Development of a host–vector system in a *Rhodococcus* strain and its use for expression of the cloned nitrile hydratase gene cluster

YOJIHIRO HASHIMOTO,¹ MAKOTO NISHIYAMA,¹ FUJIO YU,² ICHIRO WATANABE,² SUEHARU HORINOUCHI¹* and TERUHIKO BEPPU¹

¹Department of Agricultural Chemistry, University of Tokyo, Yayoi 1-1-1, Bunkyo-ku, Tokyo 113, Japan
²Nitto Chemical Industry Co., Ltd, 10-1, Daikoku-cho, Tsurumi-ku, Yokohama 230, Japan

(Received 30 September 1991; revised 2 January 1992; accepted 20 January 1992)

Two different types of plasmid were isolated from strains of *Rhodococcus rhodochrous*. Two plasmids, of the same type but from different strains, were combined with *Escherichia coli* plasmids carrying antibiotic resistance markers to develop *E. coli*–*Rhodococcus* shuttle vectors. The ampicillin and kanamycin resistance markers served for selection in *Rhodococcus*. Electroporation was used to introduce recombinant plasmid DNA into *R. rhodochrous* ATCC 12674 at a frequency of \(5 \times 10^7\) transformants per pg DNA. With these host–vector and transformation systems, the nitrile hydratase and amidase genes of a *Rhodococcus* strain were introduced into the host strain and were efficiently expressed.

Introduction

*Rhodococcus* is a versatile genus of Gram-positive coryneform bacteria with activities, such as biological transformation of nitrile compounds (Kobayashi et al., 1989, 1990, 1991; Mathew et al., 1988; Nagasawa et al., 1988; Watanabe et al., 1987) and steroids (Ferreira et al., 1984), that are useful for industrial processes. The development of genetic recombination systems for this group of bacteria will allow a better understanding of their genetics and facilitate the development of strains with enhanced activities. A lysogenic actinophage, φEC, has been physically mapped by restriction analysis, and used to develop a cloning system (Brownell et al., 1980, 1982). An *Escherichia coli–Rhodococcus* shuttle vector has been constructed by cloning a fragment from a cryptic *Rhodococcus* plasmid in pIJ30, a pBR322 derivative containing an *E. coli* origin of replication, an ampicillin-resistance determinant and a thiostrepton-resistance determinant from *Streptomyces* (Vogt Singer & Finnerty, 1988). A similar shuttle vector was constructed from a *Rhodococcus* plasmid containing an arsenic resistance gene and pEcoR251 from *E. coli* (Dabbs et al., 1990). Because these systems have some limitations, especially in transformation efficiency, a more effective host–vector system is required for the use of recombinant DNA techniques in this group of bacteria.

*Rhodococcus* sp. N-774 possesses a potent nitrile hydratase (NHase) activity, which is used for industrial production of acrylamide (Watanabe et al., 1987). Although we have cloned and sequenced genes encoding the α- and β-subunits of the NHase, expression of the cloned genes in *E. coli* resulted in accumulation of an insoluble polypeptide with no activity (Ikehata et al., 1989). These results prompted us to develop an effective host–vector system in *Rhodococcus*. We screened *Rhodococcus* strains for plasmids and used these plasmids to construct shuttle vectors. We also examined conditions for transforming *R. rhodochrous* ATCC 12674 by electroporation. The host–vector and transformation systems thus established proved to be useful for cloning and expression of the NHase genes and an amidase gene from *Rhodococcus* sp. N-774.

Methods

Bacterial strains and growth conditions. The strains of *Rhodococcus* and *E. coli*, and plasmids used in this study, are listed in Table 1. *E. coli* was grown at 37 °C in 2 × YT medium (Yanisch-Perron et al., 1985). *Rhodococcus* strains were grown at 26 °C in MYP medium containing \((g \cdot l^{-1}):\) glycerol, 10; polypeptone, 5; yeast extract, 3; malt extract, 3; KH₂PO₄, 1.0; K₂HPO₄, 1.0 (pH 7.0).

Enzymes and chemicals. Restriction endonucleases, T4 DNA ligase, and bacterial alkaline phosphatase were purchased from the Takara Shuzo Co.; IPTG and X-Gal were from Wako Pure Chemicals. Plasmid DNA from *Rhodococcus*. Plasmid DNA was isolated from *Rhodococcus* strains by the method of Vogt Singer & Finnerty (1988).
Table 1. **Bacterial strains and plasmids used**

<table>
<thead>
<tr>
<th>Strains and plasmids*</th>
<th>Characteristics†</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>R. rhodochrous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 12674</td>
<td>pDA20 (As', Cd', Cm')</td>
<td>Dabbs &amp; Sole (1988)</td>
</tr>
<tr>
<td>ATCC 4276</td>
<td>pRC1 (cryptic)</td>
<td>This work</td>
</tr>
<tr>
<td>ATCC 14348</td>
<td>pRC2 (cryptic)</td>
<td>This work</td>
</tr>
<tr>
<td>ATCC 14349</td>
<td>pRC3 (cryptic)</td>
<td>This work</td>
</tr>
<tr>
<td>IFO 3338</td>
<td>pRC4 (cryptic)</td>
<td>This work</td>
</tr>
<tr>
<td>ATCC 4001</td>
<td>pRC10 (cryptic)</td>
<td>This work</td>
</tr>
<tr>
<td><strong>E. coli TG1</strong></td>
<td>Δ(lac pro) supE thi hsdD5 F' traD36 proABlacI &amp; lacΔM15</td>
<td></td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pK1, pK2, pK3 and pK4</td>
<td>Km'</td>
<td>This work</td>
</tr>
<tr>
<td>pA3</td>
<td>Ap'</td>
<td>This work</td>
</tr>
<tr>
<td>pKRNH2</td>
<td>Km'; containing the amidase and NHase genes</td>
<td>This work</td>
</tr>
<tr>
<td>pYUK120</td>
<td>Ap'; containing the NHase gene</td>
<td>Ikehata <em>et al.</em> (1989)</td>
</tr>
<tr>
<td>pANH1101</td>
<td>Ap'; containing the amidase gene</td>
<td>Hashimoto <em>et al.</em> (1991)</td>
</tr>
<tr>
<td>pUC19</td>
<td>Ap'</td>
<td>Yanisch-Perron <em>et al.</em> (1985)</td>
</tr>
<tr>
<td>pHSG299</td>
<td>Km'</td>
<td>Takeshita <em>et al.</em> (1987)</td>
</tr>
</tbody>
</table>

* ATCC, American Type Culture Collection; IFO, Institute for Fermentation, Osaka, Japan.
† As', arsenic resistance; Cd', cadmium resistance; Cm', chloramphenicol resistance; Km', kanamycin resistance; Ap', ampicillin resistance.

Transformation of _Rhodococcus_ by electroporation. A 10 ml sample from a mid-exponential culture of _R. rhodochrous_ ATCC 12674 was centrifuged at 6500 g for 10 min at 4 °C and washed three times with 1 ml cold water. Cells were then resuspended in 1 ml cold water and kept on ice. The cell suspension (10 μl) was mixed with up to 1 μg DNA in 1 μl of TE buffer (10 mM-Tris/1 mM-EDTA, pH 8.0) in a precooled chamber (no. 11) of a Somatic Hybridizer type SSH-1 (Shimadzu Seisakusho). The chamber was kept on ice and electroporation was performed under various conditions by changing the number of pulses, the pulse width and the field strength. After electroporation, the cell suspension was kept on ice for at least 10 min, then incubated at various temperatures for 10 min, and immediately transferred to a test tube containing 1 ml of MYP medium. After 2 h at 26 °C, the cells were plated on selective medium containing 50 μg kanamycin ml⁻¹ or 10 μg ampicillin ml⁻¹.

**DNA manipulation.** Procedures with _E. coli_ were performed as described by Maniatis _et al._ (1982).

**Determination of plasmid stability.** Strains of _R. rhodochrous_ harbouring shuttle vectors were grown under non-selective conditions in MYP medium for 10 generations, and were then plated on MYP agar. A total of 500 colonies were scored by replica plating for resistance to kanamycin. The plasmid content was verified by small-scale plasmid isolation (Vogt Singer & Finnerty, 1988).

**Nitrile hydratase and amidase assays.** One- or two-day-old cultures of _R. rhodochrous_ transformants were harvested by centrifugation, and the cells were washed and resuspended in 50 mM-potassium phosphate buffer (pH 7-7). The activities of NHase and amidase were determined as described previously (Hashimoto _et al._, 1991; Nishiyama _et al._, 1991). One unit of enzyme activity catalyses formation of 1 μmol product min⁻¹ at 20 °C.

**SDS-PAGE and Western blotting.** SDS-PAGE was performed by the method of Laemmli (1970). Proteins were determined by the dye-binding method of Bradford (1976). Anti-NHase serum was prepared from a young white rabbit immunized with NHase purified from _Rhodococcus_ sp. N-774 (unpublished results). The NHase was detected by Western blotting (Burnett, 1981) with a Bio-Rad Immunoblot assay kit (GAR-HRP) using the anti-NHase serum.

**Results**

**Screening for plasmids from _Rhodococcus_ species**

Thirty-two _Rhodococcus_ strains, obtained from type culture collections, were screened for plasmid DNA by agarose gel electrophoresis. Of these, _R. rhodochrous_ ATCC 4276, ATCC 14348, ATCC 14349, IFO 3338 and ATCC 4001 each possessed a plasmid, designated pRC1, pRC2, pRC3, pRC4 and pRC10, respectively. The five plasmids were isolated and purified. Restriction analysis showed that pRC1--4 were the same size and gave the same restriction endonuclease cleavage pattern, with unique cleavage sites for BamHI, _SphI_, _PstI_, _XhoI_ and _ScaI_ (Fig. 1a). Plasmid pRC10 was 7-0 kb in length with a unique site for _SphI_ (Fig. 1b). A rough estimate of the plasmid copy numbers by the method of Projan _et al._ (1983), which is based on the intensity of plasmid DNA relative to chromosomal DNA in an ethidium-bromide-stained gel, suggested that each plasmid was present at approximately 2-4 copies per genome.

**Construction of _E. coli–Rhodococcus_ shuttle vectors**

We used pRC3 and pRC4 in preference to pRC10 for _E. coli–Rhodococcus_ shuttle vector construction, because the former plasmids were relatively small and had several
unique cleavage sites that would facilitate cloning of DNA fragments. The general strategy for constructing shuttle vectors is shown in Fig. 1(c and d). Plasmid pHSG299 (Takeshita et al., 1987) is derived from pUC19 (Yanisch-Perron et al., 1985) by replacing the ampicillin-resistance gene with a kanamycin-resistance gene. Plasmid pRC4 was digested with Clal, ligated with AccI-digested pHSG299 DNA, and introduced by transformation into E. coli TG1. Transformants were selected on 2 × YT agar medium containing 1 mm-IPTG, 0.02% X-Gal and 50 μg kanamycin ml⁻¹. The recombinant plasmid obtained in this way was named pK4 (Fig. 1c). The shuttle plasmid pK4 contains unique cleavage sites for EcoRI, KpnI, PstI, Clal and XhoI.
Plasmid pA3 (Fig. 1d) was constructed in the same way as pK4 except that pRC3 and pUC19 were used, and selection was for ampicillin resistance. The shuttle plasmid pA3 has unique cleavage sites for EcoRI, KpnI, PstI, HindIII and XhoI.

Transformation of Rhodococcus with the shuttle vectors

The chimeric plasmids isolated from *E. coli* were examined for their ability to replicate in *Rhodococcus*. When cells of numerous *Rhodococcus* strains and plasmid pK4 were subjected to electroporation according to Shigekawa & Dower (1988), only *R. rhodochrous* ATCC 12674 was transformed to grow on MYP agar medium containing 50 μg kanamycin ml⁻¹. The plasmids isolated from these transformants were identical to pK4 from *E. coli*, by restriction enzyme analyses.

Plasmid pK4 was lost at the rate of 1.5% per generation from *R. rhodochrous* ATCC 12674 during growth under non-selective conditions. When *Rhodococcus* transformants were routinely grown in medium containing 50 μg kanamycin ml⁻¹, the loss was negligible. Transformation of *R. rhodochrous* ATCC 12674 protoplasts with pK4 by the methods of Vogt Singer & Finnerty (1988) gave kanamycin-resistant colonies of *R. rhodochrous* ATCC 12674 at lower frequencies (less than 10⁴ transformants per μg plasmid DNA) than were achieved by electroporation.

Ampicillin-resistant transformants of *R. rhodochrous* ATCC 12674 were also obtained on MYP agar medium containing 10 μg ampicillin ml⁻¹ after electroporation with pA3 plasmid DNA.

Optimization of conditions for transformation of *R. rhodochrous* ATCC 12674 by electroporation

Initial conditions of electroporation were: DNA concentration, 1 μg μl⁻¹; single pulse; field strength, 12 kV cm⁻¹; pulse width, 300 μs; temperature, 0 °C; cell concentration, >10⁷ ml⁻¹; recovery incubation after electroporation, 0 °C for 10 min. The electroporation apparatus had a field strength range of 0–14 kV cm⁻¹ and a pulse duration of 0–500 μs. The effects of various factors on transformation efficiency are shown in Fig. 2. Multiple pulsing (up to ten times) did not improve transformation efficiency. From the results of these experiments, we concluded that the best conditions for transformation of the *R. rhodochrous* strain were: a single
pulse; field strength, 14 kV cm⁻¹; pulse width, 500 μs and heat treatment at 37°C for 10 min after electroporation. The number of transformants increased in proportion to the cell concentration used. Therefore, we examined the effect of DNA concentration under the optimized conditions described above. With pK4 isolated from R. rhodochrous, the maximum transformation frequency was $5 \times 10^7$ transformants per μg plasmid DNA (Fig. 2e). When plasmid DNA from E. coli was used, the transformation efficiency decreased, suggesting the presence of a restriction-modification system specific to R. rhodochrous ATCC 12674.

Expression of the NHase and amidase genes in R. rhodochrous ATCC 12674

The host-vector system was used to express the NHase (α and β subunits) and amidase genes of Rhodococcus sp.
Fig. 4. SDS-PAGE (a) and Western blotting with anti-NHase serum (b) to detect gene products of the *R. rhodochrous* transformant. Lanes 1 and 2, crude extract prepared from *R. rhodochrous* ATCC 12674 carrying pK4 cultured in the absence (lane 1) and the presence (lane 2) of 0.2% methacrylamide; lanes 3 and 4, crude extract prepared from *R. rhodochrous* ATCC 12674 carrying pKRNH2 cultured in the absence (lane 3) and presence (lane 4) of 0.2% methacrylamide; lane 5, molecular mass standards: phosphorylase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa), and α-lactalbumin (14.4 kDa); lane 6, crude extract prepared from *Rhodococcus* sp. N-774.

N-774, which had already been cloned and sequenced in our laboratory (Ikehata *et al.*, 1989; Hashimoto *et al.*, 1991). These two genes apparently give a polycistronic transcript (Hashimoto *et al.*, 1991). As shown in Fig. 3, plasmid pYUK120 (Ikehata *et al.*, 1989) contains the whole NHase gene and a part of the amidase gene on a 4.4 kb *SphI* fragment. Plasmid pANH101 (Hashimoto *et al.*, 1991) contains the whole amidase gene and a part of the NHase gene on a 5.5 kb *PstI* fragment. By combining fragments of these plasmids, of pHSG299, and of pK4, a kanamycin-resistance shuttle plasmid pKRNH2 containing the whole NHase (α and β subunits) and amidase genes was constructed (Fig. 3).

To detect the gene products in the *R. rhodochrous* ATCC 12674 transformant carrying pKRNH2, we analysed cell extracts by SDS-PAGE and Western blotting (Fig. 4). Proteins of 55 kDa and 26 kDa, of the same molecular mass as the amidase and NHase proteins, respectively (Hashimoto *et al.*, 1991; Endo *et al.*, 1989), were observed after staining the gel with Coomassie brilliant blue. Larger amounts of these two proteins were present than in the cell lysate of *R.*
Table 2. Effect of methacrylamide on amidase and NHase activities in the R. rhodochrous ATCC 12674 transformant and Rhodococcus sp. N-774

<table>
<thead>
<tr>
<th>Strain and plasmid</th>
<th>Methacrylamide (%)</th>
<th>Growth (OD₆₅₀)</th>
<th>Specific activity [units (mg dry cells)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>R. rhodochrous</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ATCC 12674</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pK4</td>
<td>0-1</td>
<td>3:1</td>
<td>3:2 trace 0:03</td>
</tr>
<tr>
<td></td>
<td>0-2</td>
<td>3:6</td>
<td>9:1 0:05</td>
</tr>
<tr>
<td>pKRNH2</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-774</td>
<td>0-1</td>
<td>2:7</td>
<td>4:5 0:50</td>
</tr>
<tr>
<td></td>
<td>0-2</td>
<td>2:1</td>
<td>152 1:49</td>
</tr>
</tbody>
</table>

Table 3. Effect of photoirradiation on NHase activities in the R. rhodochrous ATCC 12674 transformant and in Rhodococcus sp. N-774

<table>
<thead>
<tr>
<th>Strain/plasmid</th>
<th>NHase activity [units (mg dry cells)⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Light</td>
<td>Dark</td>
</tr>
<tr>
<td>ATCC 12674/pK4</td>
<td>2:2</td>
</tr>
<tr>
<td>ATCC 12674/pKRNH2</td>
<td>152</td>
</tr>
<tr>
<td>N-774/none</td>
<td>73:4</td>
</tr>
</tbody>
</table>

rhodochrous ATCC 12674 transformed with pK4. Western blotting with antibody raised against Rhodococcus sp. N-774 NHase showed that the 26 kDa protein corresponded to the NHase (26 kDa) from strain N-774. The NHase and amidase genes are constitutively expressed in the original Rhodococcus sp. N-774 (Watanabe et al., 1987), but increased amounts of both proteins were detected by SDS-PAGE when the R. rhodochrous transformant was cultured in the presence of 0:2% methacrylamide (Fig. 4).

Addition of methacrylamide to cultures markedly increased the activities of both NHase and amidase in R. rhodochrous ATCC 12674 carrying pKRNH2 (Table 2). The Rhodococcus transformant carrying pK4 as a control also showed NHase and amidase activities inducible by methacrylamide, but only at extremely low levels.

Characteristics of the NHases from Rhodococcus sp. N-774 and R. rhodochrous ATCC 12674

The NHase of Rhodococcus sp. N-774 is known to be photoactivated (Nakajima et al., 1987), whereas that of R. rhodochrous ATCC 12674 is not affected by photoirradiation (unpublished data). Therefore, we examined the NHase activity of the transformant after the cells had been cultivated with and without illumination with a fluorescent lamp. The NHase activity of the transformant harbouring pKRNH2 was increased more than 10-fold by irradiation. As expected, the activity of the transformant carrying pK4 did not show any significant difference (Table 3).

The substrate specificity of the NHases in the cells of Rhodococcus sp. N-774 and of R. rhodochrous ATCC 12674 harbouring pK4 or pKRNH2 was examined with acrylonitrile, butyronitrile and isobutyronitrile as substrates. The substrate specificity of the NHase of the transformant harbouring pKRNH2 was almost the same as that from Rhodococcus sp. N-774, but was different from that of the host strain carrying pK4 (data not shown).

These observations indicated that the NHase activity of the transformant harbouring pKRNH2 arose mainly from the cloned genes and not from the host.

Discussion

The E. coli–Rhodococcus shuttle vectors constructed from the small cryptic plasmids pRC3 and pRC4 of Rhodococcus sp. and the E. coli plasmids pUC19 and pHSG299 possess several useful cloning sites and a moderate molecular size of 5:3 kb. Transformation with chimeric plasmids obtained by combining pHSG299 and pRC4 via the BamHI, SphI or XhoI sites of pRC4 failed to give kanamycin-resistant Rhodococcus colonies (data not shown). These results suggest that the 0:6 kb (BamHI–XhoI) region possesses functions required for replication or stable maintenance of plasmid pRC4 in Rhodococcus species.

Transformation of many strains of Rhodococcus with the shuttle vector pK4 was examined by the electroporation method. The best host strain, R. rhodochrous ATCC 12674, gave 5 × 10⁷ transformants per µg DNA. This host–vector system proved useful for expressing NHase in an active form, and is a promising vehicle for both improving the production of other useful enzymes by Rhodococcus spp. and exploring the genetics of this genus.

The NHase and amidase genes in the N-774 strain appear to be transcribed in a polycistronic manner under the control of a promoter which may be located upstream of the amidase gene. Because both enzymes were constitutively produced in the original Rhodococcus sp.
N-774 strain, we had assumed that the promoter was constitutive. However, this study has demonstrated that in the new *Rhodococcus* host, expression of these genes was greatly enhanced by addition of methacrylamide to the culture medium. The endogenous amidase and NHase activities in the host strain were very weak, but were significantly induced by methacrylamide (Table 2). This suggests that *R. rhodochrous* ATCC 12674 possesses a regulatory system that controls expression of the indigenous amidase and NHase genes. The promoter on the cloned fragment may, therefore, be controlled by a regulatory protein intrinsic to the host cells. It seems possible that the putative regulatory protein responsible for induced production of NHase is mutated in the N-774 strain, leading to constitutive expression.

We found that NHase was produced at a higher level but amidase was produced at an extremely low level in the N-774 strain (Fig. 4, lane 6). This may reflect a difference in the efficiency of translational initiation between the two *Rhodococcus* strains. Alternatively, there might be another transcriptional start point just upstream of the coding region of the NHase gene functional in *Rhodococcus* sp. N-774. Clarification of the difference between the regulatory mechanisms in *Rhodococcus* sp. N-774 and *R. rhodochrous* ATCC 12674 will require further genetic and molecular studies.

We are grateful to Kengo Nakata (Department of Agricultural Chemistry, University of Tokyo) for help in the electroporation experiments.

References


