporter gene at 37 °C over the background without a promoter-bearing fragment. As the copy number of SCP2* is usually estimated as 1–2 per chromosome (Hopwood *et al.*, 1985), there seems to be little scope for attributing the difference at elevated growth temperature to change in copy number. Regarding explanations in terms of differential loss of enzyme activity at the higher temperature, there is again no indication of *in vitro* instability; and the appearance of a CO-inactivating proteolytic activity at the higher growth temperature is rendered improbable by the finding (V. J. Deeble, unpublished results) that when *S. lividans* TK54(pHL314)(pES2*strR*) is grown at 30 °C and the temperature then shifted to 37 °C for 1 or 2 d, CO activity in extracts does not consistently diminish by comparison with that for a control whose incubation is continued at 30 °C.

We suggest that the findings with *xyIE* confirm the more likely explanation in the case of the high copy number constructs with *aph*, namely that the effectiveness of the StrR-activated *PstrB1* promoter virtually disappears at

37 °C. StrR-independent expression of *PstrB1* with the 'short' fragment construct pHL728 seems to be less affected – possibly not at all – by growth at elevated temperature. The nature of this expression, shown by Lindley *et al.* (1995) to occur only when the reporter gene is *aph* and not when it is *xylE*, is still unclear; but this result may indicate that elevated temperature has its effect through the StrR activation mechanism. Many reasons for this – not mutually exclusive – are possible. The StrR protein itself may be especially heat-labile, or its mode of action may be affected by the higher temperature. Also, *strR* expression is thought to result from a regulatory cascade involving several other regulatory proteins acting at the DNA level; StrR level may therefore be affected by similar heat lability in one or more of these regulatory proteins or temperature sensitivity of their interaction with DNA. Further, at least the first of these regulatory steps involves a low molecular mass ligand, A-factor (Miyake *et al.*, 1990; Vujaklija *et al.*, 1993), and it may be that the availability of this compound is limited at elevated growth temperature. There is already an intriguing connection between A-factor and elevated temperature, in that growth of *S. griseus* at 37 °C results in the accumulation of non-A-factor-producing variants (Hara & Beppu, 1982). Finally, it has been suggested to us (T. Kieser, personal communication) that the apparent temperature effect may be due to the lower solubility of oxygen at higher temperature; this possibility will be worth pursuing.

Granted that an effect of growth at elevated temperature is to reduce StrR-activated *PstrB1* function – by whatever mechanism – to what extent does this explain the apparently complete loss of streptomycin production? It seems *a priori* likely that *PstrB1* is not the only streptomycin biosynthesis promoter to show the effect. Reduced *str* gene expression could therefore be a major, or even the sole, cause of diminished streptomycin production even if the effect on *PstrB1* and other promoters is less than 100 %, since: (a) there may be one enzyme in the streptomycin pathway whose activity is rate-determining, and expression of the gene for that enzyme may be especially sensitive to the temperature effect; or (b) even if all the *str* genes show only moderate effects, the sum total of these may be complete disappearance of flux through the pathway, a possibility predicted by metabolic control theory (Kell *et al.*, 1989). In conclusion, we suggest that at least part of the reason for depression in streptomycin production at elevated growth temperatures is a failure in expression of genes encoding enzymes of streptomycin biosynthesis.

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