Structure of a *Rhodococcus* gene encoding pigment production in *Escherichia coli*

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A 2.1 kbp DNA fragment from *Rhodococcus* strain ATCC 21145 gave rise to the production of blue and pink pigments in *Escherichia coli* when cloned downstream of a strong promoter. The sequence of this DNA fragment contains a single open reading frame with a putative ribosome-binding site, potentially coding for a single protein of M_r 42 560. Deletion analysis and *in vitro* transcription–translation experiments support the hypothesis that pigment production in *E. coli* is due to a single enzyme whose catalytic activity is still unknown. This small pigment gene may become useful for the development of a new generation of chromogenic cloning vectors which do not require expensive substrates for the detection of gene expression.

Introduction

Recently we reported the cloning and expression in *Escherichia coli* of pigment-producing genes from two strains of the nocardioform actinomycete *Rhodococcus* (Hill *et al.*, 1989). Initially, grey-blue *E. coli* colonies were detected which contained DNA from the buff-coloured *Rhodococcus* ATCC 21145 and the coral-coloured *Rhodococcus* sp. JL10. The pigments were analysed and found to contain blue and pink components, neither of which could be detected in the *Rhodococcus* strains. Introduction of the same DNA into the unpigmented actinomycete *Streptomyces griseus* did not result in pigment production. The preliminary chemical characterization of the pink and blue pigments indicated that they were different from the prodigiosin-like and actinorhodin-like pigmented compounds commonly produced by actinomycetes (Gerber & Lechevalier, 1976; Rudd & Hopwood, 1980; Tsao *et al.*, 1985).

Since the production of pigment by a *Rhodococcus* gene in *E. coli* is interesting, and as nocardioform genes have not yet been well studied, we determined the structure of the pigment gene from *Rhodococcus* ATCC 21145. Knowledge of the DNA sequence of the pigment gene will facilitate its development as a chromogenic

vector which does not require an expensive substrate (e.g. X-Gal).

Methods

Bacterial strains, plasmids and media. The *E. coli* strain LK111 was used in all experiments (K514 derivative; *lacI lacZ* Δ M15 *lacY*⁺) (Zabeau & Stanley, 1982). The plasmids used are listed in Table 1. Plasmids were designated Pig⁺ or Pig⁻ depending on whether they could or could not produce pigment in *E. coli*. *E. coli* LK111 containing pUC and 'Bluescript'-derived plasmids were grown in Luria–Bertani (LB) medium (Maniatis *et al.*, 1982) containing ampicillin (Ap) (100 μ g ml⁻¹). Transformants containing derivatives of pMC1871 were selected on LB agar containing tetracycline (Tc) (12.5 μ g ml⁻¹), X-Gal (40 μ g ml⁻¹) and IPTG (12 μ g ml⁻¹), or on lactose MacConkey agar containing Tc (12.5 μ g ml⁻¹).

Preparation of plasmid DNA. Plasmid DNA was prepared from *E. coli* LK111 by the alkaline-lysis method of Ish-Horowitz & Burke (1981). Large-scale preparations were purified by CsCl equilibrium gradient centrifugation (Maniatis *et al.*, 1982).

Subcloning and restriction fragment deletion procedures. Restriction endonucleases (Boehringer-Mannheim, Anglian Biotechnology or BRL) were used as described by Maniatis *et al.* (1982). Digested DNA fragments were gel-purified by electrophoresis in low-melting-point agarose gels (Seaplaque or BRL). DNA fragments were either excised and ligation reactions performed in the resolidified agar (Struhl, 1985), or they were separated from the agarose with 'GeneClean' (Bio 101, California, USA) according to the manufacturer's instructions. Klenow fragment of DNA polymerase I (Boehringer-Mannheim) was used to generate blunt-ended restriction fragments (Maniatis *et al.*, 1982). Ligations were performed with T4 DNA ligase (Boehringer-Mannheim) in a buffer containing 50 mM-Tris/HCl, pH 7.6, 10 mM-MgCl₂, 1 mM-DTT and 1 mM-ATP. Self-ligation of the vector plasmids was prevented by treatment with calf intestinal alkaline phosphatase (Boehringer-Mannheim) (Tabor, 1987). Competent *E. coli* cells were

Abbreviations: Ap, ampicillin; Lac, β -galactosidase; ORF, open reading frame; Pig, pigment production; TBE, tris/borate/EDTA; Tc, tetracycline, Ts, thiostrepton.

The nucleotide sequence data reported in this paper have been submitted to GenBank and have been assigned the accession number M30195.

Table 1. Description of plasmids

Plasmid	Phenotype	Reference
'Bluescript' KS	Ap ^r Lac ⁺	Stratagene, San Diego, USA
pBSLacI	Tet ^r Lac ⁻	
pHSLacI	Tet ^r Lac ⁺	
pKS1	Ap ^r Lac ⁻ Pig ⁺	This study
pKS3	Ap ^r Lac ⁻ Pig ⁺	
pMC1871	Tet ^r Lac ⁻	
pNC181	Ap ^r Lac ⁻ Pig ⁺	Casadaban (1984)
pNC184	Ap ^r Lac ⁻ Pig ⁻	
pNC185	Ap ^r Lac ⁻ Pig ⁺	
pNC186	Ap ^r Lac ⁻ Pig ⁻	This study
pNC191	Ap ^r Lac ⁻ Pig ⁺	
pNC192	Ap ^r Lac ⁻ Pig ⁺	
pNC194	Ap ^r Lac ⁻ Pig ⁺	Hill <i>et al.</i> (1989)
pNIL260	Ap ^r Ts ^r Pig ⁺	
pNIL270	Ap ^r Ts ^r Pig ⁻	
pUC18	Ap ^r Lac ⁺	Vieira & Messing (1982)
pUC19	Ap ^r Lac ⁺	

prepared and transformed by the method of Chung & Miller (1988). Electrophoresis of DNA restriction digests was performed in 0.8% agarose gels in 1 × TBE buffer (Maniatis *et al.*, 1982).

DNA sequencing. DNA was prepared for sequencing by the construction of ordered deletions using exonuclease III (Boehringer-Mannheim) (Henikoff, 1984) from two of the shortest pigment-producing plasmids pKS1 and pKS3, and plasmids containing upstream DNA sequences (Fig. 1). DNA sequencing was done by the dideoxy chain-terminating method of Sanger *et al.* (1977) with a 'Sequenase' kit (US Biochemicals Corp.). The DNA was radiolabelled with [³⁵S]dATP [>1000 Ci (37 kBq) mmol⁻¹] (Amersham, UK). The nucleotide sequences were analysed with an IBM XT computer, using the DNA Tools and Genepro (version 3.1) programs. A BASIC program based on the non-random distribution of G and C nucleotides in codons used by bacteria with a high G + C content (Bibb *et al.*, 1984) was used to identify potential open reading frames (ORFs) (R. Kirby, personal communication). Deduced amino acid sequences were analysed and compared by using the data bases of GenBank (release 60.0, 6/89) and EMBL (modified) (release 19.0, 5/89).

Construction of lac fusions. The *Hind*III/*Sma*I and *Bgl*II/*Sma*I fragments of pNC192 were subcloned into the *Sma*I site of pMC1871 to give in-frame fusions to the *lacZ* gene of pMC1871. Successful fusions were detected on LB-Tc plates containing X-Gal, confirmed by DNA sequencing and subsequently tested for transcriptional promoter activity on lactose MacConkey plates containing Tc.

In vitro transcription-translation. A prokaryotic DNA-directed *in vitro* transcription and translation kit (Amersham, UK) was used, according to the manufacturer's instructions for the *in vitro* transcription and translation of plasmid DNA. L-[³⁵S]Methionine [>1000 Ci (37 kBq) mmol⁻¹] (Amersham, UK) was used as the radiolabelled amino acid. The labelled proteins were separated by electrophoresis in a 15% (w/v) polyacrylamide gel containing 0.1% SDS by the method of Laemmli (1970). High molecular weight standards (M_r 18 500–330 000) (Pharmacia) were used to estimate the size of labelled proteins.

Results

Restriction fragment deletions and subclonings

The pigment-producing plasmid pNIL260 (Fig. 1) was shortened to obtain smaller Pig⁺ plasmids. Subcloning

the *Hind*III/*Eco*RV fragment of pNIL260 into pUC18 and pUC19 produced the Pig⁺ plasmids pNC181 and pNC191 respectively, which contained the insert DNA in both orientations relative to the *lacOP* promoter of pUC18 and pUC19 (Fig. 1). Plasmid pNC191 produced more pigment in *E. coli* cells than pNC181, and pNC191 contained the Pig⁺ DNA insert in the correct orientation to the *lacOP* promoter of pUC19. However, pigment production by pNC181 suggested that the Pig⁺ DNA insert had its own promoter. Deletion of the *Sma*I/*Eco*RV fragment of pNIL260 resulted in pNIL270, which did not produce pigment in *E. coli* cells (Fig. 1).

The plasmid pNC192 was generated by deletion of a small *Eco*RI fragment from pNC191, and the 1.8 kbp *Sph*I/*Eco*RI fragment of pNC192 was then subcloned into pUC18 and pUC19 to generate the plasmids pNC184 and pNC194 respectively. Pigment production from pNC194 was the same as from pNC191 and pNC192 but pNC184-containing transformants were not pigmented. This observation suggested that the coding sequence of the enzyme or enzymes responsible for pigment production lay between the *Sph*I and *Eco*RI sites of the *Rhodococcus* DNA insert and that the putative promoter lay between the *Hind*III and *Sph*I sites of pNC181.

Plasmids pNC185 and pNC186 were made by subcloning the *Bgl*II fragment of pNC192 into the *Bam*HI site of pUC18. The insert of pNC185 was Pig⁺ and was in the correct orientation to the *lacOP* promoter of pUC18 whereas the insert of pNC186 was in the reverse orientation and was Pig⁻. This result suggested that promoter activity originates from between the *Hind*III and *Bgl*II sites in the vector part of pNC181.

The plasmid pKS1 was prepared by subcloning the *Sph*I/*Eco*RI fragment of pNC192 into 'Bluescript' KS in the correct orientation to the *lacOP* promoter. pKS3 was made by deleting the *Bgl*II/*Bam*HI fragment at the 3' end of the pKS1 insert. Both plasmids were Pig⁺.

Nucleotide sequencing

The 2109 bp *Bgl*II fragment which was capable of pigment production (in plasmid pNC186) was sequenced in both directions. A large open reading frame (ORF) of 1653 bp was detected which began with a methionine ATG codon at position 299 of the 2109 bp sequence (Fig. 2). Five other in-frame methionine ATG codons were present at positions 305, 458, 581, 791 and 815 within the ORF. Since long stretches of open reading frame occur randomly in bacteria with a high G + C content, presumably due to the lack of A and T nucleotides which are a predominant feature of termination codons, additional evidence is necessary to assign the correct start codons in such organisms. The smallest Pig⁺

Fig. 1. Restriction maps of plasmids used in the analysis and sequencing of the pigment gene. The following plasmids were derived from pNIL260: pNIL270 (Pig⁻), deletion of *SmaI*/*EcoRV* fragment; pNC181 (Pig⁺) and pNC191 (Pig⁺), *HindIII*/*EcoRV* fragment of pNIL260 subcloned into pUC18 and pUC19 respectively; pNC192 (Pig⁺), deletion of 3' end of pNC191 up to *EcoRI* site; pNC184 (Pig⁻) and pNC194 (Pig⁺), *SphI*/*EcoRI* fragment (1.5 kbp) of pNC191 subcloned into pUC18 and pUC19 respectively; pNC185 (Pig⁺) and pNC186 (Pig⁻), *BglII* fragment (2.1 kbp) of pNC192 subcloned into pUC18 in both orientations; pHSLac1 (Lac⁺), *HindIII*/*SmaI* fragment (1.6 kbp) in pMC1871 *lac* fusion; pBSLac1 (Lac⁻), *BglII*/*SmaI* fragment (1.4 kbp) in pMC1871 *lac* fusion. Arrows denote direction of transcription from *lacOP*. Flanking open boxes indicate vector sequences; lines indicate insert *Rhodococcus* DNA; solid boxes indicate probable coding region and attached open boxes represent the rest of the ORF.

subclones, pKS1 and pKS3, contained inserts of 1.8 kbp and 1.5 kbp respectively and neither contained the first four ATG codons at positions 299, 305, 458, and 581 within the ORF. However, both pKS1 and pKS3 produced pigment in *E. coli* cells. This suggested that the translation initiation codon was at either position 791 or 815. The purine-rich sequence, GGAGGAAA₋₃-ATG, preceding the ATG codon at position 791 was a possible ribosome-binding site (Shine & Dalgarno, 1974). The ATG methionine codon at position 815 was not preceded by a purine-rich sequence.

The ORF was terminated by a TGA stop codon which was separated by 39 bp from a second TAA stop codon. An 8 bp stem, 4 base loop structure [$\Delta G = -14.0$ kcal mol⁻¹ (-58.6 kJ mol⁻¹), calculated by the method of Salser *et al.* (1977)] was present 78 bp downstream from the first stop codon. This stem-loop structure was

followed by only two T nucleotides. Rho-independent termination sequences in *E. coli* normally contain at least four T nucleotides after the stem-loop structure (Rosenberg & Court, 1979; Trifonov & Brendel, 1987).

Codon usage and the ORF

The G+C content of the *Rhodococcus* DNA ORF extending from the ATG methionine start codon at position 791 was 67.3 mol%. It has previously been established that bacterial species such as *Streptomyces* which have a high G + C content show a strong bias towards the use of G or C in the third or 'wobble' position of their codons (Bibb *et al.*, 1984). A low G + C content at the second position and an intermediate G + C level at the first position is also characteristic of the codon usage pattern of bacteria with a high G + C content

<i>Bgl</i> II	AGATCTTCACAAAGGAAGGCACGCCATGACAAACCTCGTTTCGCGCCCCGCGCCCCACGCCAACGGTCATGGTCGACGATCGCCGAGGTAGCCGCGCCCTCGCCGCGGTGAGCTGATCA	50	100
	TCCTCAGCCACCACCGCCGCTGGTGCTCGTTCGGGTGCGCGCGCGCGACCAACCGAGCAGATGGCCTTCCTGATCCGCCACACCACCGGATTTCTGAGGTGGCGCTGCACGAACGGA	150	200
	TCTGTGACCGTCTCGTCTCTGCCGAAGCGACACCACGGTGCCTGCCACTCACACTCGGCCATGGCAATGTGTACGTGACGCGCGCAGTGTACACCACGGGATCTCCGGGCGCCGACCGCGG	250	300
	GCGCACGGCCCGCATCTCTCGCGATCCGACCACCGACCCCGGGGATCTCACCAGGCCAGGGCACCTGGTTCCGGTGCGCGTGACACCCACAGATTTCCGATGCTTTGACCAACCGCGT	350	400
	CAGTCGCCCTCCCGCTCACTGACGCCACTGCAAAACCGGAACCTGGAGCGGTGTTTCGCCGACCTCGACGGCATCGCCGACCCGACCAAGTGTGCTGATCGACACCGATGCCGAAATCCCTCG	450	500
	CCGACCGGTACGGGCTCACCTCTCGTCCCGGCTCGGACCCCGAGGACAGCGTCCGCTGACACCCCGCGGTGCCGCACACGACACGCACACCCGAACCCACCAAGCATCCGACGCCCATG	550	600
	GCGGTCCCGCATGCGCTGAGCCGCGCGCATGCCACAGCACCAAGAAACAGCCACCTACACCGGAGGAAACA	650	700
<i>Sph</i> I	GAC ATC ACC CGC ACC GAA CTC	750	S-D
	M D I T R T E L M D R		823
	GTA CAC GCC CTG GTA CCG GCC TTC GCC GAA CGC GCA CAG AAG ACC GAA GAA AAC CGT GCT CCC CTC GAC GAG ACG ATC ACC GAC CTC ATC		913
	V H A L V P A F A E R A Q K T E E N R A P L D E T I T D L I		
	GAC TCG GGA ATC CTG GCC ACG CTG ACC CCG AAG GAA TAC GGC GGT CTC GAA CTC GGG CTC GAC GTC GCC GCC GAC ATC GTC CGC ACC ATC		1 003
	D S G I L A T L T P K E Y G G L E L G L D V A A A I V R T I		
	AGC GCC GTC TGC CCC TCG ACC GGC TGG GTC ACC TCC TTT TAC ATC GGA GCG GCC TGG CCG GTG AAC ATC TTC ACC GAA CAG GCC CAA CGG		1 093
	S A V C P S T G W V T S F Y I G A A W R V N I F T E Q A Q R		
	GAA GTG TTC GCC GAC AAG CCC TAC ACC CTC ACC GCC GGC ACC GCG GCC CCG CTG GGG CAG GTG CAG AAG GTT GAC GGC GGC TAC CGC ATT		1 183
	E V F A D K P Y T L T A G T A A P L G Q V Q K V D G G Y R I		
	ACC GGG CAG ACG GCA TGG AAC TCC GGG TCG GTG CAC GCG GAG TGG TTC ACC TTC GCC GGT GTG GTG TTC GAG GAG GGC AGC GCC CCC ACC		1 273
	T G Q T A W N S G S V H A E W F T F A G V V F E E G S A P T		
	CCG TTG TGG TTC CTG GTG CCC CGC GAG GAC GTC AAG GTC CTC GAC ACC TGG TAC ATC GCC GGC ATG TCC GGC ACC GGC AGC AAT GAC ATC		1 363
	P L W F L V P R E D V K V L D T W Y I A G M S G G T P G G S N D I		
	AGC GTC GAC GAC GTG TTC GTC CCG GAG TAC CGC ACC GGG CCG TTC GCC CTC GCC CTC GCC GGT ACC GCT CCC GGG CAG CTG ATC CAC CCC		1 453
	S V D D V F V P E Y R T G P F A L A L A G T A P G Q L I H P		
	AAC CCC ATG TAC CAC CTG CCG TTC CTG CCG TTC GCG ATG GCC GAG GTC ACC CCG GTC GTC GTC GGT GCC CTG CGC GGT GCG GCG GAC GCG		1 543
	N P M Y H L P F L P F A M A E V T P V V V G A L R G A A D A		
	TTC GTC CAG CGC ACC AAG GAC CGT CAA GGA ACC ATC AGT CAG GAG AAG GCC TCC GGC AAG CAG GCC GCC CAG ATG CGC CTC GGC CGG GCG		1 633
	F V Q R T K D R Q G T I S Q E K A S G K Q A A Q M R L G R A		
	CTG GCC GCC GCG GAC GCC GCC GAA ACC TTG CTC GAC GCC TTC TTC GAG CGC CTC ACG GCG CAG CGG CCC GAG CAG TCA GAC CCC CGT GAT		1 723
	L A A A D A A E T L L D A F F E R L T A Q R P E Q S D P R D		
	CGT GCC GAA ATG AAG CTC AAG GCA GCA TAT CTG GCC GAT CTG GCC CGC AAT GCG CTC AAC GAC ATG GTG CGC GGA ATC GGT GGG GAC GGG		1 813
	R A E M K L K A A Y L A D L A R N A L N D M V R G I G G D G		
	TAC CGG AAC TCC GCA CCG ATC CAG CGG TAC TTC CGT GAC CTG TCC GTG CTG TCG GTG CAC GCC TTC CTC GAC ATC GAC ACC GCC TCG GAA		1 903
	Y R N S A P I Q R Y F R D L S V L S V H A F L D I D T A S E		
	ACG ATC GGC CGG TTC ACC CTC GAC CTG CCC GTC TCC GAC CCG CTC CTC TGA CGAGCTGTTGGGATCAGGAGGAGGCGAAGTGGTGGGCAGTAACTCGACGAG		2 006
	T I G R F T L D L P V S D P L L ***		
	CGAACGCCTCGACGATGCAGCCAGACCTGCTGTTCGCGGGAGCGGAACAGTCAACAACCTTCGCATCGACCCCGGTCAGCGATGGCGAACTCGCCGAGATCT*		2 109

Table 2. Codon usage in the protein-coding region from position 791 bp to 1954 bp

TTT	Phe	1	TCT	Ser	0	TAT	Tyr	1	TGT	Cys	0
TTC	Phe	17	TCC	Ser	7	TAC	Tyr	9	TGC	Cys	1
TTA	Leu	0	TCA	Ser	1	TAA	***	0	TGA	***	1
TTG	Leu	2	TCG	Ser	5	TAG	***	0	TGG	Trp	6
CTT	Leu	0	CCT	Pro	0	CAT	His	0	CGT	Arg	5
CTC	Leu	19	CCC	Pro	11	CAC	His	5	CGC	Arg	12
CTA	Leu	0	CCA	Pro	0	CAA	Gln	2	CGA	Arg	0
CTG	Leu	15	CCG	Pro	11	CAG	Gln	13	CGG	Arg	8
ATT	Ile	1	ACT	Thr	0	AAT	Asn	2	AGT	Ser	1
ATC	Ile	16	ACC	Thr	25	AAC	Asn	6	AGC	Ser	4
ATA	Ile	0	ACA	Thr	0	AAA	Lys	0	AGA	Arg	0
ATG	Met	8	ACG	Thr	5	AAG	Lys	10	AGG	Arg	0
GTT	Val	1	GCT	Ala	2	GAT	Asp	2	GGT	Gly	6
GTC	Val	14	GCC	Ala	33	GAC	Asp	26	GGC	Gly	12
GTA	Val	2	GCA	Ala	5	GAA	Glu	11	GGA	Gly	4
GTG	Val	11	GCG	Ala	11	GAG	Glu	10	GGG	Gly	8

(Bibb *et al.*, 1984). These features of the codon usage pattern have allowed the identification of protein-coding sequences (Bibb *et al.*, 1984).

The complete *Rhodococcus* DNA sequence was analysed for these codon usage patterns. A non-random distribution of G and C nucleotides started at the ATG methionine codon at position 791 bp and continued until the first stop codon (TGA at position 1952 bp). The overall G + C content of the 1161 bp of the ORF starting at position 791 was 67.3% and that of the first, second and third codon positions was 66.9%, 47.3% and 88.1% respectively. This nucleotide distribution is typical for bacteria with a high G + C content.

The G and C distribution in the ten codons preceding the proposed start codon was 60%, 50% and 40% for the first, second and third positions respectively and 60%, 40% and 90% in the ten codons after the proposed start codon. The strong bias towards G and C in the third position was only apparent after this start codon and was not observed with the ATG methionine at position 815. The biased codon usage pattern was centred upon the start codon at position 791 bp, supporting the proposal that it was the translation start point.

The pattern of codon usage illustrates the preference for codons ending in G or C in the 'wobble' position (Table 2). This was true for all amino acids except glutamic acid, where the codon GAA was used as frequently as GAG. The distribution of the two codons for glutamic acid was polarized; the GAA codon was

predominantly utilized at the 5' end of the protein coding sequence and the GAG codon was preferred towards the 3' end. It is interesting that the occurrence of the GAA codon was also greater than expected in the only other sequenced *Rhodococcus* gene, the nitrile hydratase gene (Ikehata *et al.*, 1989).

Deduced amino acid sequence and *in vitro* transcription-translation

The deduced amino acid sequence of the 1161 bp coding sequence from the start codon at position 791 consisted of 387 residues, with a calculated M_r of 42056. The major labelled protein produced in the *in vitro* transcription-translation experiments by the insert DNA in plasmids pNC192 and pKS3 had an apparent M_r of approximately 42000 (Fig. 3), which agreed well with the predicted M_r .

The deduced amino acid sequence of the pigment gene product was compared with other known sequenced proteins using a limitation of 10 matches in a window of 30 residues. However, no significant amino acid sequence similarities were detected.

Upstream DNA sequence and *lac* fusions

Orientation of the pigment gene in reverse direction to the *lacOP* promoter, in pNC184, resulted in the inhibition of pigment production. However, pigment production was reinstated in pNC181, in which the

Fig. 2. Nucleotide sequence of the *Rhodococcus* pigment gene and deduced amino acid sequence of the ORF. The potential Shine-Dalgarno sequence is marked as S-D. The first six potential ATG start codons are boxed. The glutamic acid residues, E, are in bold lettering. An inverted complementary repeat sequence is indicated by opposing arrows. The stop codons are indicated by asterisks.

Fig. 3. Autoradiogram of the cell-free coupled transcription-translation polyacrylamide gel. Lanes: 1, 'Bluescript' KS; 2, pKS3; 3, pNC191; 4, pNC185; 5, pUC18; 6, no DNA. The bands at M_r 31 000 represent β -lactamase; those at M_r 42 000 represent the pigment gene product; those at M_r 60 000 are an artifact of the cell-free transcription-translation system (Amersham).

insert was in the same orientation to the *lacOP* promoter as that in pNC184, but which contained an additional *Hind*III/*Sph*I fragment from pNIL260 (Fig. 1). This suggested the presence of a *Rhodococcus* promoter in the upstream region on pNC181 which could initiate transcription in *E. coli*. However, the region between the *Hind*III and the *Bgl*II sites was not *Rhodococcus* DNA but part of the neighbouring vector DNA of pNIL260. The ability of the *Hind*III/*Bgl*II fragment of the vector DNA to promote transcription was confirmed by the construction of *lac* fusions with the promoter-probe vector pMC1871. pBSLac1 contained an in-frame *lac* fusion with the *Bgl*II/*Sma*I fragment of pNC192 and was Lac⁻. However, the *Hind*III/*Sma*I *lac* fusion in pHSLac1 was Lac⁺, suggesting that the promoter activity lay in the *Hind*III/*Bgl*II fragment derived from pNIL260 and not the *Rhodococcus* DNA insert.

The *Bgl*II fragment of pNC191, which contained the pigment gene and 700 bp of *Rhodococcus* upstream sequence, was subcloned into the *Bam*HI site of pUC18 in both orientations to the *lacOP* promoter. The plasmid pNC185, which contained the *Bgl*II fragment in the correct orientation for expression from the *lacOP* promoter, produced large amounts of pigment whereas pNC186, which contained the insert in the reverse orientation to the *lacOP* promoter, was not pigmented. This indicated, as did the *lac* fusions, that the *Rhodococcus* upstream sequence did not contain a promoter which functioned in *E. coli* and that a spurious promoter in the neighbouring vector DNA of pNIL260 was responsible for transcription in pNC181.

Discussion

The nucleotide sequence of the *Rhodococcus* pigment gene was located within a large ORF of 1653 bp.

Although this is a single ORF, the structural gene probably starts with an ATG start codon situated 492 bp downstream of the first ATG of the large ORF. The nucleotide sequence of the pigment structural gene therefore consists of 1161 bp with the coding capacity for a protein of M_r 42056. The conclusion that the pigment gene consists of 1161 bp is based on the following evidence: (i) the close agreement between the calculated M_r of the pigment gene product (42056) and the M_r determined by SDS-PAGE (42000); (ii) the asymmetrical distribution of G and C nucleotides observed in the codon usage pattern, which allowed the reliable prediction of the protein-coding sequence and the start point of the pigment gene; (iii) the ATG start codon of the 1161 bp sequence is the only ATG of the larger 1653 bp ORF which is preceded by a putative ribosome-binding site; (iv) deletion of the first four ATG codons of the longer 1653 bp ORF did not prevent initiation of translation of the pigment gene mRNA.

The expression of the pigment gene in both orientations in various vectors in *E. coli* initially suggested that the gene was regulated by an upstream sequence of *Rhodococcus* DNA in *E. coli* (Hill *et al.*, 1989). However, it was shown that the *Rhodococcus* upstream DNA sequence did not contain any *E. coli* promoter consensus sequences and was unable to promote the expression of the pigment gene in *E. coli*. Pigment production by *E. coli* cells containing plasmids with the pigment gene inserted in reverse orientation to the *lacOP* promoter was due to a spurious promoter in the neighbouring vector DNA sequence of pNIL260.

The codon usage for glutamic acid did not show the G + C bias in the 'wobble' position which was observed in the codon usage pattern for all other residues. The Glu codons GAA and GAG were present at the same frequency. The bias against the expected greater use of the GAG codon may be to avoid resemblances to the Shine-Dalgarno sequence (GAA codons are especially favoured at the 5' end of the gene). It has been reported that the sequences GAGG and GGAG, which are contained in the Shine-Dalgarno sequence, appear infrequently in the coding region of prokaryotes (Shpaer, 1986).

Hill *et al.* (1989) reported that the *Rhodococcus* DNA insert sequenced in this study caused production of both blue and pink pigments in *E. coli*. The nucleotide sequence of the smallest DNA fragments which gave rise to the pigments indicated that a single *Rhodococcus* gene product was involved in the biosynthesis of these pigments. The pigment gene product appears to be a novel protein since no amino acid homology was detected with other known protein sequences in the GenBank and EMBL data bases.

The small size of the pigment gene suggests its

application for the development of chromogenic vectors for detection of cloned inserts by insertional inactivation, for promoter-probe vectors and for other instances where reporter genes find application. An advantage of vectors based on this pigment gene is that no expensive substrates, such as X-Gal, are required.

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