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Published in:
Molecular Microbiology

DOI:
[10.1111/j.1365-2958.1992.tb01459.x](https://doi.org/10.1111/j.1365-2958.1992.tb01459.x)

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Document Version
Publisher's PDF, also known as Version of record

Publication date:
1992

[Link to publication in University of Groningen/UMCG research database](#)

Citation for published version (APA):

Takano, E., Gramajo, H. C., Strauch, E., White, J., & Bibb, M. J. (1992). Transcriptional regulation of the redD transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2). *Molecular Microbiology*, 6(19). <https://doi.org/10.1111/j.1365-2958.1992.tb01459.x>

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Transcriptional regulation of the *redD* transcriptional activator gene accounts for growth-phase-dependent production of the antibiotic undecylprodigiosin in *Streptomyces coelicolor* A3(2)

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Summary

Transcription of *redD*, the activator gene required for production of the red-pigmented antibiotic undecylprodigiosin by *Streptomyces coelicolor* A3(2), showed a dramatic increase during the transition from exponential to stationary phase. The increase in *redD* expression was followed by transcription of *redX*, a biosynthetic structural gene, and the appearance of the antibiotic in the mycelium, and coincided with the intracellular appearance of ppGpp. However, ppGpp production elicited either by nutritional shift-down of, or addition of serine hydroxamate to, exponentially growing cultures had no stimulatory effect on *redD* transcription. The presence of *redD* on a multicopy plasmid resulted in elevated levels of the *redD* transcript and production of *redX* and undecylprodigiosin during exponential growth; the normal growth-phase-dependent production of undecylprodigiosin appeared to be mediated entirely through the *redD* promoter, which shows limited similarity to the consensus sequence for the major class of eubacterial promoters.

Introduction

Members of the genus *Streptomyces* are notable for their ability to produce a vast array of secondary metabolites. Many of these compounds are used as antibiotics or pharmacologically active agents. In liquid medium, antibiotic production is generally limited to stationary phase, or to cultures growing at low growth rates (Demain *et al.*, 1983); in agar-grown cultures, it appears to be temporally

correlated with the onset of morphological differentiation, and some genes are required for both processes, implying at least some common elements of genetic control (Chater, 1989; Hopwood, 1988). Although repression by excess phosphate or easily assimilated sources of carbon or nitrogen appears to limit production of some antibiotics (Demain, 1989), growth-rate, and potentially growth rate control, may play a general role in determining the onset of secondary metabolism. In this context, it is interesting to note the correlation, albeit incomplete, between antibiotic production and the appearance of [p]ppGpp in several streptomycetes (Ochi, 1986; 1987a,b; 1990; Strauch *et al.*, 1991; Kelly *et al.*, 1991), and the apparent ability of [p]ppGpp to reduce growth rate in *Escherichia coli* (Sarubbi *et al.*, 1988; Schreiber *et al.*, 1991).

Streptomyces coelicolor A3(2) is by far the most studied streptomycete in terms of genetics. It produces at least four chemically distinct antibiotics: actinorhodin, undecylprodigiosin, methylenomycin and a calcium-dependent antibiotic (Hopwood, 1988). We have characterized the stringent response and the production of ppGpp in this species (Strauch *et al.*, 1991); we noted a partial correlation between ppGpp synthesis and transcription of *actIII*, one of the biosynthetic genes for actinorhodin (Hallam *et al.*, 1988). Here, we focus on undecylprodigiosin, a red-pigmented tripyrrole antibiotic made by the same species (the red pigmentation of *S. coelicolor* A3(2) is due to a mixture of biosynthetically related prodigionines, of which undecylprodigiosin is the major component (Tsao *et al.*, 1985); we refer to the mixture as undecylprodigiosin). Genetic studies indicate that there is a cluster of at least 18 genes (probably more) involved in undecylprodigiosin production (Rudd and Hopwood, 1980; Feitelson *et al.*, 1985; Coco *et al.*, 1991), and the entire biosynthetic cluster has been cloned on a DNA fragment of approximately 35.7 kb (Malpartida *et al.*, 1990). Towards, if not at, one end of the cluster lies a presumptive pathway-specific activator gene, *redD*. The function of this gene was predicted from the inability of *redD* mutants to co-synthesize undecylprodigiosin with other *red* mutant classes (Rudd and Hopwood, 1980; Feitelson *et al.*, 1985); from the lack of expression of the *redE* and/or *redF* genes required for *O*-methyltransferase

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activity in *redD* mutants (Feitelson *et al.*, 1985); and from the ability of extra cloned copies of *redD* to elicit overproduction of undecylprodigiosin in *S. coelicolor* A3(2) (Narva and Feitelson, 1990) and *Streptomyces lividans* (Malpartida *et al.*, 1990), a close relative of *S. coelicolor* A3(2) in which little undecylprodigiosin is usually made. The nucleotide sequence of *redD* has been determined (Narva and Feitelson, 1990).

Here, we define the *in vivo redD* transcriptional start site, and study the temporal and growth-phase transcription of *redD* and *redX*, a structural gene for an early step in the biosynthetic pathway (Guthrie and Chater, 1990; Malpartida *et al.*, 1990). We assess the possible correlation between transcription of *redD* and *redX*, and the production of ppGpp, which was elicited in exponentially growing cultures either by amino acid depletion (nutritional shiftdown) or by addition of serine hydroxamate, a seryl tRNA synthetase inhibitor that induced lower levels of ppGpp. We also investigated the effect of increased levels of *redD* expression during exponential growth on *redX* transcription and on undecylprodigiosin biosynthesis.

Results

Undecylprodigiosin production in liquid culture

In minimal medium supplemented with 0.2% w/v casamino acids, *S. coelicolor* A3(2) strain M145 grew exponentially with a doubling time of 2.2 h, and made a rapid transition into stationary phase, approximately 12 h after inoculation, at an OD_{450 nm} of about 2 (approximately 1 mg ml⁻¹ dry weight). Undecylprodigiosin production was readily detected as soon as the culture entered stationary phase. The low number of doublings (approximately five) limited the size of mycelial clumps, and may have served to minimize physiological heterogeneity that might have resulted from nutritional or oxygen limitation. Increasing the amount of glucose (fivefold) or phosphate (10-fold) had no effect on growth, but removal of the (NH₄)₂SO₄ led to a reduction in final biomass equivalent to the growth observed in nitrogen-limited minimal medium, indicating that the casamino acid-supplemented cultures were also nitrogen-limited. Supplementation with higher concentrations of casamino acids did not increase the growth rate, but did increase the final yield of mycelium. In minimal medium lacking casamino acids, M145 grew with a doubling time of about 4 h, and entered stationary phase in a less synchronous manner at an OD_{450 nm} of about 0.6; replacement of glucose with the same molar concentration of arabinose, fructose or galactose resulted in a lower growth rate and lower final OD_{450 nm} (about 0.2 for galactose and fructose). In the absence of casamino acids, undecylprodigiosin production occurred upon entry

into stationary phase, regardless of the carbon source used, although the amount produced was much lower than in the presence of casamino acids. Given the more rapid transition from exponential to stationary phase, the higher levels of synthesis of undecylprodigiosin, and our desire to carry out nutritional shiftdowns, all subsequent experiments used minimal medium supplemented with 0.2% w/v casamino acids.

S1 nuclease mapping of the *redD* transcriptional start site

An RNA-protected fragment was readily detected with RNA isolated from cultures of *S. coelicolor* A3(2) M145 producing undecylprodigiosin (Fig. 1A). Transcripts corresponding in size and orientation to this apparent promoter, and to the four oppositely orientated promoters (pr1–4) identified by Narva and Feitelson (1990), were also observed in *in vitro* transcription studies using the *NaeI* fragment shown in Fig. 1B and RNA polymerase isolated from a transition phase culture of M145 (data not shown), providing further evidence that the RNA-protected fragment represented an *in vivo* transcriptional start site. It is preceded by sequences in the –35 (ATGACG) and –10 (CACGAT) regions (Fig. 1B) that show limited similarity to the consensus sequences for the major class of eubacterial promoters (TTGACA and TATAAT, respectively: Hawley and McClure, 1983). This start site lies downstream of the previously assigned translational start codon of *redD* (Narva and Feitelson, 1990); based on these results, as well as FRAME analysis (Bibb *et al.*, 1984) of the *redD* sequence and the similarity of the putative *redD* product to the activators for the actinorhodin and daunorubicin pathways (the products of *actII-ORF4* (Fernandez-Moreno *et al.*, 1991) and *dnrI* (Stutzman-Engwall *et al.*, 1992), respectively), the GTG located at position 700 (Fig. 1B) probably represents the *redD* translational start codon, yielding a predicted untranslated leader sequence of 199 nucleotides and a protein of 29.6 kDa. The GUG codon would be preceded by a sequence (GGGGG) that is complementary, allowing one G:U base pair, to that found close to the 3' end of the 16S rRNA of *S. coelicolor* A3(2) (CCUCC; (Baylis and Bibb, 1987)), and that is presumed to be involved in ribosome binding.

Analysis of sequences upstream of *redD* had identified the 5' end of an oppositely orientated open reading frame of unknown function that appeared to be transcribed from four promoters, pr1–4 (Narva and Feitelson, 1990; EMBL/GenBank/DDJB Nucleotide Sequence Data Libraries Accession Number M29790). Recent searches of the translated EMBL Nucleotide Sequence Data Library indicate that the product of this gene is homologous (32% and 56% amino acid sequence identity and similarity, respectively, over the available 183-amino-acid

Fig. 2. A. Growth curve of, and ppGpp production by, *S. coelicolor* A3(2) strain M145. t_D , doubling time during exponential growth. B and C. S1 nuclease mapping of the *redD* (B) and *redX* (C) transcripts in RNA samples isolated from the culture shown in Fig. 2A at the times indicated. For A, B and C, the shaded boxes labelled RED denote the presence of undecylprodigiosin in the mycelium. For B and C: EXP and STAT indicate the exponential and stationary phases of growth, respectively, and the shaded area between them denotes the transition phase; SM, end-labelled *Hpa*II-digested pBR322 size marker.

17 h, respectively). Transcription of *redD* and *redX*, and undecylprodigiosin synthesis, occurred in the culture treated with 25 mM serine hydroxamate as soon as it entered stationary phase, 11 h after maximal ppGpp production (data not shown).

Multiple copies of *redD* result in undecylprodigiosin production during exponential growth

The stationary phase production of undecylprodigiosin might reflect transcriptional regulation of *redD* alone, or additional limitations imposed on the expression of the biosynthetic structural genes during exponential growth, either at the level of their transcription and/or translation, or by the inhibition or inactivation of the enzymes they encode. To assess this possibility, a 2.4 kb *Bgl*II fragment containing *redD* was cloned into the multicopy (approximately 150 copies per chromosome) plasmid pIJ487 (yielding pIJ6014) in M145, and the effects on transcription of *redD* and *redX*, and on undecylprodigiosin production, were assessed. The growth characteristics of M145(pIJ6014) did not differ significantly from those of M145; however, *redD* transcripts were readily detected at

the earliest point in exponential growth from which sufficient RNA could be isolated for analysis, and were present in amounts similar to those observed in stationary phase cultures of M145 (Fig. 4A). The level of *redD* transcript increased during exponential growth and continued to rise in stationary phase. Elevated levels of the *redX* transcript were also observed (Fig. 4B), and undecylprodigiosin was readily detectable in mid-exponential phase. After 65 h, the yield of undecylprodigiosin from M145(pIJ6014) ($6.1 \mu\text{g ml}^{-1}$) was 5.5 times higher than that from M145 ($1.1 \mu\text{g ml}^{-1}$).

Discussion

The production of antibiotics in stationary phase, or at low growth rates, might reflect the consumption of a nutrient that represses or inhibits synthesis during growth. We do not believe this to be the case for undecylprodigiosin in these studies for the following reasons. (i) The stationary phase production of undecylprodigiosin in minimal medium severely limited in growth by different carbon sources (e.g. galactose and fructose) strongly suggests the lack of ammonium or phosphate repression and/or

Fig. 3. A. Growth curve of *S. coelicolor* A3(2) strain M145 with (Δ) and without (O) nutritional shiftdown. The shaded box labelled O RED denotes the presence of undecylprodigiosin in the mycelium of the control culture (O), which grew exponentially with a doubling time of 2.2 h; SD, point of nutritional shiftdown; Δ , time points at which samples were taken from the culture subjected to nutritional shiftdown for RNA isolation. B. S1 nuclease mapping of the *redD* transcript in RNA samples from the culture subjected to nutritional shiftdown (Δ in Fig. 3A) at the times indicated (Δ in Fig. 3A); STAT, stationary phase; SM, end-labelled *Hpa*II-digested pBR322 size marker.

inhibition. (ii) The initial levels of ammonium and phosphate were well below those observed by Hobbs *et al.* (1990) to reduce undecylprodigiosin production significantly. (iii) Since undecylprodigiosin was not made during exponential growth in minimal medium, there is no reason to suspect that supplementation with casamino acids, necessary for studies of nutritional shiftdown, repressed or inhibited production. (iv) Increasing the amount of glucose (fivefold) or phosphate (10-fold) had no significant effect on growth or on the stationary phase onset of undecylprodigiosin production, further suggesting that the latter is not repressed or inhibited by these metabolites at the concentrations normally used. These observations are consistent with the notion that growth rate *per se*, or the cessation of growth, is important in determining the onset of undecylprodigiosin production, at least under these conditions.

Transcription of *redD* in *S. coelicolor* A3(2) M145

increases dramatically as the culture makes the transition from exponential to stationary phase, and transcription of *redX* and the production of undecylprodigiosin clearly occur after growth has finished. This differs from an earlier report (Hobbs *et al.*, 1990) in which undecylprodigiosin synthesis occurred during growth; however, in that work, growth does not appear to have been exponential, but rather was linear, and was much slower than in our experiments, again consistent with a potential role for growth rate in determining the onset of antibiotic biosynthesis. The results obtained with *redX* are similar, although not identical, to those obtained by Guthrie and Chater (1990), who used *redX::xyIE* fusions to detect

Fig. 4. S1 nuclease mapping of the *redD* (A) and *redX* (B) transcripts in RNA samples isolated from a culture of *S. coelicolor* A3(2) M145 (pJ6014) at the times indicated. The shaded boxes labelled RED denote the presence of undecylprodigiosin in the mycelium; EXP and STAT indicate the exponential and stationary phases of growth, respectively, and the shaded area between them denotes the transition phase; SM, end-labelled *Hpa*II-digested pBR322 size marker; M145-STAT indicates the use of RNA from a stationary phase culture of M145 (equivalent to the 18 h time point in Fig. 2B).

redX transcription during a relatively long period of transition into stationary phase after inoculating cultures with homogenized mycelium; the use of such inocula, rather than freshly germinated spores, may have led to larger mycelial pellets and therefore to increased physiological heterogeneity, perhaps explaining the departure from exponential growth towards the end of rapid growth (although it should be noted that a different medium was used that might equally be responsible).

Increasing the copy number of *redD* resulted in higher levels of the *redD* and *redX* transcripts, undecylprodigiosin production in exponential growth, and a considerable increase in the yield of the antibiotic. Thus the only limitation to undecylprodigiosin synthesis, at least under these conditions, appears to be the availability of enough of the RedD activator. Increases in undecylprodigiosin production after introduction of cloned copies of *redD* into *S. coelicolor* A3(2) were noted also by Malpartida *et al.* (1990) and Narva and Feitelson (1990), although it was not possible to deduce whether this reflected earlier expression of the pathway genes, or their expression at higher levels once growth had ceased; in our studies, the former clearly occurs. Similar increases in antibiotic production were observed with extra copies of the pathway-specific activator genes for actinorhodin (Fernandez-Moreno *et al.*, 1991), daunorubicin (Stutzman-Engwall *et al.*, 1992), and streptomycin (Ohnuki *et al.*, 1985); as noted by Chater (1990), extension of this principle to industrial fermentations could potentially achieve equally dramatic improvements in antibiotic titre, particularly with unimproved strains. The reason for the continuous increase in the level of the *redD* transcript during growth of M145(pIJ6014) (Fig. 4A) is unknown, but may reflect a possible increase in plasmid copy number, or possibly changes in supercoiling that might result in elevated levels of expression of the now plasmid-borne *redD* promoter.

Our results are consistent with the notion of a role for growth rate, or perhaps more specifically the cessation of growth, in determining the onset of antibiotic production. Although antibiotic biosynthesis has been observed in chemostats at low growth rates (see Bushell (1989) for references), this might reflect physiological heterogeneity within mycelial clumps, and production by cells in the interior that have stopped growing. In this context, it is interesting to note the apparent ability of ppGpp to reduce growth rate in *Escherichia coli* (Sarubbi *et al.*, 1988; Schreiber *et al.*, 1991), and the apparent correlation of ppGpp and antibiotic biosynthesis, albeit incomplete, previously observed in several streptomycetes (Ochi, 1986; 1987a,b; 1990; Strauch *et al.*, 1991; Kelly *et al.*, 1991). Although we saw a correlation between ppGpp synthesis and transcription of *redD* during the transition of a normal culture into stationary phase, this was not observed after

nutritional shutdown or after addition of serine hydroxamate. These results, and the observations of Bascarán *et al.* (1991), suggest that an increase in the level of ppGpp is not a sufficient physiological signal for the activation of transcription of antibiotic biosynthetic genes.

It is difficult to know whether the low level of *redD* transcription during exponential growth represents a basal level of expression from all cells, or reflects physiological heterogeneity within clumps of mycelium, with transcription exclusively from those that are growth-limited; the results obtained with the multi-copy construct pIJ6014 are consistent with either interpretation. Although the elevated levels of the *redD* transcript observed with this multi copy plasmid might reflect titration of a putative *redD* repressor, there is no genetic evidence to suggest the existence of a negatively acting regulatory gene.

Narva and Feitelson (1990) failed to detect a *redD* transcript by S1 nuclease mapping in experiments with RNA from 2–3 d cultures. Albeit with a different medium, this would correspond to a point in our experiments at which the level of the *redD* transcript had declined to barely detectable levels (data not shown). *redD* is transcribed from a promoter with limited similarity to the consensus sequence for the major class of eubacterial promoters. Although the preliminary experiments with RNA polymerase might indicate a role for a minor holoenzyme containing an alternative sigma factor in *redD* activation (see Buttner (1989) for a review of RNA polymerase heterogeneity in *Streptomyces*), the transcripts we observed *in vitro* might represent a basal level of *redD* transcription by the major holoenzyme in the absence of a positively acting regulatory molecule that might be required for elevated activity *in vivo*.

redD is homologous to the pathway-specific activator gene (*actII-ORF4*) required for actinorhodin biosynthesis in the same host (Fernandez-Moreno *et al.*, 1991), to the activator gene (*dnrI*) for daunorubicin production in *Streptomyces peucetius* (Stutzman-Engwall *et al.*, 1992), and to the 5' end of the putative pleiotropic regulatory gene *afsR* of *S. coelicolor* A3(2) (Horinouchi *et al.*, 1990). Alignment of the amino acid sequences they encode fails to reveal a convincing DNA-binding motif that is common to all four proteins, and previously suggested possible motifs (Stutzman-Engwall *et al.*, 1992; Horinouchi *et al.*, 1990) fall outside the regions of homology; perhaps these genes encode a family of regulatory proteins with a novel means of recognizing specific nucleotide sequences.

Experimental procedures

Bacterial strains and culture conditions

High-density spore preparations (about 10^{10} colony-forming units ml⁻¹) of *S. coelicolor* A3(2) strain M145 (SCP1⁻, SCP2⁻,

prototrophic) were obtained and pre-germinated as previously described (Strauch *et al.*, 1991). Aggregated germings were dispersed by brief sonication and inoculated into 50 ml of medium in 250 ml siliconized flasks containing coiled stainless steel springs (about 25 cm flask⁻¹, with 2.5 turns cm⁻¹). Unless otherwise stated, the medium contained 55 mM (1% w/v) glucose, 25 mM TES buffer (pH 7.2), 15 mM (NH₄)₂SO₄, 0.5 mM NaH₂PO₄, 0.5 mM K₂HPO₄, 5 mM MgSO₄, 5% w/v PEG 6000, 1% v/v Rhodorsil Antimousse 426R antifoam, and 0.1% v/v trace element solution (0.1 g l⁻¹ of each of ZnSO₄·7H₂O, FeSO₄·7H₂O, MnCl₂·4H₂O, CaCl₂·6H₂O and NaCl) supplemented with 0.2% w/v casamino acids. The inoculum was adjusted to give an OD_{450 nm} against water of about 0.1 (0.05 of which reflected medium components (largely antifoam)); this corresponded to a spore inoculum of 4 × 10⁶ ml⁻¹. Flasks were incubated at 30°C and 300 r.p.m., and growth was monitored at OD_{450 nm} against water. The conditions used were modified from those adopted and developed by Hodgson (1982); the high spore inoculum, coiled springs and PEG 6000 all contributed to dispersed growth. Nutritional shiftdown, treatment with serine hydroxamate, and assessment of undecylprodigiosin production were performed as previously described (Strauch *et al.*, 1991).

Plasmids

For analysis of *redD* transcription, a 2.4 kb *Bam*HI–*Pst*I fragment from pIJ2341 (Malpartida *et al.*, 1990) that contains all of *redD* (Narva and Feitelson, 1990) was cloned in *Bam*HI–*Pst*I-cleaved pIJ2926 (a derivative of pUC18 (Yanisch-Perron *et al.*, 1985), with *Bgl*II sites flanking a modified polylinker; G. R. Janssen, personal communication) to yield pIJ6013. For analysis of *redX* transcription, the 1.4 kb *Bam*HI fragment from pIJ2341 that is internal to a *red* biosynthetic transcript (Malpartida *et al.*, 1990; Guthrie and Chater, 1990) was cloned in pSPT18 (obtained from Boehringer Mannheim) with the promoter-proximal *Bam*HI site adjacent to the SP6 promoter of the vector; the resulting plasmid (pIJ6000) was subjected to restriction mapping and a *Sma*I site was found approximately 220 bp from the promoter-proximal end of the insert. pIJ6014 is a derivative of pIJ487 (Ward *et al.*, 1986) with the 2.4 kb *Bgl*II *redD* fragment from pIJ6013 inserted in the *Bgl*II site of the polylinker.

S1 nuclease mapping

For each S1 nuclease reaction, 30 µg of RNA was hybridized in NaTCA buffer (Murray, 1986) to about 0.02 pmol (approximately 10⁴ Cerenkov c min⁻¹) of labelled probe. For *redD*, a 1.3 kb *Cla*I–*Nde*I fragment from pIJ6013 that contains the *redD* promoter region was uniquely labelled with ³²P (Maxam and Gilbert, 1980) at the 5' end of the *Cla*I site within the *redD* coding region (Fig. 1B, nucleotide position 726) and used as probe. The results reported were confirmed using a probe uniquely end-labelled at a position 147 nucleotides downstream of the *redD* translational start codon and generated using the polymerase chain reaction (Ehrlich, 1989) and synthetic oligonucleotides; no additional transcriptional start sites were detected. For *redX*, a 990 bp *Sma*I–*Aat*II fragment from pIJ6000 uniquely labelled (Maxam and Gilbert, 1980) at the 5'

end of the *Sma*I site that lies an undetermined distance downstream of the *redX* promoter was used as probe. All subsequent steps were as described in Strauch *et al.*, (1991). Nucleotide sequence ladders were derived as described by Maxam and Gilbert (1980). Before assigning a precise RNA initiation site for *redD*, one nucleotide was subtracted from the length of the protected fragment to account for the difference in 3' ends resulting from S1 nuclease digestion and the chemical sequencing reactions (Hentschel *et al.*, 1980).

Amino acid sequence comparisons

The amino acid sequences encoded by *redD* and by the 5' end of the oppositely orientated open reading frame were used to search Release 29 of the EMBL Data Library using TFAST; alignments of the *redD* product with those of *actII*–ORF4, *dnrI* and *afsR*, and of the protein encoded by the oppositely orientated open reading frame with the *trkA* product, were achieved using BESTFIT; both programs were accessed via the UWGCG Package (Devereux *et al.*, 1984).

Reproducibility

Each experiment was performed in its entirety at least twice, and usually three times. The results shown in this paper were typical of the repeated experiments.

Acknowledgements

We thank Paco Malpartida for the gift of pIJ2341, Mark Buttner, Keith Chater, David Hopwood and Tobias Kieser for their comments on the manuscript, and David Hodgson for many useful conversations. The work was supported by grant-in-aid from the Agricultural and Food Research Council and the John Innes Foundation. E.S., H.C.G. and N.A. were recipients of postdoctoral fellowships from the Deutscher Akademischer Austauschdienst, CONICET, and The Royal Society, respectively.

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