Bacterial production of trans-dihydroxycyclohexadiene carboxylates by metabolic pathway engineering

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Homochiral-cis-cyclohexa-3,5-diene-1,2-diols are important synthons. We found a way to produce trans-configured homochiral diols using recombinant Klebsiella pneumoniae 62-1. Transformation of this mutant (Phe- Trp- Tyr-) with plasmids carrying genes involved in chorismic and isochorismic acid metabolism leads to the production of either (+)-trans-(2S,3S)-2,3-dihydroxycyclohexa-4,6-dienecarboxylic acid or (-)-trans-(3R,4R)-3,4-dihydroxycyclohexa-1,5-dienecarboxylic acid, with a yield of 70 or 90 mg (I culture broth) \(^{-1}\), respectively. The metabolic shift from one diene to the other is caused by a change in activity of isochorismate hydroxymutase and/or isochorismatase which in turn results from growth under iron deficiency or overexpression of genes (entC and/or entB) involved in chorismate metabolism.

Keywords: Klebsiella pneumoniae 62-1, pathway engineering, cyclohexadienes

INTRODUCTION

The use of cyclohexa-3,5-diene-1,2-diols in enantioselective synthesis is a field which has attracted much attention. The diols are synthons which are used in the preparation of homochiral compounds as diverse as enzyme inhibitors, inositol phosphate cell messengers, chiral cyclopentenones for prostaglandin synthesis and complex natural products (Carless, 1992; Brown & Hudlicky, 1993). The diols are prepared by a biortransformation process starting from the arenes using Pseudomonas putida strains (Gibson, 1970), and have a cis configuration. Diols with a trans configuration, however, have also been described. They can be obtained from urine after intraperitoneal injection of benzene into rabbits (Jerina et al., 1970). Syntheses of trans-configured racemic mixtures of diols are also known (DeMarinis et al., 1974; Chiasson & Berchold, 1974). In addition, homochiral diols with a trans configuration from a bacterial source have been described. They are metabolites involved in the post-chorismate steps of the shikimate pathway (Young et al., 1969a, b). The genes involved in these steps in Escherichia coli have been sequenced recently (Schrodt Nahlik et al., 1989; Ozenberger et al., 1989).

Following a previous approach which we used in the bulk preparation of isochorismic acid (Schmidt & Leistner 1995), we found a way to produce the diols (+)-trans-(2S,3S)-2,3-dihydroxycyclohexa-4,6-diene carboxylic acid (2,3-DH-2,3-DHB) and (-)-trans-(3R,4R)-3,4-dihydroxycyclohexa-1,5-diene carboxylic acid (3,4-DH-3,4-DHB) (Fig. 1) in respectable amounts using a chorismate-excreting triple mutant (Phe- Trp- Tyr-) of Klebsiella pneumoniae 62-1. Manipulation in vivo of the isochorismate hydroxymutase (EntC) and isochorismatase (EntB) activity of this mutant by different techniques led to the excretion of trans-configured diols into the culture broth.

METHODS

General techniques. Competent E. coli and K. pneumoniae 62-1 cells were prepared as described by Nishimura et al. (1991). Chromosomal DNA from E. coli MC 4100 was isolated by the method of Kohlbrecher et al. (1990). Other DNA manipulations were based on standard procedures (Sambrook et al., 1989).

Abbreviations: 2,3-DH-2,3-DHB, (+)-trans-(2S,3S)-2,3-dihydroxycyclohexa-4,6-diene carboxylic acid; 3,4-DH-3,4-DHB, (-)-trans-(3R,4R)-3,4-dihydroxycyclohexa-1,5-diene carboxylic acid; EntA, 2,3-dihydroxybenzoate synthase; EntB, isochorismatase; EntC, isochorismate hydroxymutase.
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**Fig. 1.** Metabolism of chorismic (C) and isochorismic acid (IC) to o-succinylbenzoic acid (OSB), 2,3-DH-2,3-DHB, 2,3-dihydroxybenzoic acid (2,3-DHB) and 3,4-DH-3,4-DHB.

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**Fig. 2.** Construction of the entB and the malE/entB expression plasmids. Km', kanamycin resistance; Prac, tac promoter; malE(-2-26), malE gene without its signal sequence; lacZ, β-galactosidase gene (β-fragment); bla, ampicillin resistance gene; lacI, lac repressor gene; lacIq, modified (NEB) lac repressor gene; Prac, lac promoter. See Methods for details.

Triple mutant (Phe- Trp- Tyr-) blocked in aromatic amino acid biosynthesis (Gibson & Gibson, 1964). pH5O2 containing the malE(-2-26) gene (encoding the maltose-binding protein from *E. coli* without its signal sequence) is a precursor of the pMAL-c2 vector used in the upgraded version of the protein fusion and purification system supplied by NE Biolabs. pBGS8 is a.
pRM4, i.e. pBGS8 with the menD gene (encoding 2-succinyl-6-hydroxy-2,4-cyclohexadiene-1-carboxylate synthase) was generated by PCR using primers (+)AGTCTAGAGGAGGGAATGTTGTTGCA and (−)GGGATCTAGATACACGTCGCTC and cut with XbaI and BglII. Oligonucleotides were based on the sequence of Popp (1989), entB was generated as described above and cut with XbaI and BamHI. Both menD and entB were ligated into the hydrolysed and dephosphorylated pKS3-02 and the recombinant was transformed into E. coli XL1-Blue. The resulting plasmid, pSM1, was hydrolysed in the presence of XbaI and PstI to remove menD from the plasmid. The resulting linear vector was treated with mung bean nuclease and religated. The resulting pRM4 plasmid was transformed into K. pneumoniae 62-1. The insert was checked by restriction site analysis.

**Media for production of chorismic acid and cyclohexadienes.**

Medium A (preculture) contained (1 l): Vogel & Bonner's medium (20 ml), Difco yeast extract (2 g), casamino acids (2 g), L-tryptophan (20 mg). Ten millilitres of a glucose solution (16%, w/v) and 6 ml of a solution of kanamycin (10 mg ml⁻¹) were added after sterile filtration. Medium B (production medium) contained (1 l): NaH₂PO₄·2H₂O (16.05 g), glucose (18 g), NH₄Cl (2.7 g), KH₂PO₄ (1.36 g), MgCl₂ (2 ml of a 50 mM solution, kanamycin (6 ml of a of 10 mg ml⁻¹ solution), L-tryptophan (10 ml of a 10 mM solution), L-phenylalanine (10 ml of a 10 mM solution), L-tyrosine (20 ml of a 5 mM solution) and (NH₄)₂Fe(SO₄)₂·6H₂O (1 ml of a solution of 40 mg in 10 ml H₂O).

For iron deficiency the iron content of the preculture (medium A) was reduced by 85% by extraction with a solution of 8-hydroxyquinoline in CH₃Cl₂, whereas the production culture (medium B) contained either 2,2'-dipyridyl (15 mg l⁻¹) or ferrozine (1 ml of a 0.4 M solution l⁻¹) and (NH₄)₂Fe(SO₄)₂ was omitted.

**Growth of producing strains.** K. pneumoniae 62-1 or recombinant strains harbouring either pRM7 (with entB) or pRM4 (with entB and entC) were grown in a preculture (300 ml Erlenmeyer flask, 100 ml medium A, 30°C) on a lab shaker (150 r.p.m.). After 6 h the suspension was centrifuged (6°C, 3800 r.p.m.), the cells washed (isotonic NaCl solution, 0.9%) and again centrifuged. The pellet was resuspended in isotonic NaCl solution made up to an OD₆00 of 3.35 and 1 l medium B inoculated with 50 ml of the suspension.

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Fig. 3. Construction of the entC/entB expression plasmid pRM4. Km', kanamycin resistance; P_lac, lac promoter. See Methods for details.
**HPLC analysis.** Time course studies of growth media (sample volume 5 μl) were carried out by chromatography (HPLC) on a Lichrosorb RP 18 column (4 x 30 mm, particle size 30 μm) connected to a Multispher RP-18 guard column (4 x 250 mm, particle size 5 μm). The solvent [methanol (15 %, v/v) in 1 % formic acid (85 %, v/v)] was passed over the columns at a flow rate of 1.0 ml min⁻¹. The photometer was set to 278 nm.

**Assay of EntC.** Bacteria were grown as described (Kaiser & Leistner, 1990), but, under iron deficiency succinate was replaced by glucose (5 g l⁻¹) and a minimum amount (1 μM) of FeCl₃ added to ensure growth. Bacteria were harvested by centrifugation, washed in potassium phosphate buffer (20 mM, pH 7) and lysed by sonication at 4 °C (Branson sonitier, 3 x 2 min, 50 % duty cycle). Cell debris was discarded (30 min centrifugation at 48000 g, 4 °C). The supernatant was purified by gel filtration (PD-10 columns, Pharmacia, equilibrated with phosphate buffer). Protein activity was determined in a total volume of 600 μl made up of 0.5 M Tris/HCl buffer (20 μl, pH 8.1) and 1 μl of an aqueous solution of NAD (1.5 mg in 20 μl) were incubated for 25 min at 37 °C. The reaction was stopped by addition of ice-cold MeOH (600 μl). The precipitated protein was removed by centrifugation and the supernatant analysed by HPLC as described above.

**Assay of enzymes (EntB and 2,3-dihydroxybenzoate synthase, EntA) involved in the enzymic conversion of isochorismate to 2,3-dihydroxybenzoic acid.** Bacteria were grown, harvested and protein extracted as described for EntC assays. Crude protein extract (25 μl), 3 nmol isochorismate, 0.5 M Tris/HCl buffer (20 μl, pH 8.1) and 1 μl of an aqueous solution of NAD (1.5 mg in 20 μl) were incubated for 25 min at 37 °C. The reaction was stopped by addition of ice-cold methanol (45 μl) and the mixture centrifuged. The supernatant was analysed for the presence of 2,3-dihydroxybenzoic acid by HPLC as described for isochorismate.

**Isolation of 2,3-DH-2,3-DHB.** The spent culture medium (500 ml) was acidified with HCl to pH 1.0 and extracted five times with ethyl acetate (250 ml). The ethyl acetate was dried (Na₂SO₄) and evaporated. The residue was dissolved in H₂O and residual ethyl acetate removed in a vacuum. The solution was applied to a RP-8 Lobar column (25 x 310 mm) and eluted with formic acid (1 %) in H₂O. Fractions were collected, neutralized with NH₄OH and freeze-dried. The yield was 70 mg (1 medium)⁻¹.

**Isolation of 3,4-DH-3,4-DHB.** The spent culture medium (500 ml) was acidified with HCl to pH 1.0 and extracted five times with ethyl acetate (250 ml). The ethyl acetate was dried (Na₂SO₄) and evaporated. The residue was dissolved in H₂O and residual ethyl acetate removed in a vacuum. The solution was applied to a RP-8 Lobar column (25 x 310 mm) and eluted with formic acid (1 %) in H₂O. Fractions were collected, neutralized with NH₄OH and freeze-dried. The yield was 90 mg (1 medium)⁻¹.

**Spectroscopy.** UV-spectra were taken in H₂O on a Uvikon 810/820 spectrophotometer. ¹H-NMR-spectra were obtained on a Bruker 500 MHZ AMX500. ¹³C-NMR spectra were obtained on the same instrument at 125 MHZ. IR-spectra (KBr) were performed on a Perkin-Elmer infrared spectrophotometer. EI mass spectra were taken on a Kratos spectrometer at 70 eV and an inlet temperature of 200-300 °C. CD-spectra were performed on a Jasco J-720 spectropolarimeter. Optical rotations were measured on a 241 Perkin-Elmer polarimeter.

**RESULTS**

**Effect of iron limitation on metabolites excreted by *K. pneumoniae* 62-1**

Table 1 shows that isochorismate-metabolizing enzymic activities (EntC, EntB, EntA) which are involved in the biosynthesis of the iron chelator enterobactin were detectable in crude protein preparations from *K. pneumoniae* 62-1. These activities were significantly higher than the enzyme activities from *E. coli* MC 4100. In spite of the relatively high EntC activity, *K. pneumoniae* 62-1 excreted chorisomic acid (rather than isochorismic acid or a mixture of both isomers) into the culture broth (Fig. 4a).

In contrast, when *K. pneumoniae* 62-1 was grown in the presence of an iron chelator (either dipyridyl or ferrozine), enzyme activities (Table 1) and the pattern of compounds excreted into the culture broth (Fig. 4b) changed dra-

<table>
<thead>
<tr>
<th>Strain</th>
<th>Enzymes</th>
<th>Enzyme activity [pmol product formed min⁻¹ (mg protein)⁻¹]†</th>
<th>with Fe(III)</th>
<th>without Fe(III)†</th>
</tr>
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<tr>
<td><em>E. coli</em> MC 4100</td>
<td>EntC</td>
<td>55</td>
<td>257</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>EntB + EntA</td>
<td>ND</td>
<td>56</td>
<td>355</td>
</tr>
<tr>
<td><em>K. pneumoniae</em> 62-1</td>
<td>EntC</td>
<td>406</td>
<td>4790</td>
<td>ND</td>
</tr>
<tr>
<td></td>
<td>EntB + EntA</td>
<td>355</td>
<td>1407</td>
<td></td>
</tr>
</tbody>
</table>

Mean values of three independent experiments.

† Bacteria were grown in the presence of iron [with Fe(III)] or under conditions of iron limitation [without Fe(III)].

ND, not detectable.

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Fig. 4. Time-dependent accumulation of chorismic acid (peak A) and 2,3-DH-2,3-DHB (peak B) in cultures of K. pneumoniae. (a) K. pneumoniae 62-1 grown on medium B; (b) K. pneumoniae 62-1 grown under iron limitation with dipyridyl; (c) K. pneumoniae 62-1 (pRM4) containing overexpressed genes entC and entB grown on medium B. Data points are means of two independent experiments. ■, growth; ●, chorismic acid; ▲, 2,3-DH-2,3-DHB. HPLC analysis was carried out 17.5 h after inoculation.

matically: no chorismic acid was detected but a compound appeared which was identical (UV, IR, MS, $^1$H-NMR, CD) with 2,3-DH-2,3-DHB (Young et al., 1969a, c; Beecham et al., 1971). The small coupling constants (2 Hz) of protons $H_2$ and $H_3$ were in agreement with the trans bisequatorial configuration of $H_2$ and $H_3$ in 2,3-DH-2,3-DHB (the coupling constant is subject to solvent-dependent variation; see Young et al., 1969a). $^{13}$C-NMR data taken in $D_2O$ [172.93(C$_{q}$,COOH), 138.03(CH$_{C_2}$), 135.07(CH$_{C_3}$), 130.39(C$_{q}$,C$_2$), 127.80(CH$_{C_2}$), 70.35(CH$_{C_3}$), 69.01(CH$_{C_3}$)] were in agreement with the proposed structure. The optical rotation was $[\alpha]_D^{20} = +3.89$ (C = 0.1, $H_2O$). The time course study (Fig. 4b) shows that up to 120 mg l$^{-1}$ of the cyclohexadiene accumulates in the culture broth.

Metabolites excreted after transformation with pRM4 harbouring the entC and entB genes

Data presented in Table 1 and Fig. 4 suggested that the excretion pattern encountered during growth in iron-deficient medium was due to a dramatic increase in the
EntC and EntB enzyme activities. We therefore constructed an overexpression plasmid (pRM4) carrying the entC and entB genes (Fig. 3; see Methods) and transformed K. pneumoniae 62-1 with this plasmid. A time course study (Fig. 4c) showed that both chorismic acid and 2,3-DH-2,3-DHB appeared in the medium. The recombinant strain grew to a higher density when compared to the untransformed strain grown under iron limitation (Fig. 4b) and the amount of hexadiene produced was almost doubled.

Metabolites excreted after transformation with pRM7 harbouring entB alone

We have previously shown (Schmidt & Leistner, 1995) that K. pneumoniae 62-1 harbouring pBGS8 with entC alone excretes a mixture of chorismic and isochorismic acid. We therefore constructed a plasmid (pRM7) carrying only the entB gene (Fig. 2; see Methods). The plasmid was introduced into K. pneumoniae 62-1 and a time course study was carried out (Fig. 5a). It was expected that EntB would withdraw isochorismic acid from the chorismic/isochorismic acid equilibrium resulting again in the excretion of 2,3-DH-2,3-DHB. This, however, was not observed. In addition to chorismic acid, a compound appeared with a HPLC retention time of 3.2 min compared with 2.9 min for 2,3-DH-2,3-DHB. To check the proper functioning of the entB gene we used plasmid pRM2-5 (Fig. 2) to isolate an overexpressed MalE/EntB fusion protein which was purified to homogeneity by affinity chromatography on a MalE-binding amylose column (protein fusion and purification system supplied by NEB). Indeed when this fusion protein was incubated with isochorismic acid, 2,3-DH-2,3-DHB was formed, indicating that the entB gene worked perfectly. Subsequently the unknown compound was isolated from the culture broth as described in Methods and found to be identical (UV, MS, IR, 'H-NMR, CD) with 3,4-DH-3,4-DHB (Young et al., 1969b; Beecham et al., 1971). The rather large coupling constants (10.5 Hz) for H3 and H4 and no coupling for H2 and H5 or H3 and H5 point to a trans-quasidiagonal position of protons H3 and H4. The 13C-NMR data taken in D2O [(166.04(Cp,COOH), 136.35(CH,C2), 128.08(CH,C3), 125.54(CH,C1), 119.19(CH,C0), 69.67(CH,C2), 68.82(CH,C3)] were in agreement with the proposed structure. Optical rotation was $[\alpha]_{D}^{20} = -1.60$ (C = 0.107, H2O).

Finally, we again induced entC and entB transcription by growth under iron deficiency (Fig. 5b) and found as
expected that 2,3-DH-2,3-DHB was formed. The recombinant strain carrying pRM7, however, being an over-producer (EntB) that grows under iron deprivation, showed reduced growth and the amount of 2,3-DH-2,3-DHB excreted was rather low.

**DISCUSSION**

The results presented in this paper show that _K. pneumoniae_ 62-1 can be employed in the production of cyclohexadiene carboxylic acid _trans_ diols, whereas microbial systems usually produce _cis_ diols. For the production of _cis_ diols, aromatic compounds such as toluene or benzene are used as a starting material. In the present case, however, glucose alone is the substrate. It is for this reason that the _K. pneumoniae_ 62-1 system can also be used to generate isotopically labelled metabolites of the shikimate pathway from isotopically labelled glucose. Thus, incubation of recombinant _K. pneumoniae_ transformed with pBGS8 carrying the _sbiC_ gene in the presence of [1,14C]glucose gives specifically labelled 4-hydroxy[2,6,14C]benzoic acid (Müller et al., 1995). We have also used pBGS8 carrying _entC_ and _K. pneumoniae_ 62-1 to produce 14C-labelled isochorismic acid (Schmidt & Leistner, 1995).

The experiments described herein also provide information on the metabolic flux and its regulation in the late steps of the shikimate pathway. Conclusions drawn on regulatory phenomena are usually obtained from the characterization of purified enzymes, but the _K. pneumoniae_ 62-1 system provides insight into metabolic processes that take place in _in vivo_. There are two observations reported in this paper which are consistent with the assumption that the intracellular concentration of chorismic acid prevails over isochorismic acid under normal growth conditions. Firstly, an active EntC is detectable in protein extracts from _K. pneumoniae_ 62-1 (Table 1). In spite of this the bacterium excretes chorismic acid alone but no isochorismic acid. It is known that a recombinant strain of _K. pneumoniae_ 62-1 is very well able to excrete significant amounts of isochorismic acid (Schmidt & Leistner, 1995). Thus, lack of isochorismic acid in the medium is not due to a lack of a transport system or to an undetected metabolism of isochorismic acid.

The second observation is related to the experiment in which pBGS8 harbouring _entB_ (encoding isochorismatase) was transformed into _K. pneumoniae_ 62-1. _In vitro_ EntB not only converts isochorismic acid to 2,3-DH-2,3-DHB, but also chorismic acid to 3,4-DH-3,4-DHB. The _K_ \text{m} value for isochorismatase is 14.7 μM whereas it is much larger (37 mM) for chorismatase (Rusnak et al., 1990). In spite of these kinetic constants the recombinant _K. pneumoniae_ 62-1 harbouring _entB_ excretes the metabolic product of chorismic acid (i.e. 3,4-DH-3,4-DHB) rather than the metabolic product of isochorismic acid (i.e. 2,3-DH-2,3-DHB). This may again indicate that the cellular level of chorismate significantly exceeds that of its isomer and suggests that the isochorismatase hydroxymutase is relatively inactive _in vivo_ under normal growth conditions. There are two conditions, however, under which the cellular activity of EntC increases significantly: when an overexpressed _entC_ gene is introduced into _K. pneumoniae_ 62-1 or when the enterobactin operon is derepressed by iron deficiency. The enterobactin operon contains four genes _entC_, _entE_, _entB_ and _entA_, which are transcriptionally linked and which are under negative control of the ferric uptake regulatory (Fur) protein (Brickman et al., 1990). In both cases the activity of EntC increases significantly (see Table 1) and leads to a change in the pattern of metabolites excreted by the _K. pneumoniae_ 62-1 strain. 2,3-DH-2,3-DHB, a metabolite of isochorismate, appears in the medium, indicating that the metabolic flux is shifted towards isochorismate. Thus, _in vivo_ experiments employing _K. pneumoniae_ 62-1 confirm the _in vitro_ data obtained from experiments with _E. coli_. In both cases the chorismate to isochorismate conversion was shown to be under control of iron.

The fact that 2,3-DH-2,3-DHB is excreted into the medium under iron limitation is also interesting for another reason. Since EntA, the enzyme converting 2,3-DH-2,3-DHB to 2,3-dihydroxybenzoic acid (Fig. 1), is very active (Table 1), one should expect the strain to excrete the benzoic acid rather than the cyclohexadiene. The fact that the cyclohexadiene is excreted could be explained by a limiting intracellular concentration of NAD which is required for the oxidation catalysed by EntA.

**ACKNOWLEDGEMENTS**

This work was assisted by a grant from the Fonds der Chemischen Industrie. We thank Dr E. Steckhan, Bonn, for fruitful discussions.

**REFERENCES**


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Received 7 August 1995; revised 29 November 1995; accepted 8 December 1995.