JOURNAL OF BACTERIOLOGY, Sept. 1997, p. 5511–5515 Vol. 179, No. 17
0021-9193/97/$04.00+0
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Environmental Signals Triggering Methylenomycin Production by *Streptomyces coelicolor* A3(2)

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Received 5 November 1996/Accepted 16 June 1997

Methylenomycin production by *Streptomyces coelicolor* A3(2) may be triggered by either of two environmental signals: alanine growth-rate-limiting conditions and/or an acidic pH shock. The production of this SCP1-encoded antibiotic was studied by using batch and chemostat cultures. Batch cultures indicated a role for both nutritional status and culture pH in its regulation. Steady-state methylenomycin production and transcription of an *mmy* gene under alanine but not glucose growth-rate-limiting conditions was demonstrated in chemostat culture. Transient *mmy* expression and methylenomycin production occurred following an acidic pH shock. This stimulation of methylenomycin production occurred independently of the nutritional status of the growth environment. Antibiotic production was partially suppressed under alanine compared with glucose growth-rate-limiting conditions following the acidic pH shock. A low specific growth rate was a prerequisite for both steady-state and transient production of methylenomycin.

The traditional view of antibiotic production in *Streptomyces* bacteria is that it is restricted to the stationary phase (idiophase) of the batch growth cycle and is triggered by environmental factors which give rise to this state (reviewed in reference 2). This view has arisen from the extensive use of batch culture in academic studies and industrial processes. To decipher the mechanisms whereby *Streptomyces* bacteria sense changes in their environment and transduce them into expression of specific secondary metabolic gene sets, it is essential to clearly define the environmental conditions which trigger antibiotic production. The dynamic behavior of batch cultures (23) hinders this effort.

A number of authors (1, 8, 10, 16, 18, 21, 24, 28, 31, 32) have studied antibiotic production by streptomycetes by using chemostat culture. From these studies, it is clear that secondary metabolism is not strictly associated with zero growth (22) and that antibiotic production is subject to a growth rate dependence. Also, in a number of cases, antibiotic production has been demonstrated to be influenced by the nutritional status of the growth environment and hence is dependent on a type of catabolite repression or metabolite interference (2). However, in many cases, researchers have reported the presence of metabolite interference based solely on studies using batch culture.

The antibiotic methylenomycin is one of at least five secondary metabolites synthesized by *Streptomyces coelicolor* A3(2) (33). Its biosynthetic genes (*mmy*), together with a resistance determinant (*mmr*), are all carried on a large, linear plasmid, SCP1 (17). In a previous report (12), we described a set of nutritional conditions which permitted the biosynthesis of methylenomycin in batch culture. Methylenomycin was produced toward the end of the active growth phase but prior to the complete consumption of the growth stoichiometrically limiting substrate, alanine. In these experiments, the pH was not controlled and decreased during the growth phase from 7.2 to 5.5. Low-resolution S1 nuclease assays demonstrated that the transcription of an *mmy* gene was switched on at about the same time as methylenomycin appeared in the culture broth (12). However, the precise environmental signal(s) triggering gene expression, and hence methylenomycin production, was not clear. The signal(s) may have been (i) a low specific growth rate (or a change in that rate) caused by a decrease in the growth-rate-limiting substrate concentration, pH, or a change in some other environmental factor, (ii) metabolite interference by the growth stoichiometrically limiting substrate or some other nutrient, (iii) some type of stress response induced by the change in pH, or (iv) any combination of these.

Here we report work describing the exact identification of environmental conditions which trigger methylenomycin production by *S. coelicolor* A3(2). Using batch and chemostat cultures, we have examined the effects of nutritional status and an artificial acidic pH shock on the transcription of an *mmy* gene and on methylenomycin production.

MATERIALS AND METHODS

Bacterial strains and plasmids. *S. coelicolor* A3(2) NR1 was used throughout this study. It was isolated (30) from a spore stock of *S. coelicolor* A3(2) 1147 (14). This wild-type isolate was an $SCP1^+$ $SCP2^+$ prototroph and produced the secondary metabolites actinorhodin, undecylprodigiosin, and methylenomycin. These features were heritable on subculturing to fresh R5 agar.

Plasmid pIJ518 (5) was used as a hybridization probe for transcript analysis.

Media. Cells were maintained and grown on the media previously described (11, 12). All medium components were filter sterilized (0.2-μm-pore-size Maxi capsule; Gelman Sciences) with the exception of Junlon and the trace salts solution. These were sterilized by autoclaving.

Media with glucose and alanine concentrations of 0.026 and 0.031 M (4.68 and 2.78 g $liter^{-1}$) and 0.065 and 0.011 M (11.7 and 0.98 g $liter^{-1}$) gave conditions which were growth stoichiometrically limited for glucose and alanine, respectively. Each medium yielded a maximum biomass concentration of 4 OD_{450} (optical density at 450 nm) units. The growth stoichiometrically limiting nutrient (glucose or alanine) was confirmed to be also growth rate limiting by impulse additions of nutrient to steady-state chemostat cultures (data not shown).

Inoculum preparation. Inocula for fermentation experiments were prepared by culturing a single colony, from a stored MS plate (11), in 50 ml of YEME medium (15) at 30°C in an orbital shaker for 48 h. Mycelia were then collected by centrifugation (4,000 rpm, 5 min), washed, and resuspended in 50 ml of sterile distilled water. This inoculum was used at a level of 1% (vol/vol).

Culture conditions. Shake flask cultures were prepared at 30°C in an orbital shaker at 180 rpm. These procedures used 250-ml Erlenmeyer flasks containing a length of stainless steel spring (12.7 mm by 6 turns in^{-1}; Alliance Spring Company), which aided aeration and maintenance of dispersed growth.

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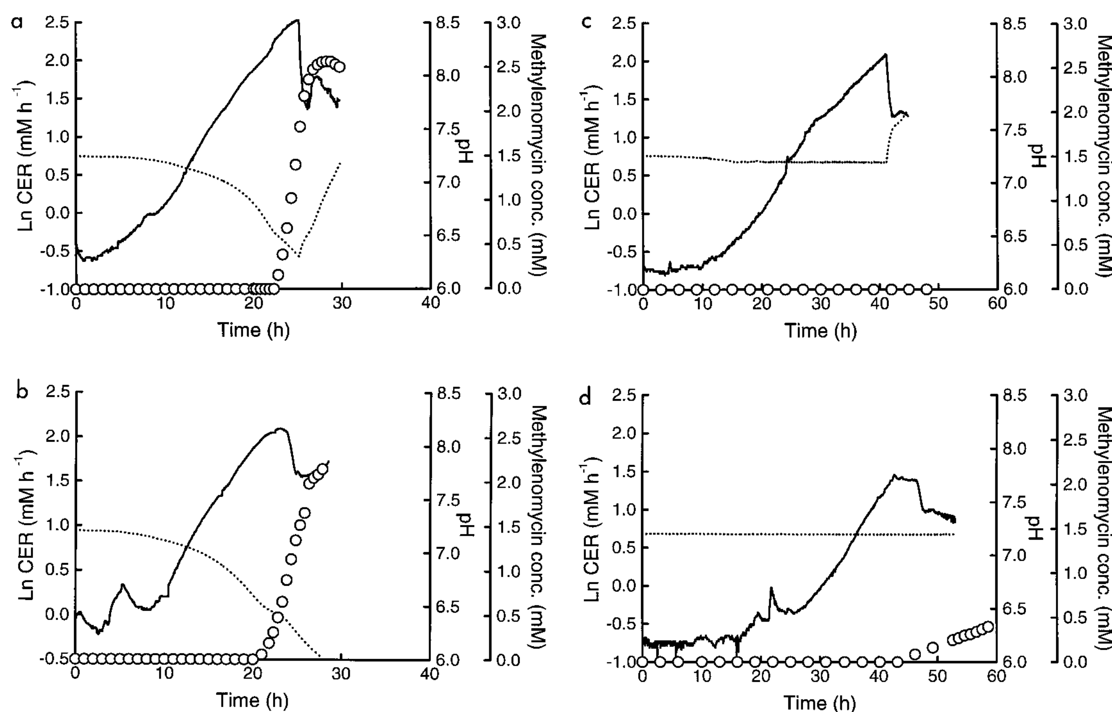

FIG. 1. Batch fermentation profiles of *S. coelicolor* A3(2) NR1 cultures grown under the following conditions: (a) glucose limited, no pH control; (b) alanine limited, no pH control; (c) glucose limited, pH control; and (d) alanine limited, pH control. Graphs show data for the profiles of CER (——), pH (· · ·), and methylenomycin concentration (○) during batch cultures of *S. coelicolor* A3(2) NR1. Data are plotted against time from inoculation. Fermentations for each condition were performed at least three times. All replicates exhibited the same key features.

Fermentations were conducted in an LKF 2000 fermentor (Bioengineering) with a working volume of 3 liters (2 liters when in continuous mode). The fermentor was equipped with a combination pH electrode and a polarigraphic dissolved oxygen probe (Ingold). Monitoring and control of fermentation variables were achieved by using a computer control system (Process Intelligence Ltd.). Cultures were aerated at 1 ± 0.02 liter min^{-1} and mixed by two six-bladed Rushton turbines at an agitation rate of 1,000 rpm. This maintained the dissolved oxygen concentration above 80% of air saturation. The culture pH was controlled (within an error from the set point of ±0.05) by automatic addition of 2.5 M HCl and/or 2.5 M NaOH. Temperature was controlled at 30 ± 0.05°C by a 200-W heater cartridge and cooling a finger with a solenoid valve. For chemostat cultures, the volume was maintained constant by using a weir-overflow device. Inlet and outlet feeds were via peristaltic pumps (Watson-Marlow). Foam production was controlled by automatic additions of antifoam at a rate of approximately 0.05 ml per retention time. Chemostat culture was initiated once a batch fermentation had reached late exponential to early stationary phase (as indicated by changes in carbon dioxide evolution rate [CER]). A steady state was deemed to have been achieved after approximately four retention times and was indicated by an approximately constant CER.

On-line analysis of fermentation products. Exhaust gases were continuously monitored for carbon dioxide concentration by using an infrared gas analyzer (ADC Instruments Ltd.).

Methylenomycin concentration was measured by high-pressure liquid chromatography (HPLC) essentially by the method of Hobbs et al. (12). HPLC analysis was performed both on-line and off-line. For on-line measurements, improved reproducibility was achieved by using a modified mobile phase, 25% (vol/vol), acetonitrile, 100 mM $NH_4H_2PO_4$, and pH adjusted to 2.2 with HCl. On-line sampling was performed by using a cross-flow filtration module and automated injection valve (Waters-Millipore). Off-line samples were prepared for HPLC by removing the supernatant after centrifugation (in an Eppendorf centrifuge). Off-line samples were stored at −20°C prior to analysis. No significant differences were detected in the measured methylenomycin concentration between on-line and off-line samples. The standard error for measuring the methylenomycin concentration was ±3%, and the detectable concentration was ca. ≥2.75 μM (0.5 mg $liter^{-1}$).

Estimation of biomass concentration. As an estimate of the biomass concentration, OD was measured at a light wavelength of 450 nm as previously described (11). Samples were diluted with fresh medium to an OD_{450} of 0 to 0.5.

RNA transcript analysis. Methods for RNA extraction and S1 nuclease mapping were essentially as described by Hopwood et al. (15). Modifications were as described by Hobbs et al. (12). The quality of RNA extracts was examined by agarose gel electrophoresis, using denaturing conditions as described by Hopwood et al. (15). RNA was visualized under UV light following ethidium bromide staining. Bands corresponding to 16S and 23S rRNA transcripts were observed in all RNA extracts. S1 hybridization reactions were carried out with 40 μg of cellular RNA and 200 ng of *Xho*I-linearized pIJ518 DNA. The probe DNA was radiolabeled with [^{32}P]dCTP (3,000 Ci $mmol^{-1}$; Amersham) by using a random primer kit (Boehringer). Typically the DNA was labeled to a specific activity of 10^7 cpm of DNA μg^{-1}. Neutral agarose gels were used to resolve the RNA-DNA hybrids, which were then visualized with a PhosphorImager (GS363 Molecular Imager system; Bio-Rad) and Phosphor Analyst/PC software (version 1.1.1; Bio-Rad).

Data analysis. CER was calculated on-line from measurements of carbon dioxide concentration as described by Butler et al. (3). The maximum specific growth rate from batch culture data was determined from the slope of the linear portion of a semilog plot of CER. The specific production rate of methylenomycin ($q_{\text{methylenomycin}}$) was calculated by using the balance equation

$$q_p = \frac{1}{x}\left(\frac{dp}{dt} + Dp\right)$$

where p is the concentration (millimolar) of methylenomycin, D is the dilution rate (flow rate of feed/culture volume/hour), x is the biomass concentration (OD), and dp/dt is the rate of change in methylenomycin concentration (millimolar) per hour. dp/dt was measured by interpolation of on-line HPLC data, using a smoothing cubic spline (6, 23). These data were then subjected to numerical differentiation using a backward difference equation.

RESULTS AND DISCUSSION

Methylenomycin production in batch culture. To investigate the influence of (i) nutritional status and (ii) medium acidification on the production of methylenomycin, batch fermentations were performed in media limited for glucose and alanine, with and without pH control. Figure 1 shows data for four representative batch fermentations. Each fermentation was performed at least three times. The mean maximum specific growth rates ± standard errors for cultures grown with and

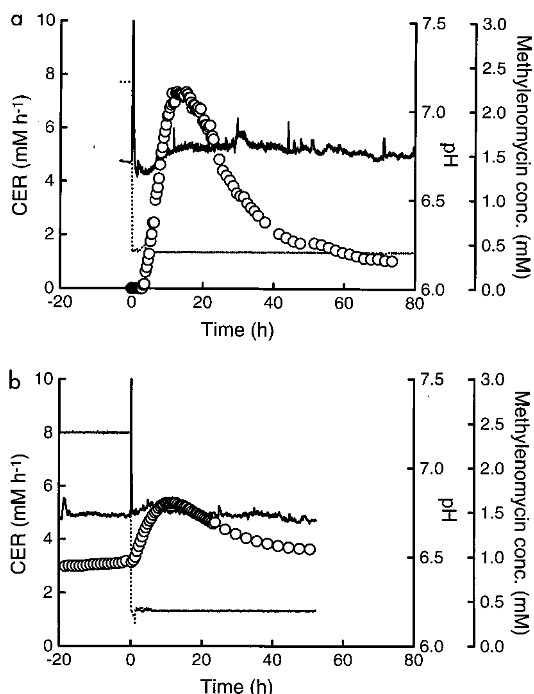

FIG. 2. Continuous fermentation profiles of *S. coelicolor* A3(2) NR1 following an acidic pH shock during growth under glucose (a) and alanine (b) growth-rate-limiting conditions. Graphs show data for profiles of CER, pH, and methylenomycin concentration following an acidic pH shock from 7.2 to 6.2 in continuous cultures at a dilution rate of 0.03 h^{-1}. Symbols are as given in the legend to Fig. 1. Data are plotted against time from the point at which the pH shock was initiated.

without pH control were 0.152 ± 0.007 h^{-1} (n = 4) and 0.107 ± 0.011 h^{-1} (n = 4), respectively.

Methylenomycin production occurred to the same degree in all batch fermentations when the pH was not controlled (Fig. 1a and b). When the pH was controlled during glucose-limited batch culture, no methylenomycin production was observed (Fig. 1c). In alanine-limited cultures with pH control, methylenomycin titers were much reduced and production occurred later in the stationary phase (Fig. 1d). These results suggested that both the culture's nutritional status and pH influenced the regulation of methylenomycin production. To further analyze and clarify these effects, experiments were performed by using chemostat culture.

Methylenomycin production during chemostat culture. We examined steady-state methylenomycin production in both glucose and alanine growth-rate-limited chemostats. *S. coelicolor* A3(2) NR1 was grown in batch culture (with no pH control) and then switched into chemostat culture with a dilution rate of 0.03 h^{-1} (doubling time, 23.1 h). The steady-state biomass concentration in chemostats was ca. 4 OD units.

All chemostat experiments were carried out at the same dilution rate (0.03 h^{-1}) and hence the same specific growth rate (μ). The absence of antibiotic production during exponential growth in batch cultures (Fig. 1) and at high dilution rates in chemostat cultures (unpublished data) suggest that growth at a low absolute μ is a prerequisite for methylenomycin biosynthesis. This feature is similar to that of other secondary metabolites produced by a variety of streptomycete species. Continual steady-state production of methylenomycin was observed under alanine growth-rate-limiting conditions (Fig. 2b), indicating that neither a change in the specific growth rate ($\Delta\mu$) nor a zero growth rate is necessary for triggering its synthesis. These data do not exclude the possibility that a $\Delta\mu$ can stimulate methylenomycin production. However, the observations that methylenomycin production was triggered neither by growth in glucose-limited batch cultures where the pH was controlled (Fig. 1c) nor following various stress conditions transiently imposed in continuous culture (see below) suggest that $\Delta\mu$ per se cannot evoke methylenomycin production.

The absence of methylenomycin production under glucose growth-rate-limiting conditions suggests that a low specific growth rate is not the sole prerequisite for triggering production of this antibiotic. These data clearly demonstrate that methylenomycin production is subject to a type of metabolite interference (4). The mechanism whereby the nutritional status influences methylenomycin production has yet to be elucidated, although transcriptional regulation of the *mmy* operon is involved (see below and reference 12).

We next investigated the effect of an artificial acid shock on methylenomycin production during steady-state glucose and alanine rate-limited chemostats. The pH was decreased from 7.2 to 6.2 over a period of ≤15 min. At least three replicate fermentations were performed. Figure 2 shows representative changes in CER and methylenomycin concentration following a pH shock in glucose and alanine growth-rate-limited chemostats. Under both nutritional conditions, some variation in the maximum concentration of methylenomycin produced was observed between replicate fermentations. However, the maximum concentration of methylenomycin achieved during glucose limitation was, in general, greater than that observed during alanine-limited fermentations. No significant changes in biomass concentration, as estimated from measurements of OD, were detected during the pH transient.

The specific methylenomycin production rate ($q_{\mathrm{methylenomycin}}$) was calculated. Given that the CER did not change significantly (Fig. 2), it was assumed that the specific growth rate was approximately equal to the dilution rate at all times and that the biomass concentration did not vary significantly from an OD_{450} of 4. The steady-state $q_{\mathrm{methylenomycin}}$ in alanine-limited continuous cultures was ca. 7.5 μM OD^{-1} h^{-1}. No detectable methylenomycin was observed in glucose-limited cultures. The maximum specific rates of methylenomycin production induced by the pH shock were ca. 90 and 30 μM OD^{-1} h^{-1} in glucose- and alanine-limited cultures, respectively. These maximum rates were approximately the same between replicate fermentations (data not shown).

The question arose as to whether methylenomycin production was a specific response to acid shock or whether any stress condition would elicit antibiotic production. A number of stress conditions were imposed during continuous culture. These were alkali (pH 7.2 to 8.2 shock), heat (30 to 37°C switch), alcohol (addition of absolute ethanol to a final concentration of 0.95% [wt/vol]), and osmotic (addition of NaCl to a final concentration of 10% [wt/vol]). None of these stresses triggered methylenomycin production (data not shown).

There are few reports on the effects of culture pH (pH_0) on secondary metabolism in *Streptomyces* species. James et al. (16) showed that the specific rate of granaticin production by *Streptomyces thermoviolaceus* in chemostat culture (D = 0.15 h^{-1}) was influenced by the absolute pH_0. Möhrle et al. (25) reported that during batch culture, acidification of the growth medium was necessary for production of nikkomycins by *Streptomyces tendae*. In this work, the production of methylenomycin by *S. coelicolor* A3(2) was stimulated by a change in the pH_0 (ΔpH_0) from 7.2 to 6.2. This response appeared to be independent of the nutritional status of the culture (Fig. 2). The constancy of the steady-state specific production rate between pH_0 7.2 and

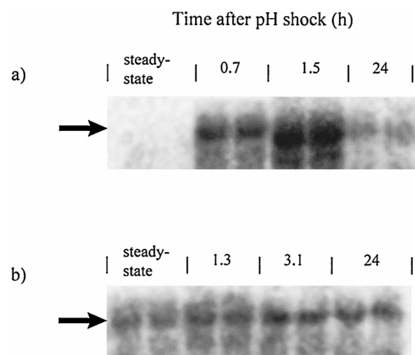

FIG. 3. S1 nuclease assays of an *mmy* gene transcript under steady-state conditions and following an acidic pH shock during glucose and alanine growth-rate-limited growth of *S. coelicolor* A3(2) in continuous culture. RNA samples were isolated from glucose (a) and alanine (b) growth-rate-limited chemostat cultures before and after an acidic pH shock as indicated. Samples from steady-state conditions were taken approximately 24 h prior to the acidic pH shock. Duplicate assays were performed on the sample from each time point. The image was generated directly from the PhosphorImager (see Materials and Methods) and shows the S1-resistant hybrid, corresponding to a methylenomycin biosynthetic gene transcript (*mmy*), generated by using linearized pIJ518 as the probe. Arrows indicate positions of the 2.4-kb transcript.

6.2 suggests that it is ΔpH_0 that is important in stimulating methylenomycin production.

Shah et al. (29) showed that penicillin G production by *Aspergillus nidulans* is subject to regulation by the medium pH. Penicillin titers were greatest under alkaline conditions. Espeso et al. (7) demonstrated that an alkaline pH leads to a bypass of the *creA*-mediated carbon catabolite repression of *ipnA* transcription. The observation that an acidic pH shock obviates metabolite interference imposed on methylenomycin production under glucose rate-limiting conditions parallels the phenomenon observed in *A. nidulans*. This work demonstrates that pH regulation has wider implications in the control of secondary metabolism.

Analysis of *mmy* transcript levels during continuous culture of *S. coelicolor* A3(2) NR1. We were interested in determining whether the nutritional status of the growth environment and an acidic pH shock would independently elicit transcription of the methylenomycin gene cluster. To this end, total cellular RNA was extracted from the mycelia of both glucose and alanine growth-rate-limited chemostat cultures of *S. coelicolor* A3(2) NR1. Samples were taken during steady-state growth (approximately 24 h before the pH shock), twice during the course of the transient response, and approximately 24 h following an artificial acidic pH shock. Figure 3 shows the results of a low-resolution S1 nuclease assay on RNA extracts. In samples taken prior to the pH shock, the *mmy* transcript was detectable during steady-state growth under alanine but not glucose growth-rate-limiting conditions. Furthermore, the acidic pH shock enhanced transcript levels under both nutritional conditions but to a lesser extent under alanine-limited conditions. These data indicate that transcription of at least one of the *mmy* genes is triggered both by certain nutritional conditions and by an acidic pH shock.

The expression of a spectrum of genes in enteric bacteria is stimulated by acidic changes in pH_0 (reviewed in reference 26). Some of the gene products have roles in pH homeostasis and development of acid tolerance (27). Two acid tolerance response systems, log phase and a stationary phase, are discernible in avirulent laboratory strains of *Salmonella typhimurium* (9, 20). Transient gene expression is involved in both systems, the longevity of the response being a function of the stability of the various protein products synthesized (19). Transient gene expression of the *mmy* operon appears to be a property of the acid shock response of *S. coelicolor* A3(2). However, it is unlikely that methylenomycin plays a role in pH homeostasis following acidogenesis since it is itself an acidic product (pK_a of 3.65). The possibility that methylenomycin production plays some other role in metabolic regulation and stress adaptation associated with a change in pH_0 cannot be ruled out. Possibly, methylenomycin production is involved in metabolic balancing, as has been suggested for prodigiosin (13). Supporting this hypothesis was the observation that methylenomycin production, triggered by an acidic pH shock, was partially suppressed under alanine growth-rate-limiting conditions, i.e., conditions where active antibiotic production occurred prior to the pH shock. Moreover, transcript levels were lower under alanine-limited conditions than under glucose limitation. Thus, methylenomycin production prior to the pH shock may provide some form of adaptation to stress such that following acidogenesis, the degree of response generated is reduced. However, it is not known whether the steady-state production of methylenomycin per se, or some other physiological factor(s) associated with growth under alanine limitation, causes the partial suppression of the acid shock response.

Significant questions remain concerning the regulation of methylenomycin production. Of most importance is the identification of the signal transduction pathway(s) involved in triggering antibiotic production and the point in the pathway(s) at which the responses to the two environmental signals, alanine growth limitation and an acidic pH shock, intersect. To answer these questions, identification of genes differentially expressed on stimulation of methylenomycin production is the focus of current and future work.

ACKNOWLEDGMENTS

This work was supported financially by a BBSRC studentship to A.H. and grants from the BBSRC awarded to S.G.O.

We gratefully acknowledge the receipt of strains and plasmids from Sir David Hopwood and Keith Chater. We are grateful to our colleagues Tony Obanye, Fiona Flett, and Dave Gardner for their help in this work.

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