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 (l 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 biotransformation 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 & Berchtold, 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).

Bacterial strains and plasmids. *E. coli* MC 4100 (Casabadan, 1976) was used as a source of chromosomal DNA. *E. coli* XL1-Blue (Bullock *et al.*, 1987) was used as recipient strain for selection of recombinant clones. *K. pneumoniae* 62-1 [formerly *Enterobacter* (syn. *Aerobacter*) *aerogenes* 62-1], ATCC 25306, is a

Abbreviations: 2,3-DH-2,3-DHB, (+)-*trans*-(2S,3S)-2,3-dihydroxycyclohexa-4,6-dienecarboxylic acid; 3,4-DH-3,4-DHB, (−)-*trans*-(3R,4R)-3,4-dihydroxycyclohexa-1,5-dienecarboxylic acid; *EntA*, 2,3-dihydroxybenzoate synthase; *EntB*, isochorismatase; *EntC*, isochorismate hydroxymutase.

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.

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

triple mutant (*Phe⁻ Trp⁻ Tyr⁻*) blocked in aromatic amino acid biosynthesis (Gibson & Gibson, 1964). pIH9O2 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

Fig. 3. Construction of the *entC/entB* expression plasmid pRM4. Km^r, kanamycin resistance; P_{lac}, *lac* promoter. See Methods for details.

derivative of pUC8 in which the β -lactamase gene has been replaced by the kanamycin resistance (Km^r) gene from Tn903 (Spratt *et al.*, 1986).

Construction of plasmids. The *entB* gene was generated by PCR using genomic DNA from *E. coli* MC 4100 and oligonucleotides (+)CAGGATCCTGAAGGAGAGAACA and (–)GATCTAGATTATTTACCTCGCGG. Oligonucleotide sequences were derived from the *entB* sequence published by Schrodtt Nahlik *et al.* (1989). PCR conditions: denaturing 5 min (95 °C); 25 cycles [denaturing 30 s (95 °C), annealing 30 s (55 °C), extension 60 s (72 °C)] and 10 cycles in which each extension step increased by 5 s. The resulting *entB* fragment was isolated by gel electrophoresis and extracted from the gel using a Jetsorb Gel extraction kit. The DNA fragment was cut (*Bam*HI/*Xba*I) and ligated (Rusche & Flanders, 1985) into vector pIH902 (Lauritzen *et al.*, 1991). The recombinant was transformed (Nishimura *et al.*, 1991) into *E. coli* XL1-Blue (Bullock *et al.*, 1987). Plasmid pRM2-4 was isolated (Jones & Schofield, 1990) and the presence of the desired insert verified.

The *entB* gene was subsequently ligated into pMalc2 (NEB), a fusion vector which allows the resultant fusion protein to be isolated on an amylose column.

Restriction of pRM2-4 (Fig. 2) with *Bam*HI and subsequent treatment with Klenow enzyme (Klenow & Henningsen, 1979) in the presence of dATP and dGTP was followed by hydrolysis with *Hind*III. Restriction of pMalc2 with *Sal*I, treatment with Klenow enzyme in the presence of dCTP and dTTP, restriction with *Hind*III and ligation of *entB* into vector pMalc2 gave plasmid pRM2-5 (Fig. 2). The polylinker region and the *entB* gene were sequenced (up to 100 bp) to verify the expected sequence between *malE* and *entB*. pRM2-5 was then hydrolysed in the presence of *Bam*HI and *Hind*III and ligated into pBGS8 (Spratt *et al.*, 1986). Transformation of the resulting pRM7 (Fig. 2) into *K. pneumoniae* 62-1 gave the recombinant strain which has kanamycin resistance. The successful ligation and transformation were verified by restriction site analysis and sequencing.

pRM4, i.e. pBGS8 with *entC* and *entB* was constructed from pSM1 carrying *entC*, *entB* and *menD* (Fig. 3). Construction of this plasmid was carried out as part of a project not discussed here. pKS3-02 (Schmidt & Leistner, 1995) containing *entC* was hydrolysed in the presence of *Bam*HI and dephosphorylated. The *menD* gene (encoding 2-succinyl-6-hydroxy-2,4-cyclo-

hexadiene-1-carboxylate synthase) was generated by PCR using primers (+)AGTCTAGAGGAGCGGCAATGTCGTGC and (–)GGCATCAGATCTAAACGCTCACGCTC and cut with *Xba*I and *Bgl*II. Oligonucleotides were based on the sequence of Popp (1989). *entB* was generated as described above and cut with *Xba*I and *Bam*HI. 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 *Xba*I and *Pst*I 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 (l⁻¹): Vogel & Bonner's (1956) 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 (l⁻¹): Na₂HPO₄·2H₂O (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 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 CH₂Cl₂, 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 (0 °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.

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 × 30 mm, particle size 30 µm) connected to a Multospher RP-18 guard column (4 × 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 sonifier, 3 × 2 min, 50% duty cycle). Cell debris was discarded (30 min centrifugation at 48000 g, 4 °C). The crude extract was treated with 0.3 vols 2% (w/v) protamine sulfate solution. The precipitate was removed by centrifugation (30 min, 16000 g, 4 °C). The supernatant was purified by gel filtration (PD-10 columns, Pharmacia, equilibrated with phosphate buffer). Protein was determined as described by Bradford (1976). EntC activity was determined in a total volume of 600 µl made up of 70 µl barium chorismate (10 nM), 70 µl Tris/HCl (0.1 M), pH 7.6, 70 µl MgCl₂ (50 nM), 70 µl extract protein, and 320 µl H₂O. After 30 min incubation 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 applied to an ion-exchange column (20 × 300 mm, Lewatit-S100-MP64), washed with H₂O and eluted with NaOH (0–0.03 M). The fractions were collected, neutralized with HCl and freeze-dried. The freeze-dried material was dissolved in H₂O (30–40 ml) and chromatographed on a RP8-Lobar (25 × 310 mm) column with formic acid (1%) as solvent.

Fractions were collected, neutralized with NH₄OH and freeze-dried. Again the solid material was chromatographed on the same column with H₂O as solvent. Collected fractions were 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 × 310 mm) and eluted with formic acid (1%) in H₂O. Fractions were collected, neutralized with NH₄OH and freeze-dried. The residue was dissolved in H₂O and again chromatographed over the same column with H₂O as solvent. Fractions were again collected 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 in D₂O 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 chorismic 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 1. Comparison of activities of enzymes involved in the conversion of chorismate to 2,3-dihydroxybenzoate in crude protein extracts from different bacterial strains

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

* 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.

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 dipyrldyl; (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, 1H -NMR, CD) with 2,3-DH-2,3-DHB (Young *et al.*, 1969b, c; Beecham *et al.*, 1971). The small coupling constants (2 Hz) of protons H_2 and H_3 were in agreement with the *trans* bisectorial 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_6), 135.07(CH , C_4), 130.39(C_q , C_1), 127.80(CH , C_5), 70.35(CH , C_2), 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

Fig. 5. Time-dependent accumulation of 3,4-DH-3,4-DHB (peak C) and chorismic acid (peak A) or of 2,3-DH-2,3-DHB (peak B) in cultures of *K. pneumoniae*. (a) *K. pneumoniae* 62-1 pRM7 containing overexpressed *entB* grown on medium B; (b) *K. pneumoniae* 62-1 (pRM7) grown under iron limitation with dipyriddy. Data points are means of two independent experiments. ■, growth; ●, chorismic acid; ▼, 3,4-DH-3,4-DHB; ▲, 2,3-DH-2,3-DHB. HPLC analysis was carried out 17.5 h after inoculation.

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, ¹H-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 H₃ or H₄ and H₅ point to a trans-quasidaxial position of protons H₃ and H₄. The ¹³C-NMR data taken in D₂O [(166.04(C_q,COOH), 136.35(CH,C₂), 128.08(CH,C₅), 125.54(C_q,C₁), 119.19(CH,C₆), 69.67(CH,C₄), 68.82(CH,C₃)] were in agreement with the proposed structure. Optical rotation was [α]_D²⁰ = -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 *ubiC* gene in the presence of [1-¹³C]glucose gives specifically labelled 4-hydroxy[2,6-¹³C]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 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_m value for isochorismate is 14.7 μ M whereas it is much larger (37 mM) for chorismate (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 isochorismate 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 isochorismic acid. 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.

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