Mol Gen Genet (1991) 227:28–32
0026892591001398

MGG

New classes of *Streptomyces coelicolor* A3(2) mutants blocked in undecylprodigiosin (Red) biosynthesis

E.A. Coco, K.E. Narva and J.S. Feitelson*

Medical Research Division, American Cyanamid Company, Pearl River, NY 10965, USA

Received June 25, 1990 / November 30, 1990

Summary. Fifteen mutants of *Streptomyces coelicolor* A3(2) blocked in both the bipyrrole branch (*redA*) and a second site specific to the undecylprodigiosin pathway were characterized. Some of the mutants were ordered biosynthetically based on cosynthesis experiments. Complementation of each of the mutants with wild-type DNA cloned in low- and high-copy number plasmid vectors allowed the mutants to be separated into 12 new classes which are physically clustered within approximately 37 kb on the *S. coelicolor* genome. Early-step biosynthetic genes are centrally located and are flanked by later-step and regulatory genes.

Key words: Recombinant DNA – Antibiotic production – Genetic organization – Directed mutant screen – Prodigiosin

Introduction

Molecular cloning of specific antibiotic production genes, identified by complementation of blocked mutants, has lead to a greater understanding of the genetic organization and regulation of antibiotic biosynthetic pathways. One of the earliest successful approaches of this type used mutants blocked in the synthesis of undecylprodigiosin, the Red antibiotic of *Streptomyces coelicolor* A3(2) (Feitelson and Hopwood 1983). Results of this study demonstrated that Red is biosynthesized from mono- and bipyrrole precursors in *S. coelicolor* by a convergent branched pathway similar to the prodigiosin biosynthetic pathway of *Serratia marcescens* (Fig. 1A). Subsequently, over 21 kb of wild-type DNA were cloned and four specific *red* loci (A, B, E, and F) involved in bipyrrole biosynthesis were identified by subcloning and complementation (Feitelson et al. 1985). Recently, the entire *red* gene cluster was isolated on 35.7 kb of *S. coelicolor* DNA as a single cloned fragment (Malpartida et al. 1990). One gene apparently involved in transcriptional regulation of Red biosynthesis (*red*D) has been characterized in detail (Narva and Feitelson 1990).

Additional *red* mutants will be useful in further studies of Red production, regulation, and resistance. For this purpose, a directed screen for new mutants was devised to identify mutations in the monopyrrole branch or precursor steps common to both branches of Red biosynthesis. In the present work, we describe the isolation and characterization of 15 new *red* mutants, and further define the overall physical and biosynthetic organization of the *red* gene cluster.

Materials and methods

Bacterial strains. Streptomyces strain TK16 (*arg*A1, *gua*A1, *act*IV117, *red*A59), a recombinational derivative of *S. coelicolor* A3(2) cured of plasmids SCP1 and SCP2, was used in mutagenesis experiments. New *red* alleles identified in TK16 UV mutants were assigned letters G through R.

Isolation of new Red mutants. Spores of strain TK16 were subjected to UV irradiation at 260 nm to give survival levels of approximately 1.2% (Feitelson et al. 1986), and plated on PGA (0.5% Difco Bacto-peptone, 1% glycerol, 2.2% agar, pH 7.1) containing 23 μM 4-methoxy-2,2′-bipyrrole-5-carboxaldehyde (MBC). MBC was purified from *Serratia marcescens* 9-3-3 (Morrison 1966) and quantitated as described by Wasserman et al. (1966). Strain TK16 typically produced Red antibiotic after 3 days at 30° C on PGA+MBC plates supplemented with arginine and guanine. Colonies that failed to produce Red were selected for further analysis.

Plasmids. All plasmids used are listed in Table 1.

Recombinant DNA methods. Growth of *Streptomyces* liquid cultures for protoplasts and plasmid DNA purifi-

* *Present address and correspondence to:* J.S. Feitelson, Mycogen Corporation, Dept. of Molecular Biology, 5451 Oberlin Drive, San Diego, CA 92121, USA

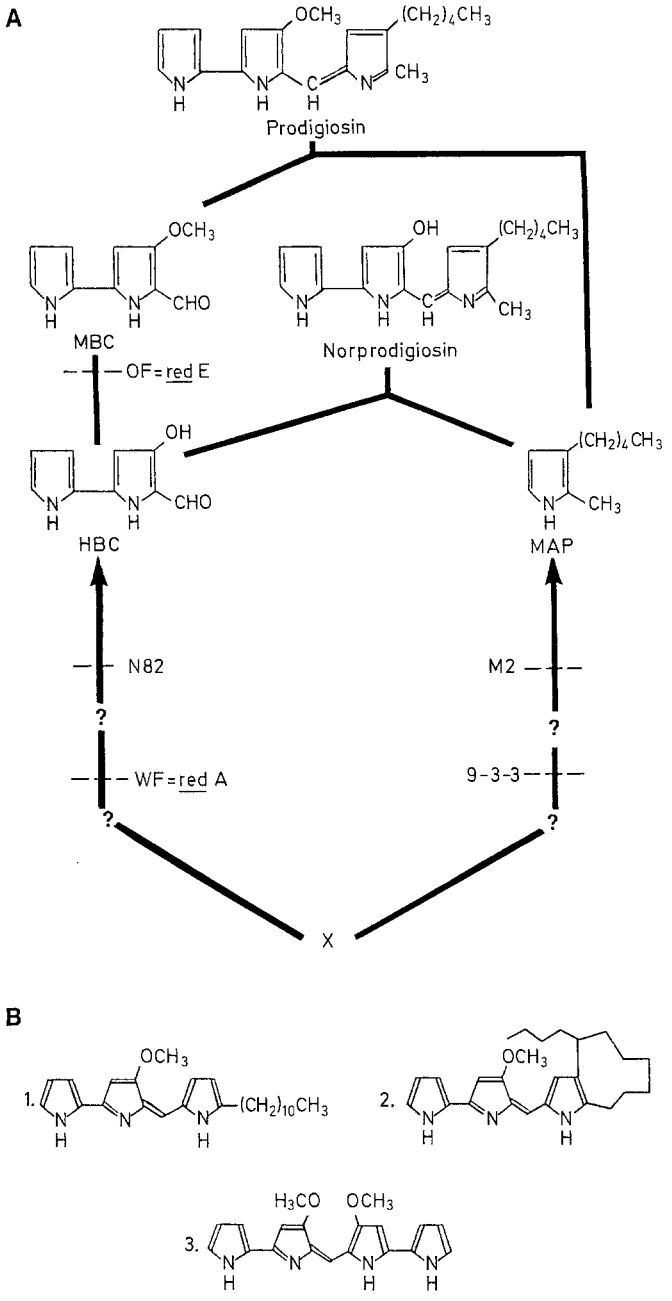

Fig. 1. **A** The convergent pathway of prodigiosin biosynthesis in *Serratia marcescens*. This scheme was developed on the basis of cosynthesis responses of *Serratia* mutants (Williams and Qadri 1980). The structures of known intermediates have been identified; unknown intermediates are indicated by *question marks*. The locations of mutants are indicated by *dotted lines*; *red*A and *red*E are loci in the *Streptomyces coelicolor* biosynthetic pathway. Mutant X represents a *Serratia* mutant believed to be blocked in a common, early branch of the monopyrrole and bipyrrole pathways. MBC, 4-methoxy-2,2′bipyrrole-5-carboxaldehyde; HBC, hydroxybipyrrolecarboxaldehyde; MAP, methyl-3-amylpyrrole. **B** Structures of the three red pigmented antibiotics of *S. coelicolor* (Tsao et al. 1985). 1, undecylprodigiosin; 2, butylcycloheptylprodiginine; 3, dipyrrolyldipyrromethane analog of prodigiosin

cation, transformation, and DNA manipulations were done by standard techniques (Hopwood et al. 1985). Thiostrepton (kindly supplied by S.J. Lucania, E.R. Squibb & Sons, New Brunswick, N.J.) was used at

Table 1. Plasmids

Plasmid	Construction	Reference
pIJ702	*Streptomyces* multicopy vector derived from pIJ350, carrying *tsr* and *mel*	Katz et al. (1983)
pIJ922	*Streptomyces* single-copy vector derived from SCP2*, carrying *tsr*	Lydiate et al. (1985)
pIJ759	pIJ922 with a 20.7 kb *Mbo*I partial insert, cloned in the *Bam*HI site, complements *red*ABEF	Feitelson et al. (1985)
pIJ755	pIJ350 with an 8.9 kb *Pst*I insert, complements *red*AE	Feitelson et al. (1985)
pIJ766	pIJ702 with a 4.0 kb *Pst*I-*Sst*I insert from pIJ755, complements *red*A	Feitelson et al. (1985)
pIJ2340	pIJ922 with a 27 kb *Mbo*I partial insert, cloned in the *Bam*HI site, complements *red*ABEF	Malpartida et al. (1990)
pIJ2342	pIJ940 with a 13 kb *Pst*I insert, carrying *hyg* and *tsr*, complements *red*CD	Malpartida et al. (1990)

hyg, hygromycin B resistance; *mel*, melanin biosynthesis; *tsr*, thiostrepton resistance

50 μg/ml, and hygromycin B (Sigma Chemical Co.) was used at 100 μg/ml, for selection of transformants.

Cosynthesis. Two methods of assaying cosynthesis were used: (i) inoculation side-by-side and (ii) pairwise co-inoculation of strains. In the first method, spores of each mutant were spread on half of a PGA+MBC plate, leaving a gap of approximately 2 mm between the cultures. Mutants which produced Red (converters) are blocked earlier in the biosynthetic pathway. Alternatively, spores of each strain tested were inoculated together on PGA+MBC plates; in these cases biosynthetic order could not be determined. Cosynthesis was scored after 3–4 days at 30° C. Triplicate assays were performed with both methods.

Results

Isolation of new Red mutants

*S. coelicolor red*A mutants are blocked at an early step in the bipyrrole pathway (Fig. 1 A) and fail to produce Red unless they are supplied with exogenous bipyrrole (MBC) (Feitelson et al. 1986). Monopyrrole biosynthesis is unimpaired in these mutants. A collection of new *red* mutants was generated by subjecting spores of TK16 (*red*A) to UV mutagenesis. Among 3053 surviving colonies plated on medium containing MBC, 15 were stably Red$^-$ (0.49%). These double mutants were expected to have a second mutation in one of the following steps:

Table 2. Cosynthesis between TK16 double *red* mutants

	N 1	K 2	M 13	G 15	O 17	I 23	R 24	L 27	N 28	H 30	M 31
N1				^		r					
K2										^	
M13				^		^				r	
G15	<		<				<		<	<	<
O17							r			r	
I23	r		<				<		<	^	<
R24				^	r	^					
L27										^	
N28				^		^					
H30		<	r	^	r	<		<			<
M31				^		^				^	

The arrowheads point to the mutants which produced Red (converters). Cosynthesizing mutant pairs requiring cell contact are indicated by the symbol, r. All pairwise combinations were tested. The following mutants failed to cosynthesize with all other mutants tested: TK16–21, -*red*J25, -*red*P29, and -*red*Q16

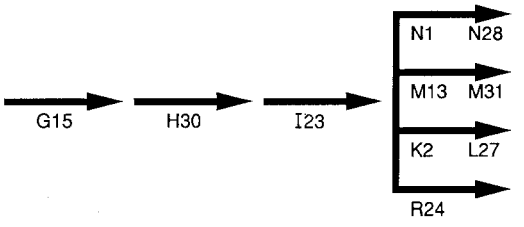

Fig. 2. Proposed biosynthetic ordering of the TK16 double *red* mutants based on cosynthesis. The mutants blocked later in the monopyrrole and/or common branch of monopyrrole/bipyrrole pathway are grouped together based on their cosynthesis patterns with TK16-*red*G15, -*red*H30, and -*red*I23

(a) monopyrrole biosynthesis, (b) condensation of the bipyrrole and the monopyrrole, or (c) possible early steps common to both biosynthetic branches.

Characterization of new Red mutants

Cosynthesis. The position of each mutational block in the Red pathway was determined by cosynthesis tests using all possible pairwise combinations on agar plates as summarized in Table 2. The proposed biosynthetic order of some of the *red* double mutants (Fig. 2) suggested that the monopyrrole branch of the *Streptomyces* Red pathway is more complex than monopyrrole biosynthesis in the *Serratia* prodigiosin pathway. This is consistent with the existence of three pigmented Red compounds (Fig. 1 B) chemically isolated and identified from *S. coelicolor* (Tsao et al. 1985).

Complementation. A total of approximately 37 kb of DNA cloned on pIJ2340 and pIJ2342 (Fig. 3) presumably encodes all of the biosynthetic and regulatory information required to produce the Red antibiotics in *Streptomyces*. When the inserts from these two plasmids were cloned together on a single plasmid construct, pIJ2355,

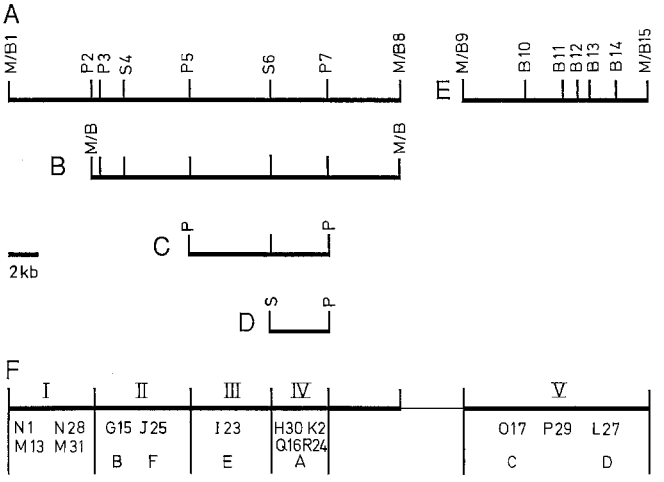

Fig. 3. A–E Restriction maps of inserts from plasmids used in this study. A, pIJ2340; B, pIJ759; C, pIJ755; D, pIJ766; E, pIJ2342. *Bold lines* represent *S. coelicolor* DNA. Restriction site abbreviations: B, *Bam*HI; M, *Mbo*I; P, *Pst*I; S, *Sst* I. Sites are numbered consecutively. **F** Genetic organization of the *red* gene cluster. Roman numerals I–V represent regions defined by cloned *red* DNA. Letters represent *red* loci within each region assigned on the basis of combined complementation and cosynthesis data

and transformed into protoplasts of *S. parvulus*, Red production occurred (Malpartida et al. 1990). *S. parvulus* is a heterologous species not known to produce any tripyrroles nor to hybridize with *red* DNA.

Five recombinant plasmids containing various inserts of wild-type *red* DNA (Table 1), were used in complementation experiments to identify the new *red* genes and to locate their positions on the physical map of the gene cluster. Detailed restriction maps of the plasmid inserts (Fig. 3) were used to localize each of the 15 new *red* mutations to five regions (I–V) in the cloned DNA. Because pIJ2342 lacked the *red*A gene, it was necessary to introduce this plasmid into TK16 mutant protoplasts already containing pIJ766, with double selection for thio^r and hyg^r, to localize mutations in region V.

The majority of complementation data were unambiguous: either the mutant in question was restored to wild-type levels of Red production or failed to produce any detectable Red on regeneration agar (Table 3). In addition, there were several other interesting findings. First, many transformations resulted in a significant fraction of sectored colonies, with clearly discernible wedge-shaped Red$^+$ regions within an otherwise Red$^-$ colony. Because of the highly non-diffusible chemical nature of Red (Williams and Qadri 1981), sectored colonies were easily scored. This level of analysis is very difficult with most other systems for analyzing gene expression during antibiotic biosynthesis.

Second, the frequency of Red$^+$ and sectored Red colonies occasionally varied with a particular insert cloned in various mutants (e.g. TK16-*red*N1, -*red*M13, -*red*N28, and -*red*M31 with pIJ2340), suggesting that plasmid integration of the insert might be necessary to regenerate a wild-type gene which is truncated on the cloned DNA insert. The frequency may depend on the distance between the mutation and an insert terminus. In the pre-

Table 3. Complementation assays

Mutant	pIJ766	pIJ755	pIJ759	pIJ2340	pIJ2342[a]	Region
N1	n.t.	n.t.	−	+[10]	−	I
K2	+	−	+	n.t.	n.t.	IV
M13	n.t.	n.t.	−	+[11]	−	I
G15	−	−	+	+	−	II
Q16	s[1]	−	s[2]	n.t.	n.t.	IV
O17	n.t.	n.t.	−	−	+[8]	V
21	n.t.	n.t.	−	−	+	V
I23	−	+[3]	+[4]	+	−	III
R24	+/−[5]	s[6]	s[7]	n.t.	n.t.	IV
J25	−	−	+	+	−	II
L27	n.t.	n.t.	−	−	+	V
N28	n.t.	n.t.	−	+[12]	−	I
P29	n.t.	n.t.	−	−	+/O[9]	V
H30	+	+	+	n.t.	n.t.	IV
M31	n.t.	n.t.	−	+[13]	−	I

n.t., not tested; s, sectored colonies

[a] Protoplasts transformed with pIJ2342 carried pIJ766 to complement the *red*A mutation in the parental TK16 strain

[1] 1 (1.4%) Red$^+$: 5 (6.9%) sectored: 66 (91.7%) Red$^-$
[2] 3 (3.3%) sectored: 89 (96.7%) Red$^-$
[3] 6 (75%) Red$^+$: 2 (25%) sectored
[4] 34 (94.4%) Red$^+$: 2 (5.6%) sectored
[5] 1 (0.4%) Red$^+$: 267 (99.6%) Red$^-$
[6] 1 (1.6%) sectored: 61 (98.4%) Red$^-$
[7] 24 (16%) sectored: 129 (84%) Red$^-$
[8] 16 (20.5%) Red^{++}: 30 (38.5%) Red$^+$: 32 (41%) Red$^-$
[9] 41 (22.1%) Red$^+$: 22 (11.9%) sectored: 2 (1.1%) Red$^-$: 120 (64.9%) Orange
[10] 93 (100%) Red$^+$
[11] 79 (96.3%) Red$^+$: 3 (3.7%) Red$^-$
[12] 73 (69.5%) Red$^+$: 20 (19%) sectored: 12 (11.5%) Red$^-$
[13] 21 (100%) Red$^+$

vious example, the mutations in TK16-*red*N1 and -*red*M31 may be further from an insert terminus than the lesions represented by TK16-*red*M13 or -*red*N28.

Third, overproduction of Red (Red^{++}) was observed in complementation tests of TK16-*red*O17[pIJ766] with pIJ2342. This phenotype results from a gene dosage effect of the regulatory gene, *red*D (Narva and Feitelson 1990), and is reminiscent of the 40-fold overproduction of actinorhodin by wild-type *actII* clones (Hopwood et al. 1986). However, TK16-*red*O17 and another region V mutant, TK16-*red*L27, cosynthesized with other *red* mutants. This result suggested that these isolates are not *red*D mutants, since *red*D strains are a regulatory class of mutants that fail to cosynthesize with other *red* mutants (Feitelson et al. 1985; Rudd and Hopwood 1980). Additional complementation studies are necessary to determine whether a third new strain with a lesion in region V, TK16-21, is allelic to *red*D. Therefore, this mutant strain was not assigned an allele designation.

Lastly, an orange pigment and an assortment of Red phenotypes were observed when strain TK16-*red*P29 contained both pIJ766 and pIJ2342. Since the *red*E gene product is required for the O-methyltransferase activity that converts the yellow precursor, undecylnorprodigiosin, to Red, these observations are consistent with the hypothesis that TK16-*red*P29 is a triple mutant, possessing lesions in *red*A, *red*E, and a mutation in region V.

Genetic organization

Combining the cosynthesis and genetic data, it was possible to correlate the proposed biosynthetic order of the *red* mutations with their physical location in the gene cluster, and to assign new *red* gene designations (Fig. 3). The three mutations blocked earliest in the pathway (*red*G15, H30, and I23) are centrally located. The mutations representing later blocks, or different branches of the monopyrrole pathway, map either to the "left" (*red*N and *red*M) or to the "right" (*red*K12, *red*L27) side of the Red gene cluster. Previously defined genes in the bipyrrole branch (*red*A, *red*BF) were also found to be nonadjacent (Feitelson et al. 1985). Thus, the genes for monopyrrole and bipyrrole biosynthesis are physically interspersed. The observation that *red*I23 and *red*E map to the same 4.9 kb fragment, while *red*H30 and *red*A map to an adjacent 4.0 kb fragment, suggests the possibility that early genes and late genes for each branch of the pathway might be clustered together; *red*H30 is bocked earlier than *red*I23, while *red*A is blocked earlier than *red*E.

Due to the low frequency of complementation using TK16-*red*Q16 and -*red*R24 as recipients, it was difficult to assign these mutants to a particular class, though the most probable location of their mutations is in region IV of the physical map, adjacent to *red*A, *red*K2, or *red*H30. Further subcloning and complementation is required to dissect the genetic organization within this 4.0 kb region.

Discussion

Previous work defined four *red* loci (A, B, E, and F) in the bipyrrole branch of Red biosynthesis in *S. coelicolor*, as well as a regulatory gene (*red*D) and a gene of unknown function (*red*C) (Feitelson et al. 1985; Malpartida et al. 1990; Narva and Feitelson 1990). At least 12 new complementation classes of *red* mutants blocked in monopyrrole biosynthesis or steps common to both branches of the Red biosynthetic pathway (*red*G–R) were defined in the present study, bringing the total number of genes required for the biosynthesis of the Red antibiotics in *S. coelicolor* to a minimum of 18.

The physical arrangement of the *red* genes is complex and does not reflect the direct biosynthetic order. Early biosynthetic genes (e.g. *red*G15, *red*H30, *red*I23) are centrally located on the physical map, and are flanked by later biosynthetic genes and regulatory genes. Since there is a branch common to both the monopyrrole and bipyrrole pathways, and the final tripyrrole product requires condensation of both mono- and bipyrrole precursors, it is reasonable to propose that genes in both arms of the pathway beyond the branch point might be co-ordinately expressed. The gene implicated in the positive activation of the Red pathway, *red*D, maps to the far right

end of the cluster, and has been characterized in detail (Narva and Feitelson 1990). This organizational complexity may be required for the regulation of a branched pathway incorporating both amino acid and fatty acid precursors. This situation contrasts to the general organization of the *S. coelicolor* actinorhodin (Malpartida and Hopwood 1986), *S. hygroscopicus* bialophos (Murakami et al. 1986), and *S. glaucescens* tetracenomycin C (Motamedi and Hutchinson 1987) gene clusters, in which early biosynthetic genes are clustered at one end and late biosynthetic gene are clustered at the other end of the complex.

Results of cosynthesis experiments using new *red* mutants blocked in monopyrrole biosynthesis suggested that monopyrrole biosynthesis in the *Streptomyces* Red pathway is more complex than the corresponding branch of the *Serratia* prodigiosin pathway. This complexity probably reflects the presence of at least three different Red compounds produced in *S. coelicolor* (Tsao et al. 1985), including a cyclized form, butylcycloheptylprodiginine (structure 2 in Fig. 1B) with the 11 carbon sidechain internally attached to the C ring. It is possible that the enzymatic functions required for cyclization may be encoded by late-step genes, such as *red*M or *red*N; however, further experimentation is required to obtain direct biochemical evidence in support of this hypothesis.

Acknowledgements. This work was submitted in partial fulfillment of the requirements for the Master of Science degree in Microbiology at Rutgers, The State University of New Jersey, by E.A.C. We thank the Medical Research Division, American Cyanamid Company for research support at Pearl River, and D.A. Hopwood for helpful discussions and for providing strains containing plasmids pIJ2340 and pIJ2342.

References

Feitelson JS, Hopwood DA (1983) Cloning of a *Streptomyces* gene for an O-methyltransferase involved in antibiotic biosynthesis. Mol Gen Genet 190:394–398

Feitelson JS, Malpartida F, Hopwood DA (1985) Genetic and biochemical characterization of the *red* gene cluster of *Streptomyces coelicolor* A3(2). J Gen Microbiol 131:2431–2441

Feitelson JS, Sinha AM, Coco EA (1986) Molecular genetics of Red biosynthesis in *Streptomyces*. J Nat Prod 49:988–994

Hopwood DA, Bibb MJ, Chater KF, Kieser T, Bruton CJ, Kieser HM, Lydiate D, Smith CP, Ward JM, Schrempf H (1985) Genetic manipulation of *Streptomyces*: A laboratory manual. John Innes Foundation, Norwich, UK

Hopwood DA, Malpartida F, Chater KF (1986) Gene cloning to analyse the organization and expression of antibiotic biosynthesis in *Streptomyces*. In: Kleinkauf H, Van Dohren H, Dornauer H, Nesemann G (eds) Regulation of secondary metabolite formation. Weinheim, VCH, pp 23–33

Katz E, Thompson CJ, Hopwood DA (1983) Cloning and expression of the tyrosinase gene from *Streptomyces antibioticus* in *Streptomyces lividans*. J Gen Microbiol 129:2703–2714

Lydiate DJ, Malpartida F, Hopwood DA (1985) The *Streptomyces* plasmid SCP2*: its functional analysis and development into useful cloning vectors. Gene 35:223–235

Malpartida F, Hopwood DA (1986) Physical and genetic characterization of the gene cluster for the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2). Mol Gen Genet 205:66–73

Malpartida F, Niemi J, Navarrete R, Hopwood DA (1990) Cloning and expression in a heterologous host of the complete set of genes for biosynthesis of the *Streptomyces coelicolor* antibiotic undecylprodigiosin. Gene 93:91–99

Morrison DA (1966) Prodigiosin biosynthesis in mutants of *Serratia marcescens*. J Bacteriol 91:1599–1604

Motamedi H, Hutchinson CR (1987) Cloning and heterologous expression of a gene cluster for the biosynthesis of tetracenomycin C, the anthracycline antitumor antibiotic of *Streptomyces glaucescens*. Proc Natl Acad Sci USA 84:4445–4449

Murakami T, Anzai H, Imai S, Satoh A, Nagoka K, Thompson CJ (1986) The bialophos biosynthetic genes of *Streptomyces hygroscopicus*: molecular cloning and characterization of the gene cluster. Mol Gen Genet 205:42–50

Narva KE, Feitelson JS (1990) Molecular cloning and transcriptional analysis of the *redD* locus of *Streptomyces coelicolor* A3(2). J Bacteriol 172:326–333

Rudd BAM, Hopwood DA (1980) A pigmented mycelial antibiotic in *Streptomyces coelicolor*: control by a chromosomal cluster. J Gen Microbiol 119:333–340

Tsao S-W, Rudd BAM, He X-G, Chang C-j, Floss HG (1985) Identification of a Red pigment from *Streptomyces coelicolor* A3(2) as a mixture of prodigiosin derivatives. J Antibiot 38:128–131

Wasserman HH, McKeon JE, Smith LA, Forgione P (1966) Studies on prodigiosin and the bipyrrole precursor. In: A Century after Kekule, Tetrahedron Suppl. No. 8, 196:647–662

Williams RP, Qadri SMH (1980) The pigment of *Serratia*. In: von Gravenitz A, Rubin SJ (eds) The genus *Serratia*. CRC Press, Boca Raton, pp 31–75

Communicated by N.D.F. Grindley