MOLECULAR GENETICS OF *RED* BIOSYNTHESIS IN *STREPTOMYCES*[1]

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ABSTRACT.—We have studied the molecular biology of undecylprodigiosin (Red) biosynthesis by *Streptomyces coelicolor* as a model system for understanding the genetic regulation of antibiotic biosynthesis. A collection of new *red* mutants was obtained using a directed screen. Regions of DNA involved in the transcriptional regulation of *red* gene expression were also isolated.

Though much is known about the metabolic pathways for antibiotic and other secondary metabolite production, relatively little is known about the relevant enzymes (1). Only very recently has the genetic regulation of these enzymes begun to be elucidated (2). To understand the mechanisms of regulation of gene expression during antibiotic biosynthesis, it is important to identify and characterize the genes involved in these processes. This approach may also lead to practical outcomes such as rational strain improvement or hybrid antibiotic formation.

Undecylprodigiosin (Red) is a pigmented tripyrrole antibiotic closely related in structure to prodigiosin, the red pigment of *Serratia marcescens* (3). A collection of blocked mutants of *Streptomyces coelicolor* unable to produce undecylprodigiosin were isolated and characterized (3). Rudd and Hopwood showed by classical genetic mapping that *red* mutations were all tightly linked on the chromosome. Subsequent cloning work (5-7) has shown that many of the *S. coelicolor* genes responsible for Red biosynthesis were physically linked on a DNA molecule of about 24 kilobase pairs (kb) in length.

Studies of the regulation of undecylprodigiosin biosynthesis were continued using two strategies resulting in the isolation of both additional biosynthetic mutants and regulatory regions of DNA. The details and results of these two approaches are described below.

RESULTS

NEW *S. COELICOLOR RED* MUTANTS.—Undecylprodigiosin is synthesized by a convergent pathway [redrawn in (5)], when a bipyrrole (1) condenses with a monopyrrole (2) to form the authentic red antibiotic. Rudd and Hopwood classified a collection of 37 *S. coelicolor red* mutants into five groups, A-E, on the basis of cosynthesis tests and genetic mapping (4). Further analysis using cloned wild-type DNA as donor genetic material in "complementation" tests uncovered a new subgroup of the *red*E class, *red*F, which mapped about 5 kb to the left of *red*E on the physical map [(6) and Figure 1]. Using this information, the following directed screen for obtaining additional *red* mutants was devised.

1   **2**

FIGURE 1.   Restriction map and gene assignments of *Streptomyces coelicolor* Red⁺ clones used for complementation tests

*S. coelicolor red*A mutants, blocked at an early step in the bipyrrole pathway, fail to produce any red pigment on standard agar media. However, monopyrrole biosynthesis is unimpaired in *red*A mutant strains, as shown by their ability to mutually cosynthesize with *Serratia* mutant 9-3-3, which has a defined block in the monopyrrole branch of the pathway (5). Growth of the *red*A mutant on minimal medium supplemented with methoxy bipyrrole carboxaldehyde (MBC) (**1**) permits the cells to take up the exogenous **1**, condense it with endogenously synthesized monopyrrole (**2**), and produce the red compound. *S. coelicolor red*A mutants that fail to produce undecylprodigiosin on MBC-supplemented media would, therefore, be expected to have a second pathway-specific mutation in one of the following steps: (a) monopyrrole biosynthesis, (b) condensation of **1** and **2**, or (c) early steps common to both branches.

The *red*A strain was subjected to mutagenesis with uv light, resulting in 0.3% survival. Among the 3,053 surviving colonies plated on MBC-minimal medium, 15 mutants were stably Red⁻ (0.49%). These putative double *red* mutants were characterized in two ways: (a) genetically, by transformation with plasmids carrying cloned inserts that "complemented" previously defined *red* mutations and (b) biochemically, by direct assay of condensation activity in crude extracts.

The mapping of mutations was based on the availability of cloned DNA from the Red⁺ strain (5). Restriction maps and current *red* gene assignments of three plasmid inserts are shown in Figure 1. All inserts contained the *red*A⁺ allele in order to complement the *red*A mutation in the parental strain. If the plasmid used for transformation restored the Red⁺ phenotype to a double *red* mutant, then the insert also contained the corresponding wild-type allele for the new, second *red* mutation. On the other hand, if the plasmid failed to complement the second *red* mutation, the mutation was presumed to be located outside of the cloned insert.

The results obtained are shown in Table 1. Nearly half of the mutations (Class I) appeared to lie outside of the *red* DNA cloned in plasmid pIJ759. These could include mutations in the *red*C or *red*D genes that are known to map to the right of pIJ759 (F. Malpartida, personal communication). Class II mutants (3/15) contain lesions that probably lie outside of the *red* region cloned in pIJ755 but within the region defined by the boundaries of pIJ759. It is presently unclear if these mutants contain lesions in the same or different genes. Class III contained only one member. The mutation in this strain, *red*-UV30, seemed to map within the 3.89 kb cloned in pIJ766 but not in *red*A. This suggested that the *red*-UV30 mutation might lie in the 2.1 kb gap between the *red*E and *red*A genes. The single mutation in class IV (*red*-UV2) was difficult to inter-

TABLE 1. The Complementation Assay for redA-UV Double Mutants

Mutant Class	No. Mutants	Isolate No.	pIJ759 redA+B+E+ F+	pIJ755 redA+E+	pIJ766 redA+	Probable Lesion
I	8	1,13,17, 21,27,28, 29,31	−	n.t.[a]	n.t.	outside of cloned 24 kb in pIJ759
II	3	15,24,25	+	−	−	outside of PstI redA,E region
III	1	30	+	+	+	within redA PstI-SstI region
IV	1	2	+	−	+	possible negative gene within redE PstI-SstI region
V	2	16,23	sectored	sectored	sectored	?

[a]n.t. = not tested.

pret. This mutation was complemented by the small insert in pIJ766 but not complemented by the larger and completely overlapping insert in pIJ755. One possibility is that the 4.67 kb region between redB+F and redE contains a negatively acting regulatory gene that prevents red-UV2 expression when present in high copy number. Finally, the two mutants in class V gave rise to sectored Red+ colonies at a frequency of 1-5%, after transformation with any of the three plasmids tested. This partial complementation probably required recombination resulting in the observed low-frequency mixed Red phenotype. Successive subculturing of the Red+ and Red− cells yielded colonies that "bred true."

The second test used to characterize the new red mutants was based on a biochemical assay for bipyrrole-monopyrrole condensation (8). Crude cell-free extracts were prepared from each mutant by sonication, centrifugation, and filtration. Extracts were incubated in the presence of 1 (purified from S. marcescens 9-3-3), 2,4-dimethyl-3-ethyl-pyrrole (a monopyrrole analog obtained from Aldrich Chemical Co.), Mg^{+2}, ATP, dithiothreitol, and glycerol. An extract from the redA strain served as a positive control for condensation activity. Only one mutant, red-UV17, failed to produce red pigment in vitro. Therefore, this mutant was tentatively identified as a condensation mutant. Because O-methyltransferase is the only other assay available for enzymes in the Red pathway (4), the 14 other red-UV mutants were not further classified biochemically.

ISOLATION AND CHARACTERIZATION OF RED PROMOTERS.—The isolation and characterization of a number of red DNA sequences involved in the initiation and control of transcription would greatly extend our understanding of genetic regulation during antibiotic biosynthesis. The availability of promoter-probe vectors and the ability to make gene fusions provided tools with which to accomplish this task (9,10). Previous studies have shown that there is considerable heterogeneity among Streptomyces promoter sequences (11,12), suggesting the possiblity that promoters involved in secondary metabolism may have special structural features that distinguish them from promoters involved in "housekeeping" functions.

The plasmid pARC1 (Figure 2), constructed by Horinouchi and Beppu (10), was used to identify and isolate red promoters. This vector allowed the chromogenic identification of DNA fragments containing functional promoter activity by the activation of plasmid-borne genes encoding brown pigment production. Transformants contain-

ing recombinant pARC1 with a functional promoter cloned in the correct orientation at the unique *Bam*HI site produced an easily detectable brown pigment. Transformants containing intact pARC1 or pARC1 without a functional promoter at the *Bam*HI site failed to produce brown pigment.
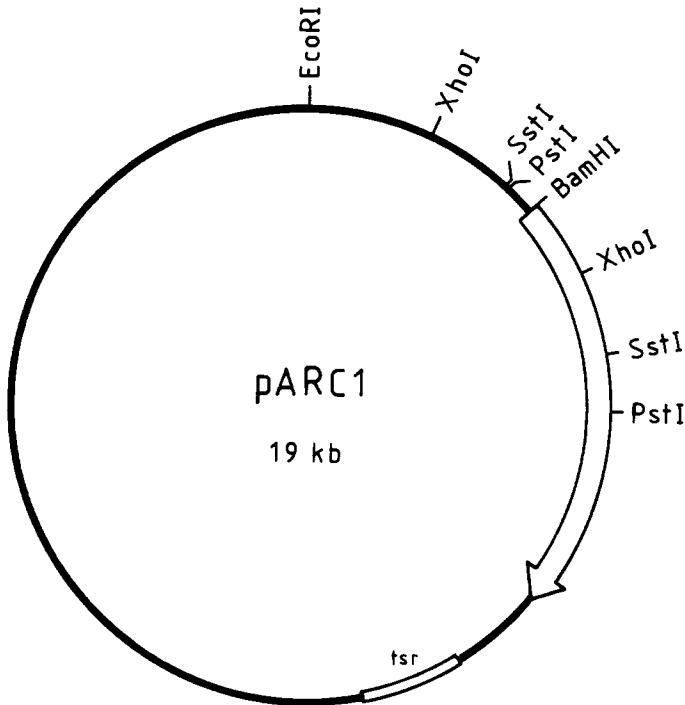

FIGURE 2.   Restriction map of promoter-probe plasmid vector pARC1 redrawn from (10). The double line with an arrow represents gene(s) involved in brown pigment production with the direction of transciption indicated; *tsr*, the *Streptomyces azureus* gene encoding thiostrepton resistance.

Inserts from two *Pst*I subclones of the *red* region, pIJ769 and pIJ770 (Figure 3), were used as donor DNA to search for promoter-active fragments. Upon digestion with *Sau*3A, pIJ770 (5.9 kb–*red*B+F) yielded 17 fragments ranging in size from 1.15 kb to 70 base pairs (bp). *Sau*3A digestion of pIJ769 (9.8 kb–*red*A+E) yielded 26 fragments ranging in size from 0.9 kb to 72 bp. In two separate experiments, these fragments were ligated into *Bam*HI-digested, dephosphorylated pARC1, and introduced by transformation into *Streptomyces lividans* protoplasts (13). Of the approximately 4,500 thiostrepton-resistant transformants obtained from the pIJ770 ligation mix, ten were Brown[+]. Of approximately 6,000 transformants obtained from the pIJ769 ligation mix, five were Brown[+]. The insert sizes and relative time and strength of pigment production in these 15 strains are shown in Table 2.

Plasmid pCLL-pr7a was found to have the strongest promoter activity and to express its activity at the earliest time in the cell growth cycle. The 160 bp *Sau*3A insert in this plasmid was subcloned into the promoter probe vector, pIJ486 (9). Functional promoter activity is assayed in pIJ486 by the activation by transcriptional fusion of resistance to neomycin. Half of the thiostrepton-resistant *S. lividans* transformants obtained were also resistant to 10 µg/ml neomycin, suggesting a unidirectional strong promoter. The insert was sequenced by the Maxam-Gilbert method (14). As seen in Figure 4, one end of the insert showed very strong homology to a promoter involved in
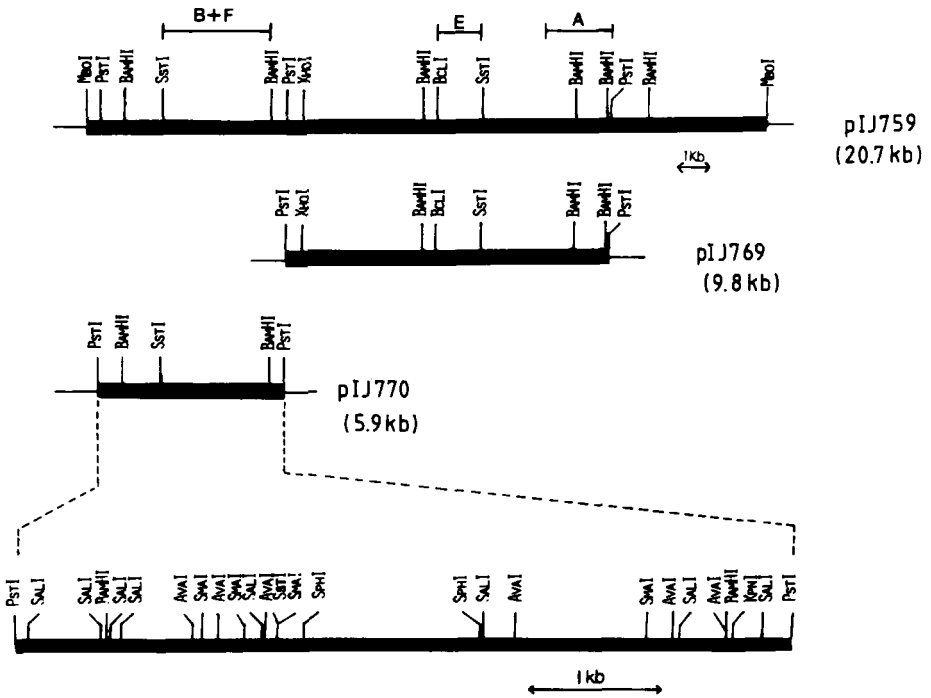
FIGURE 3.   Restriction map and gene assignments of *Streptomyces coelicolor* Red⁺ clones used for the
             isolation of promoter-active fragments

neomycin resistance: the *Streptomyces fradiae* aphP2 promoter (16). Fourteen of 18 bp
matched perfectly; the -10 and -35 boxes were also exactly aligned with a 12 bp spacer.
This striking homology to a promoter known to be involved in antibiotic resistance and

TABLE 2.   Cloning of *red* Promoters in pARC1

DNA Source	Clone Number	Insert Size[a] (kb)	Expression[b]
9.9 kb fragment with redA+E (pIJ769)	1	0.57	middle strong
	2	0.04	late weak
	4	2.89	middle strong
	6a	0.12	middle strong
	6b	0.50	late weak
6.0 kb fragment with redB+F (pIJ770)	2a	0.04	late weak
	2b	1.40	late weak
	3a	0.12	middle strong
	3b	0.22	middle strong
	4	0.22	late strong
	5b	1.02	late weak
	5c	2.69	late weak
	7a	0.16	early strong
	7b	0.08	late weak
	23	1.00	middle strong

[a]Insert sizes were estimated by comparison of SstI fragment mobility of the Brown⁺ clones to that of
pARC1.

[b]Expression levels were determined qualitatively by inspection of colonies containing each plasmid on
Bennett's agar with thiostrepton (10). Relative time of appearance after plating of spores (early=1 day;
middle=2 to 3 days; late=4 to 7 days), and strength of pigmentation (weak=slightly brown;
strong=deeply brown) were determined.

FIGURE 4.   Restriction map of pIJ486 (9) with the partial sequence of the 160 bp insert derived from
promoter-active clone 7a. DNA sequence homologies between the pr7a insert and the *Strep-
tomyces fradiae aph*P2 promoter (16) are identified by boxes, with mismatches indicated (*).
Presumed direction of transcription mediated by the insert is counterclockwise (↦→).

the strong and early expression of pr7a suggest that pr7a may be involved in some aspect
of Red resistance.

We are currently studying other promoters listed in Table 2.

DISCUSSION

The expanded collection of new *red* mutants (Table 1) should help to define further
the gross genetic architecture of *S. coelicolor* DNA involved in undecylprodigiosin pro-
duction and resistance. It appears likely that several genes corresponding to these muta-
tions map in the gaps of unknown function within the cloned *red* region. Cosynthesis
tests and chemical characterization of accumulated precursors or shunt products should
shed light on the nature of these mutations and provide more information about the
biosynthetic pathway. If any of the mutants are affected in an early step common to
both branches of the pathway, this mutant may provide a means with which to study
the switch between primary and secondary metabolism. The Red system may be par-
ticularly suitable for exploring this issue because proline, an essential primary metabo-
lite, is directly incorporated into **1** during Red biosynthesis (3), and cloned *red* DNA is
available for anaylsis (6-7).

Of special interest is the collection of *red* promoters. Current work is focused on the
purification, sequencing, and detailed characterization of several promoter-active frag-
ments (Table 2). If there are structural features specific to promoters involved in second-
ary metabolism, they may emerge from studies such as these. This could have impor-
tant implications in understanding the basis for RNA polymerase heterogeneity in

Streptomyces (15) and the general control circuits involved in differentiation and secondary metabolism. Such knowledge could be applied to developing specialized tools for overexpressing gene products by manipulating naturally occurring signals controlling gene expression.

LITERATURE CITED

1. C.R. Hutchinson, *Nat. Prod. Repts.*, **3**, 133 (1986).
2. D.A. Hopwood, M.J. Bibb, K.F. Chater, G.R. Janssen, F. Malpartida, and C.P. Smith, in: "Regulation of Gene Expression—25 years on Symp. Soc. Gen. Micbrobiol." Ed. by I. Booth and C.F. Higgins, Cambridge University Press, Cambridge, U.K., 1986, pp. 251-276.
3. R.P. Williams and S.M.H. Qadri, in: "The genus Serratia." Ed. by A. von Graevenitz and S.J. Rubin, CRC Press, Boca Raton, 1980, pp. 31-75.
4. B.A.M. Rudd and D.A. Hopwood, *J. Gen. Microbiol.*, **119**, 333 (1980).
5. J.S. Feitelson and D.A. Hopwood, *Molec. Gen. Genet.*, **190**, 394 (1983).
6. J.S. Feitelson, F. Malpartida, and D.A. Hopwood, *J. Gen. Microbiol.*, **131**, 2431 (1985).
7. J.S. Feitelson, A.M. Sinha, F. Malpartida, and D.A. Hopwood, in: "Biological, Biochemical, and Biomedical Aspects of Actinomyces: Proceedings of the 6th International Symposium on the Biology of the Actinomycetes." Ed. by G. Szabó, S. Biró, and M. Goodfellow, Akadémiai Kiadó, Budapest, Hungary, 1986, pp. 91-93.
8. P.P. Mukherjee, M.E. Goldschmidt, and R.P. Williams, *Biochem. Biophys. Acta*, **136**, 182 (1967).
9. J.M. Ward, G.R. Janssen, T. Kieser, M.J. Bibb, M.J. Buttner, and M.J. Bibb, *Molec. Gen. Genet.*, **203**, 468 (1986).
10. S. Horinouchi and T. Beppu, *J. Bacteriol.*, **185**, 406 (1985).
11. G.R. Janssen, M.J. Bibb, C.P. Smith, J.M. Ward, T. Kieser, and M.J. Bibb, in: "Microbiology-1985." Ed. by G. Hegemon and C.L. Hershberger, A.S.M., Washington, 1985, pp. 392-396.
12. M.J. Buttner and N.L. Brown, *J. Mol. Biol.*, **185**, 177 (1985).
13. D.A. Hopwood et al., in: "Genetic Manipulation of Streptomyces: A Laboratory Manual," John Innes Foundation, Norwich, England, 1985, pp. 104-114.
14. A.M. Maxam and W. Gilbert, *Meth. Enzymol.*, **65**, 499 (1980).
15. J. Westpheling, M. Ranes, and R. Losick, *Nature*, **313**, 22 (1985).
16. M.J. Bibb, M.J. Bibb, J.M. Ward, and S.N. Cohen, *Mol. Gen. Genet.*, **199**, 26 (1985).