Shikimic Acid Production by a Modified Strain of *E. coli* (W3110.shik1) Under Phosphate-Limited and Carbon-Limited Conditions

Louise Johansson,[1] Anna Lindskog,[1] Gustav Silfversparre,[2] Christian Cimander,[2] Kristian Fog Nielsen,[3] Gunnar Lidén[1]

[1]Department of Chemical Engineering, Lund University, P.O. Box 124, 221 00 Lund, Sweden; telephone: +46 46 22 28 278; fax: +46 46 149 156; e-mail: Louise.Johansson@chemeng.lth.se
[2]Novozymes Biopharma AB, St. Lars väg 47, 220 09 Lund, Sweden
[3]Center for Microbial Biotechnology, Technical University of Denmark, Building 221, DK-2800 Lyngby, Denmark

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Abstract: Shikimic acid is one of several industrially interesting chiral starting materials formed in the aromatic amino acid pathway of plants and microorganisms. In this study, the physiology of a shikimic acid producing strain of *Escherichia coli* (derived from W3110) deleted in *aroL* (shikimic acid kinase II gene), was compared to that of a corresponding control strain (W3110) under carbon- and phosphate-limited conditions. For the shikimic acid producing strain (referred to as W3110.shik1), phosphate limitation resulted in a higher yield of shikimic acid (0.059 ± 0.012 vs. 0.024 ± 0.005 c-mol/c-mol) and a lower yield of by-products from the shikimate pathway, when compared to carbon-limited condition. The yield of the by-product 3-dehydroshikimic acid (DHS) decreased from 0.076 ± 0.028 to 0.022 ± 0.001 c-mol/c-mol. Several other by-products were only detected under carbon-limited conditions. The latter group included 3-dehydroquinic acid (0.021 ± 0.021 c-mol/c-mol), quinic acid (0.012 ± 0.005 c-mol/c-mol), and gallic acid (0.002 ± 0.001 c-mol/c-mol). For both strains, more acetate was produced under phosphate than the carbon-limited case. Considerable cell lysis was found for both strains but was higher for W3110.shik1, and increased for both strains under phosphate limitation. The advantages of the latter condition in terms of an increased shikimic acid yield was thus counteracted by an increased cell lysis, which may make downstream processing more difficult.

Keywords: shikimic acid; cell physiology; *Escherichia coli*; chemostat; carbon limitation; phosphate limitation

INTRODUCTION

Shikimic acid is one of the primary intermediates in the shikimate pathway (aromatic amino acid synthesis pathway (Fig. 1), which is found in most organisms except for mammals (Haslam, 1993; Neidhardt et al., 1996). Shikimic acid and other intermediates in the pathway (Bongaerts et al., 2001) contain several functional groups and are thus industrially interesting chiral starting material for production of many different chemical compounds (Schreiber et al., 2002), such as the neuraminidase inhibitor Tamiflu® used for treatment of influenza (De Clercq, 2002; Ghisalba, 1985; Kim et al., 1997). Shikimic acid derivatives are furthermore interesting as herbicides and antibacterial agents as they can block the shikimate pathway without negative effects in mammals (Jiang et al., 1999; Song et al., 2001). Shikimic acid can also potentially be used for a non-benzene derived production of phenol (Gibson et al., 2001). However, a significant drawback is that shikimic acid is still to a large extent produced by a multistep and low-yielding extraction process of the plant *Illicium* (Haslam, 1993). New ways of production have been proposed to increase production capacity and lower production costs, including organic chemical synthesis (Mirza and Harvey, 1991; Takeuchi et al., 2000) and methods based on genetically modified *Escherichia coli* strains (Chandran et al., 2003; Draths et al., 1999; Knop et al., 2001).

Previous fermentation studies have focused on maximizing the shikimic acid yield by genetic modifications of targets in—or closely connected to—the aromatic amino acid pathway. Reported strain constructs have been deleted for both the *aroK* and the *aroL* genes, which completely turns off the flow through the aromatic amino acid pathway (Fig. 1); consequently, it has been necessary to add vitamins and aromatic amino acids that are produced downstream of shikimic acid in this pathway.

In the current study, an *E. coli* strain deleted for only *aroL* (but not *aroK*), was studied. The gene *aroL* encodes the main enzyme (shikimic acid kinase II) responsible for the

Correspondence to: Louise Johansson
Contract grant sponsor: Swedish Agency for Innovation Systems (Vinnova)

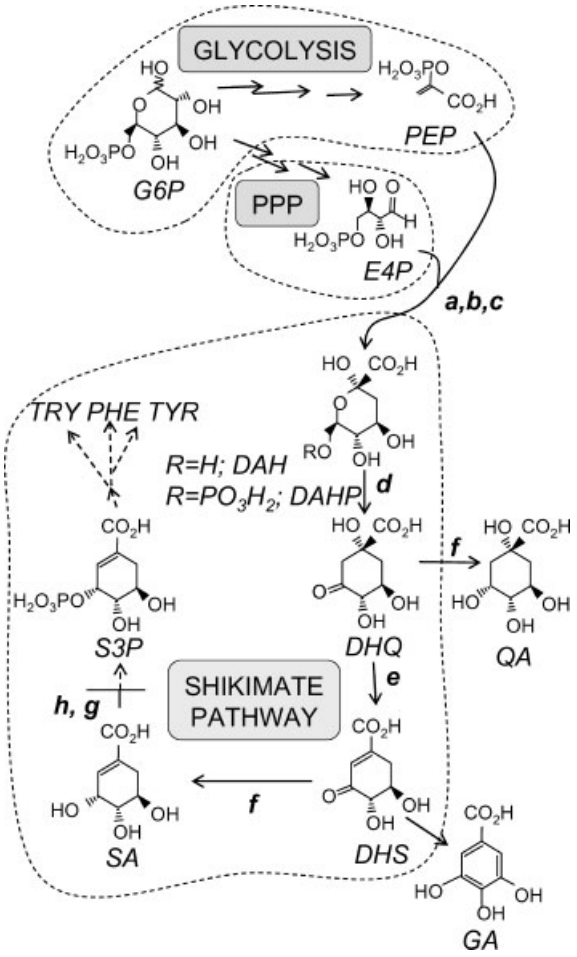

Figure 1. The production of shikimic acid in *E. coli.* Intermediates (abbreviations): glucose-6-phosphate (G6P), phosphoenolpyruvic acid (PEP), 3-deoxy-*D*-arabino-heptulosonic acid (DAHP), 3-dehydroquinic acid (DHQ), 3-dehydroshikimic acid (DHS), shikimic acid (SA), shikimic acid-3-phosphate (S3P), quinic acid (QA), gallic acid (GA). Enzymes (encoding genes): (a, b, c) DAHP synthase (a = *aroF*, b = *aroG*, c = *aroH*). (d) 3-dehydroquinate synthase (*aroB*), (e) 3-dehydroquinate dehydratase (*aroD*), (f) shikimic acid dehydrogenase (*aroE*), (g) shikimic acid kinase I (*aroK*), (h) shikimic acid kinase II (*aroL*), PPP = pentose phosphate pathway.

phosphorylation of shikimate needed for further conversion in the pathway. Maintaining *aroK* allows for a small bleed through the pathway past shikimate, and addition of vitamins or amino acids formed downstream of shikimic acid is therefore potentially not needed.

The engineered strain (W3110.shik1) and the control strain (W3110), were characterized using chemostat cultivations to enable a determination of product yields at well-defined growth conditions at a given specific growth rate. Both glucose-limited and phosphate-limited conditions were studied. The latter was chosen primarily as a means to study glucose-rich conditions, which have previously been shown to give higher yield of shikimic acid (Chandran et al., 2003; Knop et al., 2001).

MATERIALS AND METHODS

In both carbon-limited and phosphate-limited cultivations, *E. coli* W3110 modified to produce shikimic acid was compared with W3110.

Strains

W3110 was received from American Type Culture Collection (ATCC 27325, LGC Promochem, Teddington, UK) and was also used as host strain for the shikimic acid producing strain (referred to as W3110.shik1), with the following genetic modifications: *ΔaroL*, tryptophan, and phenylalanine feedback resistant $aroG^{FBR}$, tryptophan feedback resistant $trpE^{FBR}$ and *tnaA*. In addition, W3110.shik1 was cloned with plasmid pSGs26 (derived from pBR322) containing tyrosine and phenylalanine feedback resistant $aroF^{FBR}$ and two antibiotic resistance markers amp^R and $tetc^R$. The *ΔaroL* was introduced by transduction of a p1 lysate carrying the *aroL* allele *aroL478::Tn10* (Ely and Pittard, 1979). The transductants were then selected for $tetc^R$ phenotype. The resulting phenotype was then selected for deletion of the transposon containing the $tetc^R$ and *aroL* allele.

Inoculum Preparation

The medium used for inoculum preparation, 100 mL, was the same for carbon-limited and phosphate-limited experiments. For W3110, the medium contained per liter: 20 g glucose (VWR International, Stockholm, Sweden), 7.5 g K_2HPO_4, 0.3 g NH_4Fe(III)-citrate, 2.1 g citric acid monohydrate, 0.1 mL $MgSO_4 \cdot 7H_2O$ (1M), 0.0037 g $(NH_4)_6(Mo_7O_{24}) \cdot 4H_2O$, 0.0029 g $ZnSO_4 \cdot 7H_2O$, 0.0247 g H_3BO_3, 0.0025 g $CuSO_4 \cdot 5H_2O$, 0.0158 g $MnCl_2 \cdot 4H_2O$ and 0.055 mg thiamine (Sigma-Aldrich, Steinheim, Germany), 1.2 mL H_2SO_4 (96%) (VWR International). For W3110.shik1 the medium additionally contained: 0.010 g *p*-hydroxybenzoic acid, 0.010 g potassium *p*-amino benzoate, 0.010 g 2,3-dihydroxybenzoic acid (vitamins), and 0.015 g tetracycline hydrochloride (antibiotic) (Sigma-Aldrich). Inoculum cultures were grown in baffled E-flasks of 250 mL at 37°C at a shaker speed of 200 rpm up to an OD-value of 1.5–2.5, to ensure exponentially growth. The fermenter was inoculated with 40 mL of the inoculum culture (W3110.shik1) or 20 mL (W3110).

Culture Media

The glucose and mineral solutions were sterilized separately at 120°C for 20 min and thereafter mixed. Antibiotic solution, vitamins and trace metals and $MgSO_4$-solution were added by sterile filtration through a 0.2 μm Minisart cellulose acetate filter (Sartorius AG, Goettingen, Germany). All solutions were prepared using deionized water. 1.5 L of medium was prepared for the batch cultivation. The working volume of the chemostat was therefore around 1.3 L, and pH was adjusted to 7.0 by addition of NH_4OH (25%) before and after sterilization.

Carbon-Limited Cultivations

The composition of the C-limitation medium for the batch phase and the chemostat phase of the W3110 was the same as for the preculture medium, except for some minor changes. The medium contained 0.13 g/L antifoam 286 (Sigma-Aldrich). In addition, the glucose concentration was 10 and 25 g/L in batch and chemostat phase, respectively. In the two first chemostats, however, the glucose concentration was 30 g/L. The composition of the medium for growth of W3110.shik1 was the same as that for W3110 except that it also contained 0.015 g/L tetracycline hydrochloride.

Phosphate-Limited Cultivations

The composition of the P-limited medium in batch phase was per liter: 20 g glucose, 92 μL H_3PO_4 (85%), 5.39 g NH_4SO_4, 3.32 g NaOH, 1.66 g KOH (Sigma-Aldrich), 0.52 g $MgSO_4 \cdot 7H_2O$, 3.73 g, 0.133 g antifoam 286, H_2SO_4 (96%), trace metals; 0.093 g $FeSO_4 \cdot 7H_2O$, 0.079 g citric acid monohydrate, trace metal, i.e., 0.0073 g $CoCl_2 \cdot 6H_2O$, 0.00207 g $MnCl_2 \cdot 4H_2O$, 0.00103 g ZnCl (Sigma-Aldrich). The medium of W3110.shik1 also contained 0.015 g/L tetracycline hydrochloride.

In chemostat operation, the phosphate solution and the solution of the remaining medium components were fed separately to the fermenter. The phosphate feed contained 0.9 mL/L H_3PO_4 (85%), whereas the second feed solution contained all other components. Since the phosphate solution contributed extra volume and thereby diluted the second feed solution the latter was up concentrated 1.3 times, e.g., the glucose concentration was 32 g/L giving a concentration of 25 g/L into the reactor.

Fermentation Methods

All fermentations were carried out in a Biostat® CT (Sartorius BBI Systems GmbH, Melsungen, Germany) culture vessel with a maximum working volume of 3.5 L. Temperature (37°C), pH (7.0), stirring rate (750 rpm) and airflow rate (0.75 slpm) were controlled by the program MFCS/win shell 2.0 (Sartorius BBI) via the control unit DCU Biostat C (Sartorius BBI). pO_2 was measured with an InPro® 6000 (Mettler Toledo Gmbh, Giessen, Germany). The pH was measured with a pH-meter of type 405-DPAS-SC-K8S/120 (Mettler Toledo). For maintaining the pH at 7.0 H_2SO_4, (2M) and NH_4OH (25%) were used in the C-limited case and H_2SO_4 (1M) and NH_4OH (12.5%) in the P-limited case. O_2 and CO_2 contents in the off gas were measured by an Innova 1311 (INNOVA Air Tech Instruments, Ballerup, Denmark). Two balances and pumps were also coupled to the equipment allowing precise measurement of the feed rate during chemostat operation. Data from all equipment except for one of the pumps were routinely logged by the data acquisition software.

The chemostat was in the C-limited case started at a dilution rate of 0.2 h^{-1} at the time when the glucose was finished (indicated by a rapid reduction of the CO_2 in the off-gas). In the P-limited case the chemostat was started when the phosphate was consumed (also seen as a small peak in the pO_2, Fig. 2). Phosphate limitation was ensured by measuring glucose and phosphate concentration and keeping the glucose at ∼5 g/L. Establishment of steady-state was confirmed from measurement of the off-gas composition. At least five retention times were allowed to pass before each steady-state. Sampling of OD and for metabolite analyzes were carried out at fermentation start, end of batch, and at steady state during chemostat operation. Samples for dry weight were taken at the end of the batch cultivation and at steady state during chemostat cultivation.

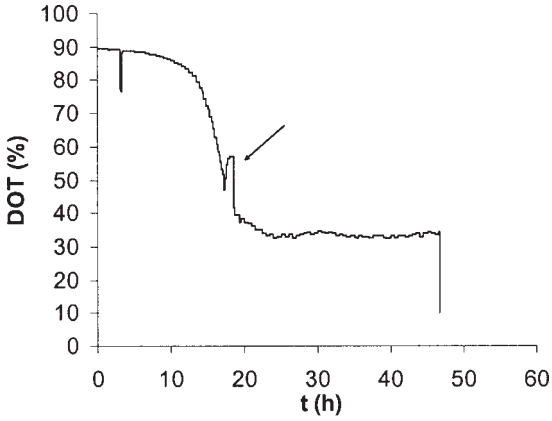

Figure 2. Measured dissolved oxygen tension (DOT) in a batch cultivation of *E. coli*. At the time indicated by the arrow, the phosphate in the medium is depleted.

Analytical Methods

Metabolites

Samples for metabolite analysis were taken in 1.5 mL eppendorf tubes and cells were removed by centrifugation for 5 min at 12,000*g*. The samples were stored at −20°C until analysis. Small metabolites related to the shikimate pathway were analyzed with a Waters Breeze™ HPLC system (Waters Corporation, Milford, MA) controlled with the GPS Breeze™ Software, and equipped with a Waters 2410 refractive index (RI) detector and Spectroflow 757 (Applied Biosystems, Foster City, CA) UV-detector of set at 210 nm. The column used was a HPX-87H (Bio-Rad Laboratories, Inc., Hercules, CA) maintained at 60°C. The internal temperature of the RI-detector was 45°C. The eluent used was 0.005M H_2SO_4 (99.99% H_2SO_4, Sigma-Aldrich) dissolved in ultra pure water (MQ-water).

Compounds not available as standards were quantified by RI from the shikimic acid calibration curve. Shikimic acid was also analyzed using an Agilent 1100 series LC system (Agilent, Palo Alto, CA) equipped with a UV detector, set at 210 nm, and Supelcosil LC-NH_2 column (Chromtech AB, Stockholm, Sweden), using acetonitrile/25 mM H_3PO_4 (60/40 v/v).

Glucose

In the P-limited case, glucose was analyzed enzymatically using a SIRE^{TM} Biosensor P100 (Chemel AB, Lund, Sweden) based on glucose oxidase as described in Kriz et al. (1998). The analysis range of the instrument was 0.1–10 mM, and the samples were therefore diluted 25–50 times prior to analysis. In both the C-limited and the P-limited case glucose was also measured with the Breeze HPLC system mentioned above.

Phosphate

Phosphate concentration measurements were performed according to a modified pyrophosphate/phosphate protocol (Boehringer Mannheim, 1989; Gawehn, 1974; Kraak, 1974; Langner, 1964). In this method phosphate forms a colored complex with ammonium molybdate, which is detected by absorption at 578 nm. 0.05 mL of medium sample was mixed with 0.25 mL distilled water and 0.7 mL phosphate reagent in an eppendorf tube. The tube was centrifuged for 10 min at 12,000*g* (Z 160 M, Hermle Labortechnic GmbH, Wehingen, Germany) and the absorbance was then measured with a spectrophotometer (PRIM Light & Advanced, Secomam, Domont Cedex, France). A reference sample was prepared following the procedure above, but by mixing 0.3 mL distilled water with 0.7 mL phosphate reagent. The concentration was finally obtained from the absorbance difference between medium and reference samples using a calibration curve.

Biomass Concentration

Dry weight (DW) was determined from quadruple 10 mL samples which were centrifuged 10 min at 2,000*g* and 4°C. The cells were washed once with 5 mL ice-cold water and were then dried at 105°C overnight.

The optical density (OD) was measured at 620 nm with a spectrophotometer (PRIM Light & Advanced, Secomam, Domont Cedex, France). When the OD was higher than 0.3 the samples were diluted with distilled water.

Total Organic Carbon (TOC)

TOC was determined by combustion of samples to CO_2 and its subsequent quantification by an infrared gas analyzer on a Shimadzu TOC-5050A (Shimadzu Corporation, Kyoto, Japan).

Supernatant samples were thawed and filtered through 0.20 μm Sartorius AG Minisart polypropylene housing filters. The resulting filtrates were diluted 50 times and measured according to the instruction manual of the instrument.

Endotoxin Analysis

For endotoxin analysis a commercial kit (Endpoint Chromogenic LAL Kit R160, Charles River Laboratories, Wilmington, MA) was used.

Protein Analysis

Total biomass protein content and protein content in the medium (from lysed cells) were analyzed according to a modified form of the Biuret method (Schultze, 1995). Duplicates of 1 mL samples were taken for total analysis of biomass protein, cooled on ice and the centrifuged for 5 min at 12,000*g*. The supernatant was removed and the cells were washed twice with NaCl (0.9 %) and stored at −80°C until analysis. Bovine serum albumin (Sigma-Aldrich) was used for calibration, concentrations between 0 and 2.5 mg/mL were produced by dilution with 1M NaOH. The same kinds of samples as taken for metabolite analysis were used for analysis of the protein content of the supernatant. The supernatant samples, however, had to be treated somewhat differently. These samples were first filtered through a 0.2 μm Minisart cellulose acetate filter (Sartorius AG). Subsequently, 250 μl of the filtrate of each sample was transferred to a 1.5 mL tube, 250 μL NaOH (2M) was added, and finally the samples were diluted to 1 mL by adding NaOH (1M). Because glucose (in the P-limited experiments) and by-products (using W3110.shik1) interfered in the supernatant samples, an extra set of reference samples was prepared in which 300 μL MQ-water was added instead of the protein complexing $CuSO_4 \cdot 5H_2O$ (2.5% (w/v)). In this way, the background absorbance from glucose and by-product derived substances could be measured and subtracted from the samples giving the absorbance of the proteins.

Identification of By-Products

LC-MS

In order to identify the products formed by W3110.shik1, which gave rise to unidentified peaks on the HPX-87H column not observed in the chromatograms of W3110, the samples were analyzed by LC-UV-High Resolution Mass Spectrometry (LC-UV-HR MS). These were performed on an Agilent HP 1100 Liquid Chromatograph with a diode array detector coupled to a time of flight (TOF) mass spectrometer of model LCT (Waters-Micromass, Manchester, UK) with Z-spray electrospray source (ESI) and a LockSpray probe (Nielsen and Smedsgaard, 2003). The MS was operated in the negative ESI mode and tuned to a resolution >5,000 FWHM, and MS spectra were collected as centroid from m/z 70 to 900, with a scan time of 1 s. The capillary was held at 2,000 V, potential between skimmers was 10 V, desolvation temperature was 450°C, and nitrogen flow 550 L/h. The LockSpray sprayed a solution of 2,6-dihydroxy benzoic acid, where the $[M\text{-}H]^-$ ion at m/z 153.0188 was used for on-line mass correction every 3 s.

Separations were performed on two different columns: Polar RP, 150 × 2 mm, 4 μm (Phenomenex, Torrance, CA), phenyl column with embedded polar groups, and a Thermo Electron Hypercarb, 100 × 2.1 mm, 5 μm, graphite column. Both columns were operated at flow of 0.3 mL/min with water-acetonitrile gradient, starting with 100% water, maintaining this for 2 min before raising the acetonitrile

content to 50% in 10 min. Formic acid, 200 ppm, was added to the water.

Two types of samples were analyzed by LC-UV-HR MS. The first type consisted of fractions collected from the HPX-87H column, in which 200 μL had been injected in the Breeze system and a fraction was collected at the outlet for about 0.8 min at the relevant peak residence time. Five microliter of these samples were analyzed by LC-UV-HR MS on the RP column.

The second type consisted of crude fermentate and medium blank samples, which were filtered through Ultrafree-MC 5 000 NMWL centrifugal filters (Millipore, Billerica, MA) for 30 min at 15,000*g* to remove macro molecules. One microliter was analyzed by LC-MS. The compounds available as reference in these analyzes were citric acid, shikimic acid, gallic acid, quinic acid, and protocatechuic acid.

Chemical Identification of DHS

The identification of DHS was based on a slightly modified method from Kambourakis et al. (2000). In this method gallic acid is formed chemically from DHS, but the conversion is not complete. Supernatant, 1 mL, was added to a 3 mL solution of pure acetic acid. The reaction was started by adding 150 μL of 0.082 g/mL $CuSO_4$ solution and the mixture was held at 40°C for 45 h. The sample was analyzed on the HPX-87H column before and after chemical treatment and the peaks from gallic acid and the suspected DHS compared.

Plasmid Stability

The plasmid stability of W3110.shik at steady-state was controlled by comparing the colony forming unit (CFU) on Luria-Bertani agar plates medium (LB), LB-agar containing 0.015 g/L tetracycline hydrochloride (LBT), and on LB-agar containing 0.015 g/L tetracycline hydrochloride and 0.10 g/L Ampicillin (LBTA). LB medium contained per liter: 10 g Bacto-tryptone, 5 g yeast extract (VWR International), and 10 NaCl (Sigma-Aldrich). The concentration of agar was 1.5%.

RESULTS

The presented results are based on a total of 17 experiments, of which 9 were carbon-limited chemostats (5 W3110 and 4 W3110.shik1) and 8 were phosphate-limited chemostats (4 of each strain). The batch phase of each experiment was analyzed in order to determine the maximum specific growth rates (μ_{max}) of the strains in the different media used. Typical examples of cultivation characteristics are shown in Figure 3.

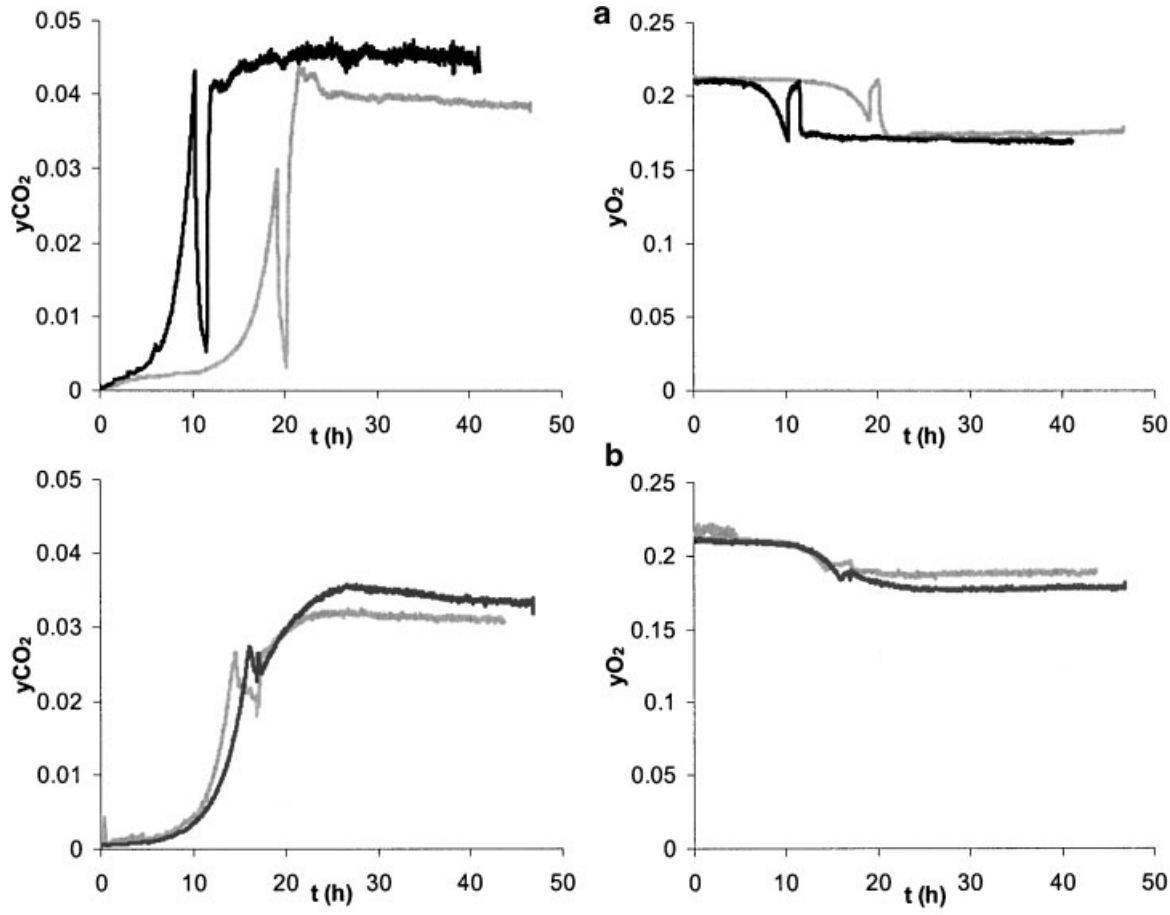

Figure 3. Measurement of mole fractions of CO_2 (yCO_2) and O_2 (yO_2) in the off-gas as a function of fermentation time in (**a**) carbon-limited cultivations (**b**) phosphate-limited cultivations. Black lines indicate W3110 and gray lines indicate W3110.shik1.

Table I. Measured specific growth rates from batch cultivations, and measured/calculated dilution rates, glucose-phosphate feed ratios, carbon dioxide evolution rates, oxygen uptake rates, and RQ's from chemostat cultivations of a shikimic acid producing mutant strain (W3110.shik1) and a wt strain W3110 of *E. coli*.

		Batch cultivation	Chemostat cultivation				
Strain	Type of medium[a]	μ_{max} (h^{-1})	D (h^{-1})	Fg/Fp	CER (mmol/lh)	OUR (mmol/lh)	RQ
W3110. shik1	Carbon	0.33 (±0.04)	0.22 (+0.02)		59 (±2)	50 (±5)	1.2 (±0.2)
	Phosphate	0.39 (±0.04)	0.23 (±0.01)	3.4 (±0.1)	48 (±3)	48 (±3)	1.0 (±0.1)
W3110	Carbon	0.58 (±0.03)	0.22 (+0.02)		68 (±4)	61 (±6)	1.1 (±0.2)
	Phosphate[b]	0.71 (±0.06)	0.25 (±0.01)	2.6 (±0.1)	59 (±8)	51 (±4)	1.2 (±0.1)

[a]The type of medium used for creating phosphate and carbon-limited conditions respectively in the chemostat phase.
[b]One outlier experiment with a specific growth rate of 0.34 h^{-1} was excluded.

Specific Growth Rates

The maximum specific growth rate, μ_{max}, of W3110.shik1 was significantly lower than that of W3110, regardless of medium used (Table I). This was clear from the mean values of μ_{max} for the different types of cultivations. The medium used for the phosphate-limited cultivations had to be modified, since phosphate for obvious reasons could not be used as a buffer. The analysis showed that, μ_{max} was somewhat higher in the medium used for phosphate limitation than in the medium used for carbon limitation experiments (see Table I). In one of the four batch cultures of W3110 using the ''phosphate-limited medium,'' the specific growth rate ($\mu_{max} = 0.34\ h^{-1}$) was significantly lower than in the other repeats. This batch was excluded from the calculation of mean values.

Chemostat Cultivations

Chemostat experiments were carried out at dilution rates of 0.22–0.25 h^{-1}. The carbon dioxide evolution rate, CER, was generally higher in the W3110 cultivations compared to the W3110.shik1 cultivations regardless of medium (Table I). Furthermore, CER was higher under carbon than under phosphate limitation. The same trend as for CER could be seen also for the oxygen uptake rate, OUR. The overall mean RQ was 1.1 ± 0.2 and there was no significant difference between the cultivations except for W3110.shik1 under phosphate-limited conditions, for which the RQ was somewhat smaller than in the other cases (1.0 ± 0.1). Phosphate limitation was verified by measurements of the residual glucose concentration and phosphate concentrations in the first four phosphate-limited chemostats (two of each strain). The steady-state phosphate concentration was below the detection limit in all these chemostats. It was considered sufficient to measure the residual glucose concentration in the following phosphate-limited experiments (Fig. 4). The ratio of the glucose to the phosphate feed in the phosphate-limited cultivations, Fg/Fp (Table I), was somewhat higher for W3110.shik1 than for W3110 giving also a somewhat higher steady-state glucose concentration (8.3 ± 0.2 and 5.5 ± 1.2 g/L, respectively, for the two strains). A plasmid stability test (see Table II) showed no plasmid loss at the time of sampling at steady-state.

Identification of By-Products

Analysis of the crude fermentate showed that DHS, shikimic acid, DHQ, quinic acid, and citric acid were almost unretained on the Polar RP column. However, since very sharp peaks were obtained, the separation was still sufficient to obtain resolution of the peaks from each other (results not shown). Compounds with the same nominal masses, like citric acid and DHQ, could furthermore be resolved in a ±10 mDa window as their different molecular compositions yields $[M\text{-}H]^-$ ions of m/z 191.0192 and 191.0556, respectively.

For identification of peaks from the HPX-87H column, it would appear obvious to directly subject the effluent from the column directly to the MS. However, the solvent composition (0.005M H_2SO_4) is not compatible with electrospray MS. Fortunately, the samples collected after HPX-87H separation could be analyzed by LC-MS as it was just possible to resolve the analytes from the sulfuric acid peak. It was, however, not possible to up-concentrate the metabolites by freeze drying of the extracts because of formation of oxidization products.

The presence of DHS was confirmed by LC-UV-HR MS analysis of the fraction of HPX-87H RT 13.2 peak. The base peak in the total ion chromatogram (Fig. 5) had m/z of 171.0324 which was 3 mDa away from the calculated m/z 171.0294 of the DHS $[M\text{-}H]^-$ ion. The presence of DHS was further confirmed by partial chemical conversion to gallic

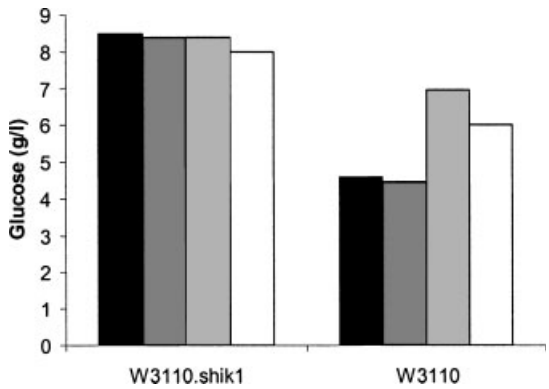

Figure 4. Measured glucose concentration in the medium at steady-state in phosphate-limited chemostat cultivations of *E. coli*. The left hand bars show values for cultivations of W3110.shik1 and the right hand show values for cultivation of W3110.

Table II. Summary of plasmid stability measurements.

Groups	Replicates	Sum CFU*10^{-11} all replicates	Average CFU*10^{-11}	Variance*10^{-23}
LB	3	21	7.0	0.78
LBT	3	25	8.3	2.61
LBTA	3	22	7.3	4.41

acid which gave a yield of 35% (mol gallic acid/mol DHS). This was in the same range as reported by Kambourakis et al. (2000). LC-MS analysis of the fraction obtained from HPX-87H at a retention time of 21.98 min showed that the base peak was gallic acid. DHQ was found in the RT 8.9 min peak from the HPX-87H column (Fig. 5), where it co-eluted with compound(s) from the media. A compound which was co-eluting with quinic acid in the ion-exchange method showed an intensive ion at m/z 155.010 $[M-H]^-$, a predominant ion at m/z 111 [M-H-CO_2] and a predominant ion at 311 [2M-H]. It was also found in the crude fermentate, but not in the unfermented medium indicating that it was a metabolite formed. However, this compound could not be identified.

On the graphite column all the target compounds were strongly retained, but with broad peaks (up to 1 min), giving poor detection limits. Analysis using this method was only possible on the crude fermentates. In this system DHQ eluted at RT 7.59 min, which was about 1.32 min later than quinic acid, which in turn was eluted 0.05 min before citric acid. Furthermore DHS was eluted at 10.32 which was about 0.17 min sooner than shikimic acid.

Yields of Shikimic Acid and Other Metabolites

Extracellular metabolites directly derived from the shikimate pathway were only produced in W3110.shik1 (Fig. 6). In the phosphate-limited case, the shikimic acid yield was twice that of the carbon-limited case (Table III). The DHS yield was about one third, whereas quinic acid and gallic acid were not produced in detectable amounts. The ''loss of shikimic acid'' via DHS in the carbon-limited cultivations may in fact be even higher than the measured DHS concentration, since DHS can be degraded in the presence of phosphate (Kambourakis et al., 2000).

With respect to metabolites derived from the central catabolic pathways, there was a clear increase in acetate yield under phosphate-limited condition, with a higher acetate yield for W3110 than W3110.shik1 (Fig. 7), despite the fact that the glucose concentration was somewhat higher for

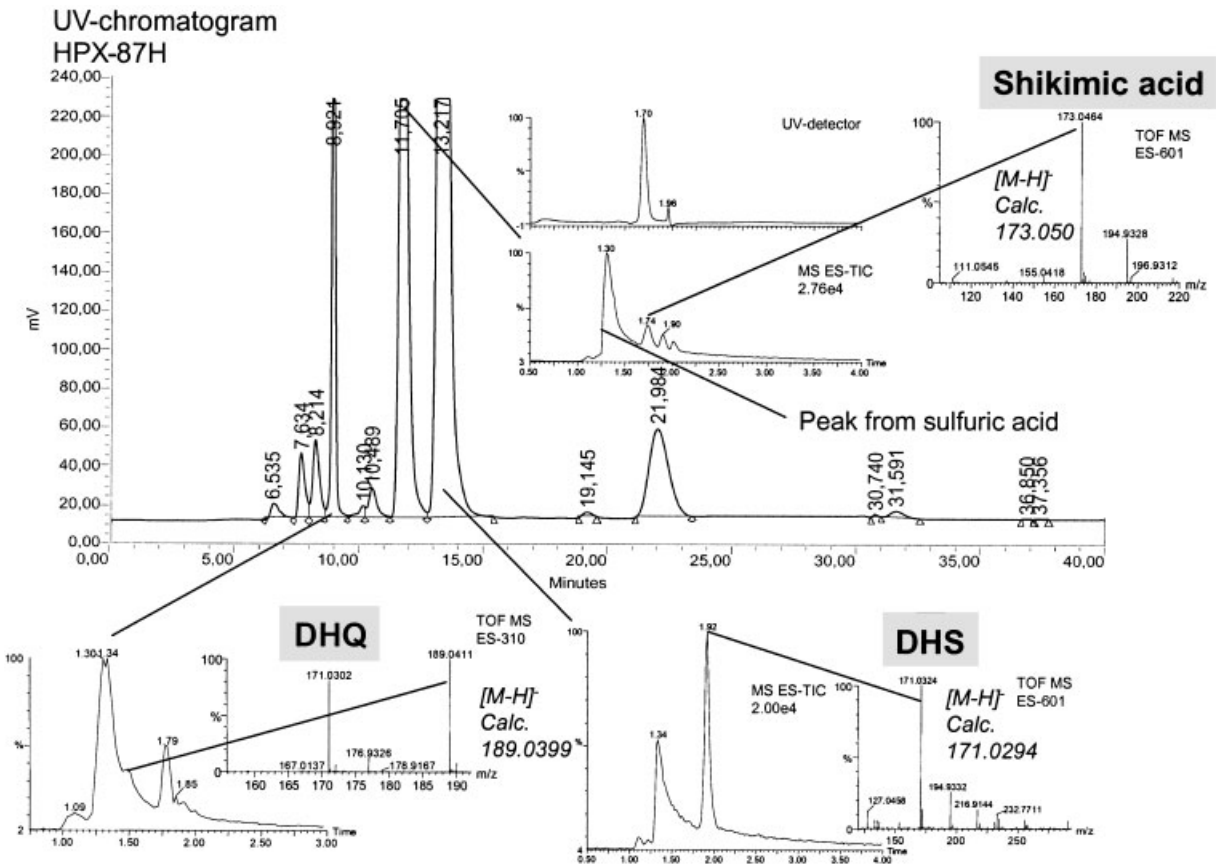

Figure 5. HPLC ion-exchange chromatogram (UV-detection) of fermentation medium from W3110.shik1 fermentation, together with LC-MS analyzes of characteristic peaks. The H_2SO_4 from the ion-exchange solvent was resolved from the later eluting target peaks. Shikimic acid identified as its $[M-H]^-$ ion with a mass deviation of 1.6 mDa and correct retention time in this system also demonstrates proof of concept. Also identified in the figure are DHQ and DHS.

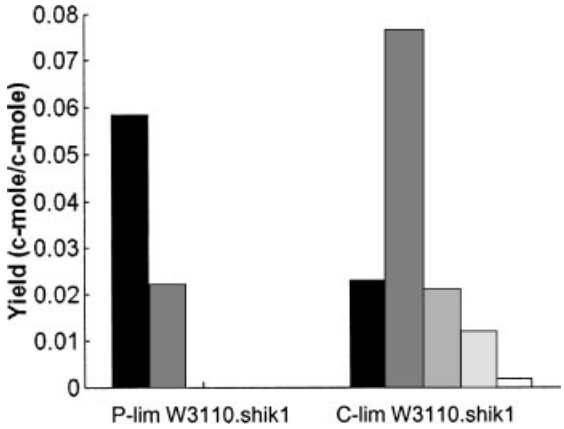

Figure 6. Average product yields in the shikimate pathway obtained in chemostat cultures of the shikimic acid producing *E. coli* strain (W3110.shik1). Symbols: black, shikimic acid; dark gray, DHS; medium gray, DHQ; light gray, quinic acid; white, gallic acid. None of the metabolites were produced in W3110.

W3110.shik1. Formic acid was also produced in small amounts by W3110. More formic acid was formed under phosphate-limited condition than under carbon-limited condition. In addition small amounts of ethanol and succinic acid were produced by W3110 under phosphate limitation.

Carbon Balances

The carbon balances for most chemostat cultivations closed reasonably well (see Table III). However, it was necessary to consider cell lysis in these balances. The fraction of lysed cells was high, up to 30% for W3110.shik1 in some cases. It was not obvious at first that substantial cell lysis would take place, as this had not been reported previously for shikimic acid over-producing strains. Therefore, the lysed cells initially appeared as carbon missing in the carbon balance calculations. The missing carbon was found by an analysis of the total carbon content (TOC) of the supernatant (see Table III). However, this method only gives a gross measurement of the total carbon content without information about the origin of the carbon. In addition, the efficiency of oxidation is only about 80% in samples like the ones in the present study, giving a lower value of TOC than expected (Raczyk-Stanislawiak et al., 2003).

To test for possible cell lysis, the protein content of the supernatant as well as the total protein content of the cells was measured. Substantial amounts of protein were indeed found in the supernatant for the cultivations which had a low carbon recovery. The lysed cell mass was estimated by back-calculating from the measured protein content of the supernatant. The total protein content in biomass analyzed was 0.59 (±0.08) g/g for all strains, with no systematic difference found for the different cultivation conditions. A cellular protein content of 0.59 g/g was therefore used in the calculations. The proportion of lysed cells was clearly higher for W3110.shik1 than for W3110, under both carbon limitation and phosphate limitation. The amount of lysed cells of W3110.shik1 and W3110 was, furthermore, higher under phosphate limitation for both strains. Interestingly, addition of the cell yields and the yield of lysed cells gave an overall yield of 0.54 (±0.07) g/g in all cases (Table III). The large difference in cell yield thus appears to be predominantly explained by the difference in cell lysis. In batch phase the

Table III. Carbon balances for W3110.shik1 and W3110.

	Yields[a] in chemostat steady-state (c-mol/c-mol glucose)			
	W3110.shik1		W3110	
Metabolite	Carbon limitation	Phosphate limitation	Carbon limitation	Phosphate limitation
Shikimic acid	0.023 (±0.003)	0.056 (±0.009)	n.d.	n.d.
DHS[b]	0.077 (±0.028)	0.022 (±0.001)	n.d.	n.d.
DHQ[b]	0.021 (±0.021)	n.d.	n.d.	n.d.
Gallic acid	0.002 (±0.001)	n.d.	n.d.	n.d.
Quinic acid	0.012 (±0.006)	n.d.	n.d.	n.d.
Acetic acid	0.004	0.026 (±0.010)	0.001	0.044 (±0.004)
Formic acid	n.d.	n.d.	0.003 (±0.001)	0.005 (±0.002)
Succinic acid	n.d.	n.d.	0.003	0.004 (±0.001)
Ethanol	n.d.	n.d.	n.d.	0.005 (±0.001)
Endotoxin	0.005 (±0.002)	n.d.	0.018 (±0.003)	0.003 (±0.001)
Carbon dioxide	0.32 (±0.01)	0.37 (±0.02)	0.36 (±0.03)	0.38 (±0.04)
Dry weight	0.32 (±0.03)	0.26 (±0.03)	0.50 (±0.07)	0.34 (±0.03)
Lysed cells[c] (±−0.06)	0.21 (±0.02)	0.31 (±0.02)	0.06 (±0.04)	0.25
Carbon balance	0.89 (±0.10)	1.04 (±0.04)	0.94 (±0.07)	1.03 (±0.08)
TOC[d]	0.11 (±0.03)	0.15 (±0.03)	0.09 (±0.02)	0.04 (±0.06)

[a]Mean values.
[b]The concentration of DHS and DHQ were estimated using shikimic acid as standard.
[c]The amount of lysed cells was calculated from measurements of the protein content of the supernatant and the total protein content of the cells.
[d]Total carbon analysis corrected for known carbon containing compounds present in the broth.

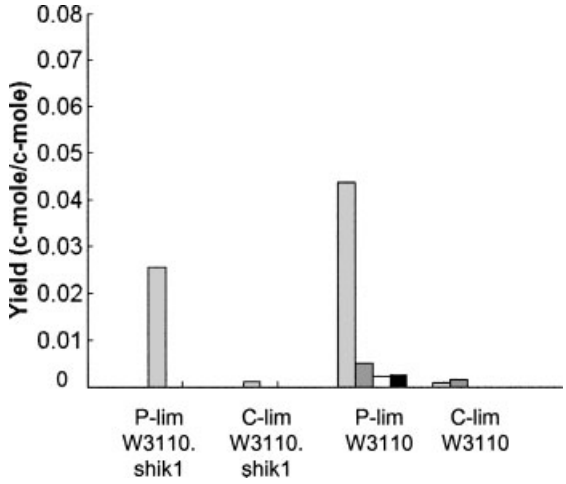

Figure 7. Yields of metabolite production in the central carbon metabolism obtained in chemostat cultivation of the two strains of *E. coli*. Symbols: light gray, acetic acid; dark gray, formic acid; white, succinic acid; black, ethanol.

cell lysis was less pronounced. The balances closed within 90 % without correction for cell lysis. For W3110 the cell lysis was about 5% (data not shown).

The amount of endotoxins produced was also measured at the different conditions. It was found to be generally somewhat higher under phosphate than carbon-limited conditions.

The CO_2 yield was significantly higher under phosphate-limited condition than under carbon-limited condition for both strains. This indicates a higher maintenance requirement in the phosphate-limited case. The CO_2 yield was also higher for W3110 than for W3110.shik1 under carbon limitation.

DISCUSSION

Phosphate-limited conditions clearly favored the production of shikimic acid in the mutant strain used in the present study and the yields of the by-products quinic acid, DHQ, DHS, and gallic acid all decreased. There are relatively few reports on phosphate-limited cultivations of *E. coli*. However, dual phosphate and carbon limitation has been used for production of lysine, which resulted in a higher yield of this amino acid (De Hollander et al., 1998). Although the yield of shikimic acid increased in the present study, it is interesting to note that the sum of the yield of shikimic acid and shikimate by-products was in fact slightly higher in the carbon-limited case. The effect of phosphate limitation may be compared to studies with glucose-rich fed-batch cultivations of shikimic acid producing *E. coli*, which have been reported to give reduction of by-products (Draths et al., 1999; Knop et al., 2001; Yi et al., 2002). In the present study the ratio of formed DHS to shikimic acid was higher than in those studies. This is probably due to the fact that the feedback inhibition of shikimic acid on AroE (Snell et al., 1996) has not been fully compensated for in the strain used in the present study—in contrast to the previous studies mentioned where *aroE* was over-expressed with a strong promoter (Draths et al., 1999; Knop et al., 2001; Yi et al., 2002). In the glucose-limited case, on the other hand, DHS, DHQ, quinic acid, and gallic acid were reported also in the previous studies. The formation of these compounds, except for gallic acid which is probably formed by chemical oxidation, has previously been attributed to the so-called hydroaromatic equilibration (Knop et al., 2001). This theory can be described as follows; at very low glucose concentrations, shikimic acid can be taken up by the cells through the H^+-symporter (MHS) encoded by *shiA*, which is a member of the MFS (major facilitator superfamily) (Pao et al., 1998; Pittard and Wallace, 1966; Whipp et al., 1998). It has been shown by Pittard and Wallace (1966) and by Whipp et al. (1998) that *E. coli* can use shikimate as a precursor in the synthesis of aromatic amino acids in the absence of glucose (Fig. 8a). At high glucose concentrations, on the other hand, the uptake of shikimic acid has been reported to be inhibited (Knop et al., 2001). Consequently, no hydroaromatic equilibration should occur in the presence of glucose.

A perhaps more likely explanation for by-product formation in the present study is that the transport out of the cell of shikimic acid (and possibly that of shikimate by-products) is insufficient under carbon-limited conditions. This would give rise to a build up of the intracellular pools, which in turn gives rise to equilibration of the reversible reactions (Fig. 8b). To explain the lack of by-product formation at phosphate-limited conditions, an enhanced shikimic acid excretion in the presence of excess glucose is needed (Fig. 8c). Some DHS formation is still to be expected because of the feedback inhibition on *aroE* from shikimic acid. This is in good agreement with the results found here.

The maximum specific growth rate of W3110.shik1 was lower than for W3110, 0.33/h (carbon-limited medium) and 0.39/h (phosphate-limited medium) compared to 0.58 and 0.71/h, respectively. This lower specific growth rate is not surprising and may be explained by the deletion of aroL limiting the flow of carbon to essential compounds. It could also be explained by the increased metabolic burden caused by over-expression of genes situated on plasmids (Stephanopoulos et al., 1998). The μ_{max} value of W3110 in the two media are in fair agreement to previously reported values (Chang et al., 1999; Xu, 1999; Xu et al., 1999), considering the differences in media composition.

The acetate yield was, as to be expected, higher during phosphate limitation. The acetate production in the phosphate-limited case is most likely due to the well-known overflow metabolism, occurring at high glycolytic rates caused by high glucose concentrations in the medium (Chang et al., 1999; Delgado and Liao, 1997; Holms, 1986; Neidhardt et al., 1996; Xu, 1999). In W3110, the yields of formic acid, ethanol, and succinic acid also increased during phosphate limitation. The reason for formation of these metabolites is less clear. In the first two experiments, the dissolved oxygen level dropped to a minimum value below

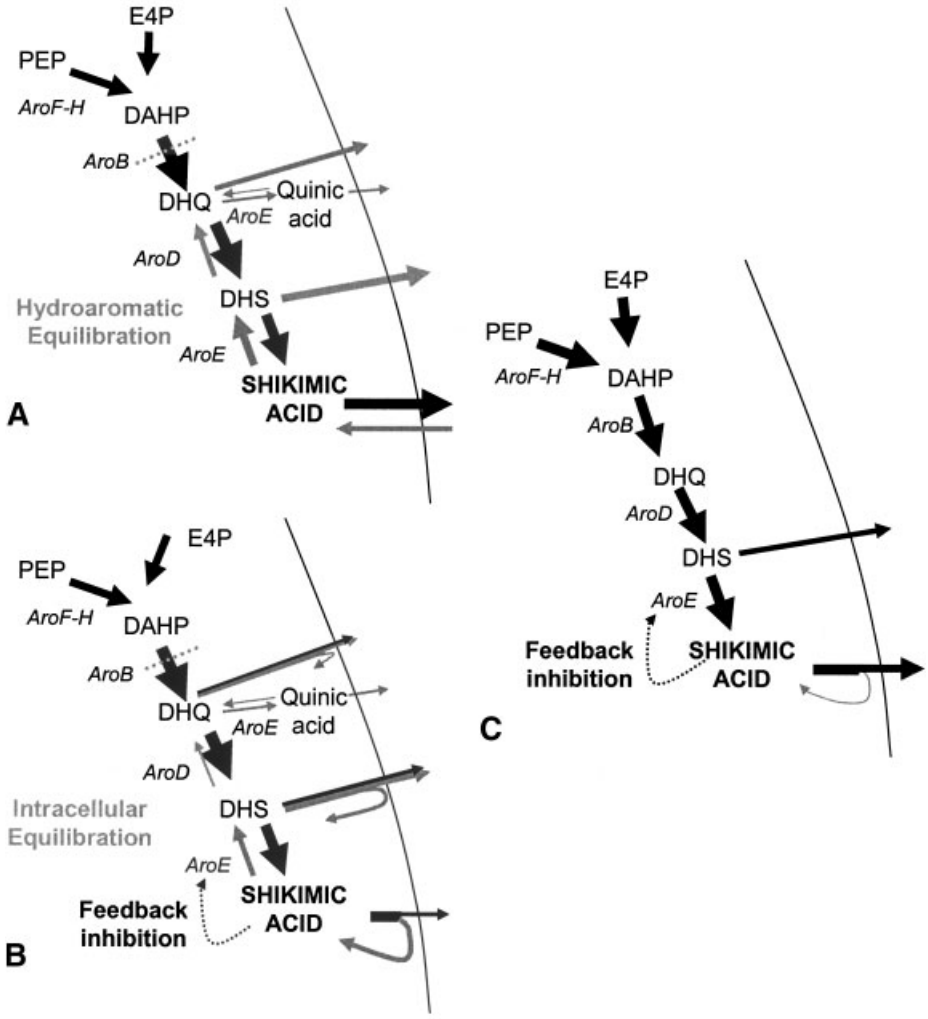

Figure 8. (**A**) Schematic representation of the hydroaromatic equilibrium in carbon-limited chemostat (Knop et al., 2001). The reaction between DAHP and DHQ is irreversible, preventing formation of DAHP from DHQ. (**B**) Schematic representation of the intracellular equilibrium. Here it is assumed that the transportation of shikimic acid and DHS limits the excretion which results in a build up of pools of shikimic acid, DHS and DHQ inside the cell. AroE is in addition feedback inhibited by shikimic acid (Snell et al., 1996), which in turn increases the pool of DHS. That may be an explanation to why DHS is produced in a larger amount compared to shikimic acid. (**C**) Schematic representation of metabolic consequences caused by repression of shikimic acid uptake at glucose rich condition (phosphate-limited condition). Alternatively, excretion of shikimic acid and DHS is increased, which would decrease the size of the intracellular pools. The DHS produced is probably due to the feedback inhibition of AroE by shikimic acid.

10%, which might give induction of mixed acid fermentation. However, in the other experiments it stayed well above 30%. Castan and Enfors (2002) observed in high density fed-batch fermentation a correlation between DNA release and formic acid production. They suggested that the DNA released caused oxygen limitation by increasing the diffusion resistance around the cells, and thereby induced mixed acid fermentation. In the present study, cell lysis, especially in the phosphate-limited case, would cause release of DNA and other cell fragments, however, the amount is probably not high enough to induce mixed acid fermentation as described above and in addition the W3110.shik1 did not form any formic acid although it lysed to a larger extent than did W3110. Therefore the formic acid production is probably due to processes that are yet to be examined.

A surprisingly large fraction of cell lysis was found to occur for W3110.shik1 at all conditions, but also for W3110 in the phosphate-limited cultivations. Cell lysis has previously been reported in high density *E. coli* fed-batch cultures for recombinant protein production (Park et al., 1999). The reported fractions of cell lysis in the current work are higher than previously reported for chemostat cultivations of *E. coli* (Emmerling et al., 2002; Hommes et al., 1991). However, comparable studies, especially for phosphate-limited chemostat conditions are very few, and concern other *E. coli* strains. A large maintenance requirement was reported in phosphate-limited chemostats (at dilution rates 0.15 and 0.3/h (Hommes et al., 1991). This large maintenance, which was indicated by a large CO_2-yield (0.67 and 0.56 c-mol/c-mol for $D = 0.15\ h^{-1}$ and $D = 0.3\ h^{-1}$, respectively), point to a stressed condition. In the current study, as mentioned above, an increased CO_2-yield was indeed observed in the phosphate-limited cultivations compared to the carbon-limited case. However, it was not

as pronounced as in the previous studies. Tentatively, the cell lysis of W3110.shik1 in phosphate limitation could be caused by stress induced by a lack of necessary metabolites downstream of shikimic acid, e.g., tryptophan, phenylalanine, and tyrosine. However, at present the reason for the surprisingly high cell lysis remains unclear.

To conclude, the use of phosphate limitation when cultivating W3110.shik1 resulted in a higher shikimic acid yield and lower yields of by-products from the shikimate pathway than carbon limitation. However increased cell lysis and acetate production were also observed, which may counteract the economical benefits by increasing the costs for downstream processing.

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References

Boehringer Mannheim GmbH B. 1989. Pyrophosphate/phosphate colorimetric method for the determination of inorganic pyrophosphate and inorganic phosphate in foodstuffs. Biochemical analysis, food analysis, instruction for enzymatic kit.

Bongaerts J, Kramer M, Muller U, Raeven L, Wubbolts M. 2001. Metabolic engineering for microbial production of aromatic amino acids and derived compounds. Metab Eng 3(4):289–300.

Castan A, Enfors S-O. 2002. Formate accumulation due to DNA release in aerobic cultivations of *Escherichia coli*. Biotechnol Bioeng 77(3):324–328.

Chandran SS, Yi J, Draths KM, Von Daeniken R, Weber W, Frost JW. 2003. Phosphoenolpyruvate availability and the biosynthesis of shikimic acid. Biotechnol Progr 19(3):808–814.

Chang D-E, Shin S, Rhee J-S, Pan J-G. 1999. Acetate metabolism in a pta mutant of *Escherichia coli* W3110: Importance of maintaining acetyl coenzyme A flux for growth and survival. J Bacteriol 181(21):6656–6663.

De Clercq E. 2002. Strategies in the design of antiviral drugs. Nature Reviews/Drug Discovery 1:13–25.

De Hollander JA, Eswilder FR. Noordover JAC. 1998. (Triple-A B.V. p/a Produkschap voor Veevoedor. Neth.). assignee. Amino acid fermentation processes. Us patent 5763230.

Delgado J, Liao JC. 1997. Inverse flux analysis for reduction of acetate excretion in *Escherichia coli*. Biotechnol Progr 13(4): 361–367.

Draths KM, Knop DR, Frost JW. 1999. Shikimic acid and quinic acid: Replacing isolation from plant sources with recombinant microbial biocatalysis. J Am Chem Soc 121(7):1603–1604.

Ely B, Pittard J. 1979. Aromatic amino acid biosynthesis: Regulation of shikimate kinase in *Escherichia coli* K12. J Bacteriol 138: 933–943.

Emmerling M, Dauner M, Ponti A, Fiaux J, Hochuli M, Szyperski T, Wuthrich K, Bailey JE, Sauer U. 2002. Metabolic flux responses to pyruvate kinase knockout in *Escherichia coli*. J Bacteriol 184(1):152–164.

Gawehn K. 1974. Methods of enzymatic analysis In: Bergmeyer HU, editor. Methods of enzymatic analysis. New York and London: Verlag Chemie, Weinheim/Academic Press, Inc. pp 2239–2242.

Ghisalba O. 1985. Biosynthesis of rifamycins (ansamycins) and microbial production of shikimate pathway precursors intermediates. and metabolites. Chimia 39(4):79–88.

Gibson JM, Thomas PS, Thomas JD, Barker JL, Chandran SS, Harrup MK, Draths KM, Frost JW. 2001. Benzene-free synthesis of phenol. Angew Chem Int Ed 40(10):1945–1948.

Haslam E. 1993. Shikimic acid: Metabolism and metabolites. New York: Wiley & sons.

Holms WH. 1986. The central metabolic pathways of *Escherichia coli*: Relationship between flux and control at a branch point, efficiency of conversion to biomass and excretion of acetate. Curr Top Cell Regul 28:69–105.

Hommes RWJ, Simons JA, Snoep JL, Postma PW, Tempest DW, Neijssel OM. 1991. Quantitative aspects of glucose metabolism by *Escherichia coli* B/r, grown in the presence of pyrroloquinoline quinone. Antonie van Leeuwenhoek 60(3–4):373–382.

Jiang S, Singh G, Boam DJ, Coggins JR. 1999. Synthesis of 3-deoxy-3.3-diflouroshikimic acid and its 4-epimer from quinic acid. Tetrahedron: Assymetry 10:4087–4090.

Kambourakis S, Draths KM, Frost JW. 2000. Synthesis of gallic acid and pyrogallol from glucose: Replacing natural product isolation with microbial catalysis. J Am Chem Soc 122(37):9042–9043.

Kim CU, Lew W, Williams MA, Zhang L, Liu H, Swaminathan S, Bischofberger N, Chen MS, Tai CY, Mendel DB, Laver WG, Stevens RC. 1997. Influenza neuraminidase inhibitors possessing a novel hydrophobic interaction in the enzyme active site: Design, synthesis, and structural analysis of carbocyclic sialic acid analogs with potent anti-influenza activity. J Am Chem Soc 119(4):681–690.

Knop DR, Draths KM, Chandran SS, Barker JL, von Daeniken R, Weber W, Frost JW. 2001. Hydroaromatic equilibration during biosynthesis of shikimic acid. J Am Chem Soc 123(42):10173–10182.

Kraak J. 1974. Enzymatischer nachweis von anorganischem diphosphatzusätzen. Fleischwaren. Fleischwirtschaft. pp 1661–1664.

Kriz D, Berggren C, Johansson A, Ansell RJ. 1998. SIRE technology. Part I. Amperometric biosensor based on flow injection of the recognition element and differential measurements. Instrum Sci Technol 26(1):45–57.

Langner HJ. 1964. Die quantitative Bestimmung von phosphat mit hilfe von anorganischer pyrophosphatase. Fleischwirtschaft. pp 784–786.

Mirza S, Harvey J. 1991. Synthesis of shikimic acid and its phosphonate analog via Knoevenagel condensation. Tetrahedron Lett 32(33):4111–4114.

Neidhardt FC, Curtiss R, Ingraham John L, Lin ECC, Low KB, Magasanik B, Reznikoff SW, Riley M, Schaechter M, Umbarger HE. 1996. *Escherichia coli* and *Salmonella* cellular and molecular biology, 2nd edn. Washington DC: ASM Press.

Nielsen KF, Smedsgaard J. 2003. Fungal metabolite screening: Database of 474 mycotoxins and fungalmetabolites for de-replication by standardised liquid chromatography-UV detection-mass spectrometry methodology. J Chromatogr A 1002:111–136.

Pao SS, Paulsen IT, Saier MH, Jr. 1998. Major facilitator superfamily. Microbiol Mol Biol R 62(1):1–34.

Park SJ, Georgiou G, Lee SY. 1999. Secretory production of recombinant protein by high cell density culture if a protease negative mutant of *Escherichia coli* strain. Biotechnol Prog 15:164–167.

Pittard J, Wallace BJ. 1966. Gene controlling the uptake of shikimic acid by *Escherichia coli*. J Bacteriol 92(4):1070–1075.

Raczyk-Stanislawiak U, Swietlik J, Kasprzyk B, Nawrocki J. 2003. The efficiency of different oxidation methods in total organic carbon analysis. Chem Anal (Warsaw) 48(2):243–254.

Schreiber SL, Shair MD, Tan DS, Foley MA, Stockwell BR. 2002. (President and Fellows of Harvard College, USA). assignee. Synthesis of combinatorial libraries of compounds reminiscent of natural products. Us patent 6448443.

Schultze U. 1995. Anaerobic physiology of *Saccharomyces cerevisiae*. Copenhagen: Technical University of Denmark.

Snell KD, Draths KM, Frost JW. 1996. Synthetic modification of the *Escherichia coli* chromosome: Enhancing the biocatalytic conversion of glucose into aromatic chemicals. J Am Chem Soc 118(24):5605–5614.

Song C, Jiang S, Singh G. 2001. Syntheses of (6S)-6-fluoro- and (6R)-6-hydoxyshikimic acids. Tetrahedron: Assymetry 42:9069–9071.

Stephanopoulos GN, Aristidou AA, Nielsen J. 1998. Metabolic engineering principles and methodologies. San Diego: Academic Press.

Takeuchi M, Taniguchi T, Ogasawara K. 2000. Ring-closing metathesis protocol for a diastereocontrolled synthesis of (−)-shikimic acid. Synthesis 10:1375–1379.

Whipp MJ, Camakaris H, Pittard AJ. 1998. Cloning and analysis of the shiA gene. which encodes the shikimate transport system of *Escherichia coli* K-12. Gene 209(1/2):185–192.

Xu B. 1999. Modeling of overflow metabolism in batch and fed-batch cultures of *Escherichia coli*. Biotechnol Progr 15:81–90.

Xu B, Jahic M, Blomsten G, Enfors SO. 1999. Glucose overflow metabolism and mixed-acid fermentation in aerobic large-scale fed-batch processes with *Escherichia coli*. Appl Microbiol and Biotechnol 51(5):564–571.

Yi J, Li K, Draths KM, Frost JW. 2002. Modulation of phosphoenolpyruvate synthase expression increases shikimate pathway product yields in *E. coli*. Biotechnol Progr 18(6):1141–1148.