Cloning and Expression of Rhodococcus Genes Encoding Pigment Production in Escherichia coli

By RUSSELL HILL, STEPHEN HART, NICOLA ILLING, RALPH KIRBY AND DAVID R. WOODS*

Department of Microbiology, University of Cape Town, Rondebosch 7700, South Africa

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Pigment was produced by Escherichia coli cells carrying recombinant plasmids pNIL100, pNIL200 and pNIL400 containing DNA from Rhodococcus sp. E. coli cells containing pNIL100 or pNIL200 (with DNA inserts from Rhodococcus sp. JL10 and Rhodococcus sp. ATCC 21145 respectively) produced both blue and pink pigments, while cells containing pNIL400 (with a DNA insert from Rhodococcus sp. ATCC 21145) produced only pink pigment. Colonies of E. coli(pNIL100) and E. coli(pNIL200) were dark blue, whereas E. coli(pNIL400) colonies were pink. No pigment was detected in Streptomyces griseus transformants containing pNIL100, pNIL200 or pNIL400. Restriction endonuclease mapping indicated that the cloned DNA fragments were different. The pigment gene(s) in pNIL200 producing both the blue and pink pigments were contained within a 2.8 kb DNA fragment. The pigments produced by E. coli transformants containing pNIL200 were characterized by visible and UV spectroscopy. No similar pigments were detected in Rhodococcus sp. ATCC 21145.

INTRODUCTION

The genus Rhodococcus comprises aerobic Gram-positive actinomycetes that show considerable morphological diversity. Colonies are usually pigmented buff, orange or red, although colourless strains do occur (Goodfellow & Cross, 1984). Rhodococci produce antibiotics, perform useful chemical modifications of complex compounds (Peczyńska-Czoch & Mordarski, 1984) and degrade pollutants (Kurane et al., 1979; Apajalahti & Salkinoja-Salonen, 1986), but have been relatively poorly studied at the genetic level.

Among the actinomycetes, pigment production has been best studied in Streptomyces spp. The biosynthesis of actinorhodin and prodigine (prodigiosin-like) pigments has been reported (Gerber & Lechevalier, 1976; Rudd & Hopwood, 1980; Tsao et al., 1985). Recently a novel tyrosine-derived pigment, designated SL-1, has been isolated from a mutant of Streptomyces lavendulae (Mikami et al., 1987). A particularly well characterized bacterial pigment system is the biosynthesis of prodigiosin by Serratia marcescens (Williams & Qadri, 1980). The cloning and expression in Escherichia coli of S. marcescens genes for prodigiosin synthesis has been reported (Dauenhauer et al., 1984) but no pigmented E. coli transformants were isolated in which the entire prodigiosin biosynthetic pathway was expressed.

In this work, genomic libraries of Rhodococcus sp. JL10 and Rhodococcus sp. ATCC 21145 were constructed in E. coli as an initial step in gene cloning studies in rhodococci. The pigmented E. coli transformants that were observed in these genomic libraries were investigated as they provided an interesting example of the expression of genes from a Gram-positive species in a Gram-negative organism. The isolation of genes encoding pigment production in E. coli could be useful in the construction of chromogenic vectors. Preliminary chemical characterization of one of the pigments was undertaken to determine whether this pigment was novel or belonged to a class of pigments previously described.

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al., et al., & coding for pigment production were designated Pig+. E. coli sp. ATCC 35691 and Rhodococcus sp. ATCC 21145 were previously classified as Nocardia corallina JL10 and Nocardia sp. ATCC 21145 respectively. Developments in nocardioform taxonomy (Goodfellow & Stanely 1982) make it clear that both strains belong to the genus Rhodococcus not Nocardia. Rhodococcus sp. JL10, which forms pink to coral red colonies, was isolated from soil and harbours a 2-7 kb plasmid designated pKU100 (Kirby & Usdin 1985). Rhodococcus sp. ATCC 21145 forms buff-coloured colonies and is described by Raymond (1971) in a patent for the production of hydroxyphenylketobutyric acids by the microbial oxidation of naphthalene.

Preparation of DNA. Rhodococcus chromosomal DNA was prepared as described by Hopwood et al. (1985) for Streptomyces total DNA (procedure 1). The positive selection Streptomyces—E. coli shuttle vector pLR591 (Hill et al., 1989) was prepared from E. coli K514Δ by the method of Ish-Horowicz & Burke (1981) and purified by CsCl equilibrium gradient centrifugation. This procedure was also used for the preparation of recombinant plasmids coding for pigment production. Restriction endonucleases (Boehringer-Mannheim) were used according to the conditions of Maniatis et al. (1982). Ligations were performed with T4 DNA ligase (Boehringer-Mannheim) according to the manufacturer's instructions, using the ligation buffer of King & Blakesley (1986).

Construction of Rhodococcus genomic libraries. Chromosomal DNA of Rhodococcus sp. JL10 and Rhodococcus sp. ATCC 21145 was partially digested with Sau3A endonuclease and DNA fragments of 5–10 kb were obtained by sucrose gradient centrifugation. The vector pLRS91 was digested with BglII endonuclease; vector and insert DNA were mixed in ratios between 1:1 and 50:1 at a final DNA concentration of 0.1–1.3 µg µl⁻¹. Competent E. coli DL1 and E. coli KL111 cells were transformed by the method of Cohen et al. (1972) as modified by Dagert & Ehrlich (1979). E. coli transformants were expressed at 42 °C in LB medium with vigorous shaking for 1 h and plated on LB agar containing 100 µg ampicillin ml⁻¹. The expression step ensures efficient killing of transformants containing pLR591 plasmids without inserts as the intact lethal EcoRI gene is efficiently expressed at 42 °C in these parental plasmids. Plasmids containing DNA inserts in the BglII cloning site within the EcoRI gene will not code for EcoRI endonuclease and cells containing recombinant plasmids will therefore survive (Hill et al., 1989).

Southern blot hybridization. PstI endonuclease digested chromosomal DNA from Rhodococcus sp. JL10 and Rhodococcus sp. ATCC 21145 was separated by gel electrophoresis (Maniatis et al., 1982), transferred to filters by blotting (Southern, 1975), and dried at 80 °C for 2 h under vacuum. Plasmids pNIL100 and pNIL200, nick-translated with [α-32P]dATP (Amersham), were used as hybridization probes. Blotted filters were prehybridized for 2 h at 65 °C in a solution containing 2 × SSC, 0.1% (w/v) SDS, 1 × Denhardt's solution and 100 µg denatured herring sperm DNA ml⁻¹ (1 × SSC is 0.15 M-NaCl, 0.015 M-trisodium citrate, pH 7.0; 1 × Denhardt's solution is

**METHODS**

**Bacteria, plasmids and growth conditions.** The bacterial strains and plasmids used are listed in Table 1. Plasmids coding for pigment production were designated Pig+. E. coli cells were grown at 37 °C in Luria-Bertani (LB) medium (Maniatis et al., 1982). Rhodococcus strains were grown in LB medium at 30 °C. Rhodococcus sp. JL10 and Rhodococcus sp. ATCC 21145 were previously classified as Nocardia corallina JL10 and Nocardia sp. ATCC 21145 respectively. Developments in nocardioform taxonomy (Goodfellow & Alderson, 1977; Goodfellow & Cross, 1984) make it clear that both strains belong to the genus Rhodococcus not Nocardia. Rhodococcus sp. JL10, which forms pink to coral red colonies, was isolated from soil and harbours a 2-7 kb plasmid designated pKU100 (Kirby & Usdin, 1985). Rhodococcus sp. ATCC 21145 forms buff-coloured colonies and is described by Raymond (1971) in a patent for the production of hydroxyphenylketobutyric acids by the microbial oxidation of naphthalene.

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**Table 1. Bacterial strains and plasmids**

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<th>Strain or plasmid</th>
<th>Geotype or genetic markers</th>
<th>Reference or source</th>
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<tbody>
<tr>
<td>Rhodococcus sp. JL10</td>
<td>Wild-type</td>
<td>Authors' laboratory</td>
</tr>
<tr>
<td>Rhodococcus sp. ATCC 21145</td>
<td>Wild-type</td>
<td>ATCC 21145</td>
</tr>
<tr>
<td>E. coli K514Δ</td>
<td>Δ lysogen of K514</td>
<td>Zabeau &amp; Stanley (1982)</td>
</tr>
<tr>
<td>E. coli DK1</td>
<td>K12 derivative Δ(srl-recA)</td>
<td>ATCC 35691</td>
</tr>
<tr>
<td>E. coli sp. ATCC 21145</td>
<td>K514 derivative lacI lacZ M15 lacY&lt;sup&gt;+&lt;/sup&gt;</td>
<td>Zabeau &amp; Stanley (1982)</td>
</tr>
<tr>
<td>S. griseus</td>
<td>Wild-type</td>
<td>ISP 5236</td>
</tr>
<tr>
<td>pLR591</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Ts&lt;sup&gt;+&lt;/sup&gt; EcoRI</td>
<td>Hill et al. (1989)</td>
</tr>
<tr>
<td>pNIL100</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Ts&lt;sup&gt;+&lt;/sup&gt; Pig&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
</tr>
<tr>
<td>pNIL200</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Ts&lt;sup&gt;+&lt;/sup&gt; Pig&lt;sup&gt;+&lt;/sup&gt;</td>
<td>This study</td>
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<tr>
<td>pNIL300</td>
<td>Ap&lt;sup&gt;+&lt;/sup&gt; Ts&lt;sup&gt;+&lt;/sup&gt; Pig&lt;sup&gt;+&lt;/sup&gt;</td>
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<td>pNIL400</td>
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<td>pUC18</td>
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Rhodococcus genes encoding pigments in E. coli

0.02% bovine serum albumin, 0.02% Ficoll, 0.02% polyvinylpyrrolidone). Blotted filters were hybridized overnight and washed twice in 2x SSC, 0.1% (w/v) SDS, and once in 1x SSC, 0.1% (w/v) SDS at 65°C for 15 min. Kodak XAR-5 film was used for autoradiography.

Preparation and transformation of Streptomyces griseus. Protoplasts of S. griseus were prepared and transformed with the plasmids pNIL100, pNIL200 and pNIL400 according to the methods of Hopwood et al. (1985). Thioestrepton-resistant (Ts') transformants were screened for pigment production.

Extraction, purification and characterization of pigments. Pigments were extracted from pigmented E. coli strains grown in 100 ml LB broth containing 50 μg ampicillin ml⁻¹. Cells were harvested by centrifugation, resuspended in 10 ml water and disrupted by sonication. Suspensions were shaken with an equal volume of chloroform for 2 h and centrifuged at 16000 g for 10 min. Chloroform extinction was repeated and the extracts were pooled, evaporated to dryness and resuspended in chloroform. Pigment extracts were fractionated by preparative partition chromatography on a 60 x 3.4 cm glass column packed with silica gel 60 (Merck) using chloroform as the eluant. Extracts were prepared and fractionated from cells of Rhodococcus sp. JL10 and Rhodococcus sp. ATCC 21145 by the same procedure. Pigment was analysed by visible and UV light spectroscopy on a Beckman DU-40 spectrophotometer.

RESULTS

Construction of Rhodococcus genomic libraries. The genomic libraries of Rhodococcus sp. JL10 and Rhodococcus sp. ATCC 21145 consisted of 8900 and 25000 E. coli clones respectively. The size of DNA inserts in the vector pLR591 was estimated by linearizing pools of the recombinant plasmids with NotI endonuclease, which has an eight base pair recognition sequence. The vector pLR591 contains a single NotI site. The majority of linearized plasmids were between 13 and 20 kb in size, as expected for DNA inserts of approximately 4-11 kb in the 8.9 kb vector pLR591. Assuming an average insert size of 7-5 kb, the probability of having a DNA sequence represented in the libraries was determined using the formula of Clarke & Carbon (1976); it was greater than 99.9% in both cases.

Identification of pigment-producing clones. One pale blue colony of the Rhodococcus sp. JL10 genomic library in E. coli DK1 was clearly distinguishable, after 18 h incubation at 37°C, from the other E. coli transformants, which formed translucent pale cream colonies. This colony became more pigmented after a further 24 h at room temperature and appeared dark blue. Two colonies of the Rhodococcus sp. ATCC 21145 genomic library in E. coli LK111 were dark blue after 24 h at 37°C and remained this colour after 24 h at room temperature. One E. coli colony of the Rhodococcus sp. ATCC 21145 genomic library was pale pink after 24 h at 37°C and after a further 24 h at room temperature.

Plasmid preparation, restriction mapping and transformation studies. The plasmid prepared from the pigmented colony of the Rhodococcus sp. JL10 genomic library in E. coli DK1 was clearly distinguishable, after 18 h incubation at 37°C, from the other E. coli transformants, which formed translucent pale cream colonies. This colony became more pigmented after a further 24 h at room temperature and appeared dark blue. Two colonies of the Rhodococcus sp. ATCC 21145 genomic library in E. coli LK111 were dark blue after 24 h at 37°C and remained this colour after 24 h at room temperature. One E. coli colony of the Rhodococcus sp. ATCC 21145 genomic library was pale pink after 24 h at 37°C and after a further 24 h at room temperature.

Plasmid preparation, restriction mapping and transformation studies. The plasmid prepared from the pigmented colony of the Rhodococcus sp. JL10 genomic library was designated pNIL100. Plasmids prepared from the dark blue colonies of the Rhodococcus sp. ATCC 21145 genomic library were designated pNIL200 and pNIL300, and the plasmid from the pale pink colony of this library was designated pNIL400.

Mapping of these plasmids with a range of restriction endonucleases indicated that pNIL200 and pNIL300 were identical, and pNIL300 was not studied further. Restriction endonuclease maps of pNIL100 and pNIL400 are shown in Fig. 1 and the map of pNIL200 in Fig. 2. Plasmids pNIL100, pNIL200 and pNIL400 contained DNA inserts of 10, 15.1 and 5 kb respectively. The DNA inserts in these three plasmids were different since they had different restriction endonuclease maps. The plasmids pNIL100, pNIL200 and pNIL400 transformed E. coli LK111 cells at a high frequency and pigment production was always associated with transformation to Ap⁻.

Southern hybridization. Labelled pNIL100 showed homology to four bands of Rhodococcus sp. JL10 chromosomal DNA digested with PstI endonuclease, and pNIL200 showed homology to six bands of Rhodococcus sp. ATCC 21145 chromosomal DNA digested with PstI (results not shown). No DNA homology was detected when E. coli chromosomal DNA digested with PstI was hybridized with labelled pNIL100 and pNIL200. This confirmed that the DNA inserts in
Fig. 1. Restriction endonuclease maps of pNIL100 and pNIL400. Bold lines represent chromosomal DNA inserts from *Rhodococcus* sp. JL10 (in the case of pNIL100) and *Rhodococcus* sp. ATCC 21145 (in the case of pNIL400). Thin lines represent DNA derived from the vector pLR591.

Fig. 2. Restriction endonuclease maps of pNIL200 and shortened derivatives of pNIL200. Bold lines represent chromosomal DNA inserts from *Rhodococcus* sp. ATCC 21145 and thin lines represent DNA derived from pLR591.

pNIL100 and pNIL200 were derived from *Rhodococcus* sp. JL10 and *Rhodococcus* sp. ATCC 21145 respectively. *PstI* digestion of pNIL100 and pNIL200 produced six and eight fragments respectively. The vector pLR591 contains three *PstI* sites (Hill *et al.*, 1989) and *PstI* digestion of pNIL100 and pNIL200 would therefore result in two fragments in each digest consisting of pLR591 DNA only. Hybridization of pNIL100 to four bands of *PstI*-digested
Rhodococcus sp. JL10 chromosomal DNA and of pNIL200 to six bands of PstI-digested Rhodococcus sp. ATCC 21145 chromosomal DNA is therefore consistent with the presence of one copy of the DNA insert in pNIL100 and pNIL200 in each of the Rhodococcus sp. chromosomal DNA digests. Under the stringent washing conditions used, cross-homology between pNIL100 and Rhodococcus sp. ATCC 21145, and pNIL200 and Rhodococcus sp. JL10 was not detected.

Shortening and subcloning of plasmid pNIL200. The plasmid pNIL200, with an insert size of 15.1 kb, was shortened by restriction fragment deletion and religation to produce the plasmids pNIL240, pNIL250 and pNIL260, with insert sizes of 8.7, 5.2 and 2.8 kb respectively (Fig. 2). These plasmids all produced pigmented colonies after transformation into E. coli LK111.

Subcloning of the pigment-producing DNA insert from pNIL250 into pUC18 and pUC19 to produce the plasmids pNC18 and pNC19 resulted in this insert being present in both orientations relative to the lacOP promoter present in pUC18 and pUC19. Pigment production by E. coli cells containing the plasmids pNC18 and pNC19 was determined. Both plasmids coded for pigment production, but cells containing pNC19 produced markedly more pigment (results not shown).

Transformation of S. griseus. The plasmids pNIL100, pNIL200 and pNIL400 were transformed into S. griseus but did not cause detectable pigment production in the (non-pigmented) S. griseus strain.

Extraction, purification and characterization of pigment. Purification of the pigment extract from E. coli cells by preparative column chromatography yielded pink and blue pigments from pNIL100 and pNIL200 and a pink pigment only from pNIL400. Presumably, cultures and colonies of E. coli cells containing pNIL100 and pNIL200 appeared blue because the blue pigment was masking the pink pigment. No pigment was detected in chloroform extracts of Rhodococcus sp. JL10 or Rhodococcus sp. ATCC 21145.

Cultures of E. coli LK111(pNIL200) were used for the preparation of large amounts of pigments for chemical analysis, as cultures containing this plasmid yielded the largest amounts of pigments (24 mg of the blue pigment and 12 mg of the pink pigment were obtained from a 400 ml culture). The blue and pink pigments purified from E. coli LK111(pNIL200) exhibited absorption maxima of 560 nm and 610 nm respectively. Both compounds were soluble at low concentrations in chloroform and insoluble in water, ethanol and methanol. The compounds flocculated at concentrations above 20 mg ml\(^{-1}\) in chloroform. Addition of acid or alkali to the compounds did not cause any colour change.

DISCUSSION

This appears to be the first report of the cloning and expression of genes which result in pigmented E. coli colonies. The cloning and expression in E. coli of Rhodococcus genes to produce pigmented E. coli cells is interesting and important from three aspects: (1) it provides a convenient system for the study of nocardiform gene expression; (2) the production of large amounts of pigment for chemical characterization and industrial production is facilitated; and (3) the cloning of a relatively small piece of DNA encoding pigment production in E. coli will permit the development of chromogenic vectors which do not require expensive substrates (e.g. X-Gal).

Pigment production by the DNA insert from pNIL250 cloned in both orientations with respect to the lacOP promoter in pUC18 and pUC19 indicated that pigment production was expressed from a Rhodococcus regulatory region in E. coli. Pigment production was greater in E. coli cells containing pNC19 than in cells containing pNC18. The Rhodococcus DNA insert in pNC19 is probably in the correct orientation relative to the lacOP promoter of pUC19, and the higher level of pigment production would then be a result of gene expression from both the lacOP promoter and a putative Rhodococcus promoter present in the insert. The lower level of
pigment production found when the insert is in the opposite orientation to the lacOP promoter would be a result of gene expression from the putative Rhodococcus promoter alone.

The preliminary chemical characterization of the pink and blue pigments produced by E. coli cells containing pNIL200 indicates that these pigments are different from the prodigiosin-like or actinorhodin-like compounds commonly produced by actinomycetes. Neither the pink nor the blue pigment could be detected in chloroform extracts of Rhodococcus sp. ATCC 21145. The E. coli recombinants could be making shunt metabolites not seen in the Rhodococcus sp. Alternatively, if the DNA insert in pNIL200 does code for production of the pink and blue pigments in Rhodococcus sp. ATCC 21145, pigment production could be at too low a level for detection. A large number of copies of pNIL200 would be expected in E. coli as the high-copy-number vector pLR591 is present in approximately 50–100 copies per cell in this organism (Hill et al., 1989). Pigment production would therefore be at a higher level in E. coli carrying pNIL200 than in Rhodococcus sp. ATCC 21145.

The plasmids pNIL100, pNIL200 and pNIL400 did not cause pigment production in the non-pigmented actinomycete S. griseus. It is not known whether the regulatory regions of the Rhodococcus genes were not functional in S. griseus or whether the precursors or correct metabolic conditions for pigment production were lacking in S. griseus.

Both pink and blue pigments were expressed in E. coli from 2.8 kb of DNA in pNIL260. The sequencing of this DNA insert in pNIL260 is in progress and will elucidate whether both pigments are the result of one gene product or whether more than one gene is involved.

REFERENCES


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