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Biotin production under limiting growth conditions by *Agrobacterium/Rhizobium* HK4 transformed with a modified *Escherichia coli bio* operon

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The *E. coli* biotin (*bio*) operon was modified to improve biotin production by host cells: (a) the divergently transcribed wild-type *bio* operon was re-organized into one transcriptional unit; (b) the wild-type *bio* promoter was replaced with a strong artificial (*tac*) promoter; (c) a potential stem loop structure between *bioD* and *bioA* was removed; and (d) the wild-type *bioB* ribosomal binding site (RBS) was replaced with an artificial RBS that resulted in improved *bioB* expression. The effects of the modifications on the *bio* operon were studied in *E. coli* by measuring biotin and dethiobiotin production, and *bio* gene expression with mini-cells and two-dimensional polyacrylamide gel electrophoresis. The modified *E. coli bio* operon was introduced into a broad host-range plasmid and used to transform *Agrobacterium/Rhizobium* HK4, which then produced 110 mg L^{-1} of biotin in a 2-L fermenter, growing on a defined medium with diaminononanoic acid as the starting material. Biotin production was not growth-phase dependent in this strain, and the rate of production remained high under limiting (maintenance) and zero growth conditions.

Keywords: biotin production; *E. coli bio* operon; *Agrobacterium/Rhizobium* HK4; limiting growth conditions

Introduction

Biotin is a commercially important vitamin, with pharmaceutical, feed and cosmetic applications and a market valued at more than $100 million per year. It is currently synthesized on an industrial scale by multi-step chemical processes [17,26]. However, biotechnological production could be advantageous in terms of cost, simplicity and environmental considerations. Biotin is synthesized by microorganisms and plants, and functions biologically as an essential cofactor for carboxylase-catalyzed reactions [21]. The genes and pathway for biotin biosynthesis have been studied in detail in bacteria, especially in *Bacillus sphaericus* [28] and *E. coli* [23], and synthesis is tightly regulated. In *E. coli*, the *bioH* and *bioC* genes code for enzymes involved in the synthesis of pimeloyl CoA, and the *bioF*, *bioA*, *bioD* and *bioB* genes code for the remaining enzymes in the pathway (Figure 1). The *bioA*, *bioB*, *bioF*, *bioC* and *bioD* genes are grouped together in an operon, which is divergently transcribed towards *bioA* and an ORF of unknown function in one direction, and towards *bioB*, *bioF*, *bioC* and *bioD* in the other direction. One gene *bioH*, is located elsewhere on the chromosome [29].

Wild-type cells of *E. coli* excrete only about 10–15 ng L^{-1} (0.04–0.06 nM) of biotin into the growth medium, whereas de-repressed strains excrete about 5–15 μg L^{-1} (20–60 nM) [6,30]. Two approaches have been used to produce a microorganism that makes increased amounts of biotin. The first involves cloning and over-expressing the genes of biotin biosynthesis in wild-type or de-repressed host cells and the second involves selecting microorganisms with biotin anti-metabolites. For example, the *E. coli bio* genes have been over-expressed in *E. coli* under the control of both of the wild-type *bio* promoters and under the control of strong artificial promoters [23], and the *Bacillus sphaericus bio* genes have been over-expressed in *E. coli* [15]. A combination of the two approaches has also been used, where the biotin synthetic genes from *Serratia marcescens* have been over-expressed in host cells of the same bacterium, which had been selected with anti-metabolites for increased biotin production [24]. In addition to strain design, many other factors, such as medium components and fermentation conditions, play a role in biotin production [33].

It is generally accepted that a commercially viable fermentative process for biotin will require cells able to produce more than 1 g L^{-1} day^{-1}. Some groups have been able to obtain biotin concentrations in the 100 × mg range by fermentation, but this requires several days, and the use of growth media that are not always compatible with downstream processing. In addition, it is usually observed that biotin is produced only by actively growing cells, which limits the possibility for maximizing their specific productivity.

Agrobacterium/Rhizobium HK4 is characterised by its very high metabolic activity at very low growth rates, and a derivative of this strain is used for the biotechnological

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[*]Hans Kulla died in a mountaineering accident in 1993. He contributed much to this project, and we dedicate this paper to his memory
Abbreviations: CAT, chloramphenicol acetyl transferase; DAPA, 7,8-diaminopelargonic acid (diaminononanoic acid); KAPA, 7-keto-8-aminopelargonic acid; PLP, pyridoxal phosphate; SAM, S-adenosylmethionine; 2-D PAGE, two-dimensional polyacrylamide gel electrophoresis
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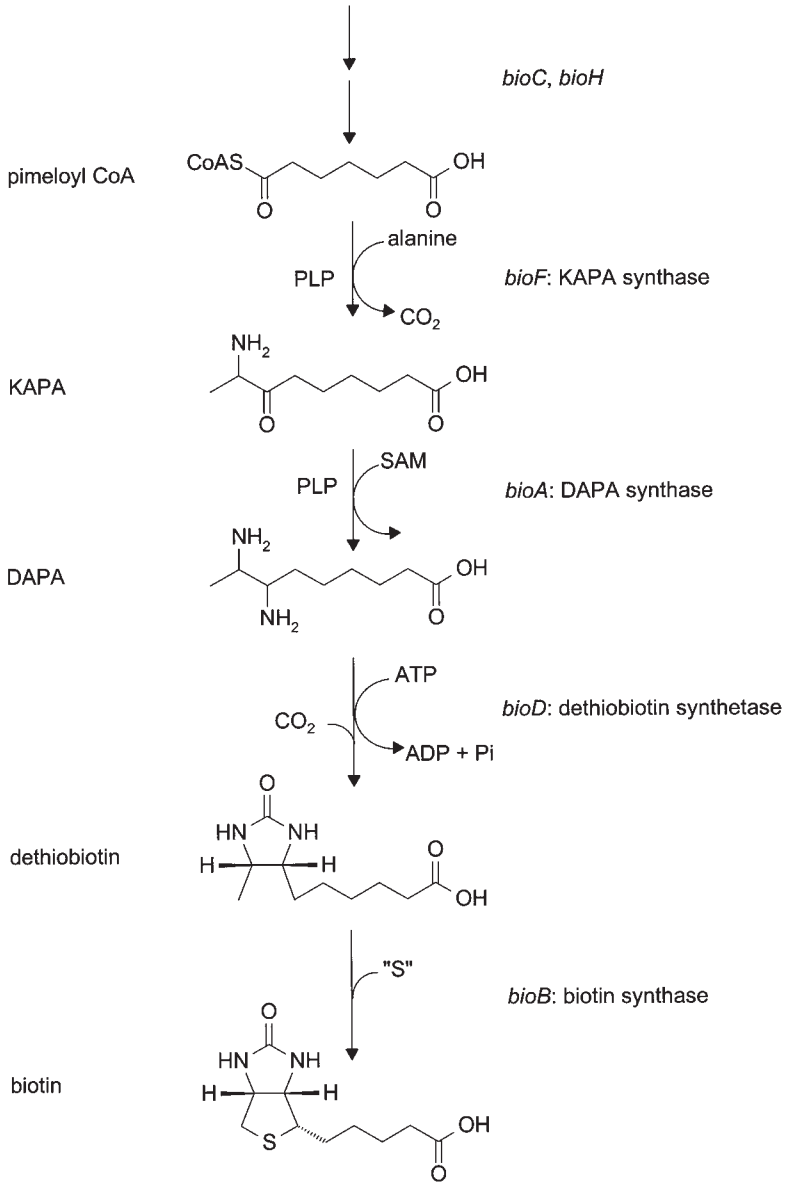

Figure 1 The biotin biosynthetic pathway in *E. coli*.

production of multi-ton amounts of l-carnitine, starting from exogenously supplied butyrobetaine [46]. Our approach to solving the problem of strains that only produce biotin during phases of growth and high metabolic activity was to modify the *E. coli bio* operon to improve biotin production, introduce it into a broad host-range plasmid and use that to transform *Agrobacterium*/*Rhizobium* HK4. The rate of biotin production for *Agrobacterium*/*Rhizobium* HK4 transformed with the plasmid carrying the modified *bio* operon remained high on a defined medium under maintenance and zero growth conditions. *Agrobacterium/Rhizobium* HK4 is therefore a promising candidate as a host organism for biotin production.

Methods

Bacterial strains and growth

The *E. coli* strains and the vectors and plasmids used in this study are listed in Tables 1 and 2. Details of bacterial growth and fermentation were described previously [2,3]. OD measurements were made with a Perkin-Elmer (Norwalk, CT, USA) Lambda 1 spectrophotometer, with cells with a 1-cm light path. Cultures were diluted with water where necessary.

Agrobacterium/Rhizobium HK4 is a strain that was selected for growth on 4-butyrobetaine, crotonobetaine and l-carnitine as sole carbon, nitrogen and energy source under aerobic conditions. The strain has been phenotypically

Table 1 *E. coli* strains

E. coli strain	Genotype	Origin/reference
DSM498	K12 'Wild type'	DSM Braunschweig
SA291	F⁻Δ (*gal-bisA*) *his strR*	[10]
BM2661	F⁻*araD*139 Δ*lacU*169 rpsL thi⁻ *ϕ*(*bioFC-lac*)501	[1]
BM4062	F⁻*araD*139 Δ*lacU*169 rpsL thi⁻ *ϕ*(*bioFC-lac*)501 *birA*85	[1]
JM109	*recA*1 *endA*1 *gyrA*96 *thi hsdR*17 *supE*44 *relA*1 λ⁻ Δ(*lac-pro*AB [F′ *traD*36 *proAB lacI*^{q}ZΔM15]	[45]
RR28	*hsdR hsdM recA pheS*12 *thi leu pro lac gal ara mtl xyl supE*44 *endA*	[19]
XL1-Blue	*recA*1 *endA*1 *gyrA*96 *thi hsdR*17 (r⁻m⁻) *supE*44 *relA*1 λ⁻ *lac*⁻ [F′ *proAB lacI*^{q}ZΔM15 TN10 (tc^R)]	[5]
DS410	*thi min ara lacY xyl malA mtl tonA rpsL* azi^{R-} λ⁻	[13]

Table 2 Plasmids

Plasmid/vector	Genotype	Origin/reference
pBR322	ap^R.tc^R	[4]
pACYC184	cm^R.tc^R	[9]
pHE3	cm^{R}pheS	[19]
M13mp18/19	*lacZ′*	[45]
pUC18/19	ap^R*lacZ′*	[45]
pBluescript SK⁺/KS⁺	ap^R *lacZ′*	Stratagene, La Jolla, CA
pMC1871	tc^R *lacZ′YA*	[8]
pME81	ap^R *oriV lacI*^{q} *ptac-lacZ*	[40]

characterised, but has not, despite extensive studies, been assigned to one genus. It is taxonomically related to the common soil microorganisms *Agrobacterium* and *Rhizobium*, and 16s rRNA characterisation showed that it is closely related to *Rhizobium meliloti*. The strain is assumed to belong to a new, as yet undefined, genus [46].

Chemicals

Chemicals were obtained from Fluka (Buchs, Switzerland). Dethiobiotin and materials for the assay of biotin synthase were as previously described [3]. Diaminononanoic acid (DAPA, Figure 1) was synthesised by Dr L Duc, Lonza AG.

Cloning

Standard methods for the manipulation of DNA [36] were used. Extra-chromosomal DNA from *E. coli* was isolated using Qiagen packs from Qiagen, Dusseldorf, Germany. Total DNA from *E. coli* was isolated using the procedure described previously [18]. For the transformation of bacterial cells with plasmid DNA, cells were made competent for plasmid uptake [11]. For DNA sequence analysis the chain termination method was used [37]. DNA linkers for the construction of new restriction sites were from Boehringer Mannheim, Mannheim, Germany. Specific oligonucleotides for DNA/DNA hybridisation, for primers for DNA sequencing and for cloning were synthesised by Microsynth, Windisch, Switzerland.

Details concerning the steps involved in the construction of pBO3, pBO30, pBO30-A15, pBO30-A15/9, pBO30-A15/9ΔORF1 and pBO47 have been described previously [2].

Derivatives of pBO30 with deletions in *bioA*, *bioC* and *bioD* were also constructed. To form pBO30ΔA, a 1.2-kb fragment was removed from *bioA* with endonucleases *Sal*I and *Bam*HI. For pBO30ΔC, a 150-bp *Bgl*II fragment was removed from *bioC* and pBO30ΔD was constructed in an analogous way to pBO30ΔA, using *Sal*I and *Sno*I.

Specific expression of genes from plasmids in E. coli mini-cells

Mini-cells from derivatives of *E. coli* DS410 containing various plasmids were isolated, newly synthesised proteins labelled with L-[^{35}S]methionine (>1000 Ci mmol^{-1}, NEN Life Science Products, Boston, MA, USA) and analysed by SDS-PAGE followed by autoradiography [14].

Two-dimensional electrophoresis of proteins

Two-dimensional polyacrylamide gel electrophoresis (2-D PAGE) was carried out as described previously [32].

Measurement of enzyme activities

E. coli strains with *bio::lacZ* plasmids were grown in liquid culture to an OD_{600} of 0.5 and β-galactosidase activity measured [27].

Measurement of dethiobiotin and biotin production

Biotin was measured using the *Lactobacillus plantarum* agar plate assay [12]. Because this method can, under certain conditions, give erroneous results, TLC analysis was also carried out. Medium samples (500 μl) were diluted to 5 ml with 10 mM ammonium formate buffer pH 5.5 then loaded on disposable C_{18} solid-phase extraction columns (Macherey-Nagel, Oensingen, Switzerland; Chromabond C_{18} ec, 100 mg) that had been equilibrated with methanol (1 ml), water (1 ml) and the ammonium formate buffer (1 ml). Dethiobiotin and biotin were eluted with water and the sample acidified by the addition of 12.5% w/v TCA. Dethiobiotin and biotin were then purified further on C_{18} solid-phase extraction columns that had been equilibrated with methanol (1 ml), water (1 ml) and 1% v/v acetic acid (1 ml). After washing with 1% v/v acetic acid (1 ml) and water (1 ml), dethiobiotin and biotin were eluted with 0.5 ml of methanol. The samples were dried under vacuum and resuspended in 20 μl methanol: water: acetic acid (65:25:10). Dethiobiotin and biotin were detected on TLC with *p*-dimethylaminocinnamaldehyde [25] and quantified by comparison with standards. Recovery of biotin and dethiobiotin was estimated by means of radioactive tracers.

Fermentation of Escherichia coli XL1 Blue pBO30A-15/9 and Agrobacterium/Rhizobium HK4 pB047

Details were described previously [2].

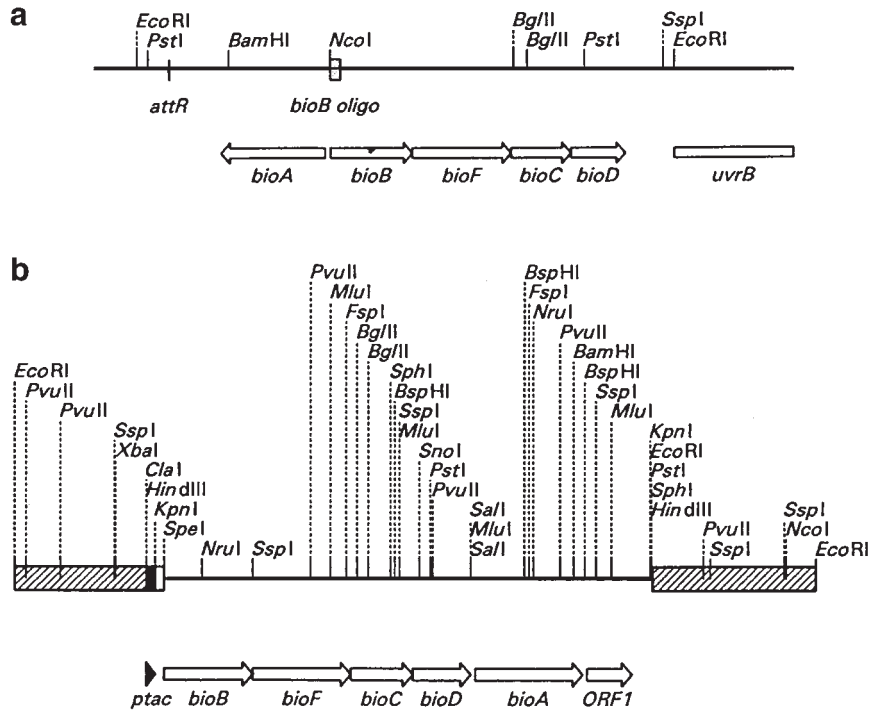

Figure 2 Restriction maps for the *bio* operon and pBO30A-15/9. (a) The *bio* operon. Known restriction sites [42] are shown. (b) pBO30A-15/9. The hatched boxes indicate vector DNA, the filled box the *tac* promoter and the open box the improved RBS in pBO30A-15/9.

Results

Improved biotin production by modification of the E. coli bio operon

Cloning of the wild-type *E. coli bio* operon (Figure 2a) and expression from a multi-copy plasmid alone (pBO3) in *E. coli* resulted in a 2000-fold improvement in biotin production compared with a wild-type (non-derepressed) *E. coli* strain (Table 3). The *bio* operon was then modified to improve biotin production. The two *bio* transcriptional units were fused to form one (*bioB::F::C::D::A::ORF1*), and the wild-type promoter was replaced by the *tac* promoter to give pBO30, in which biotin production through the biotin biosynthetic pathway (*de novo* synthesis), or from dethiobiotin supplemented to the growth medium, increased by about 17- and 30-fold respectively compared with pBO3 (Table 3).

A potential stem loop structure downstream of *bioD* in pBO30 was then removed, resulting in pBO30A-15, in which two amino acids were deleted from the 3′ end of the *bioD* gene and 13 were added, but this had no adverse effect on the synthesis of dethiobiotin (Table 3).

The last step in biotin biosynthesis is catalysed by the enzyme biotin synthase, which has a very low turnover number *in vitro* [38,39]. There was also only a small increase in biotin production from cells containing pBO30 when the growth medium was supplemented with dethiobiotin, and a build-up in dethiobiotin levels from cells containing the plasmids for biotin production listed in Table 3, indicating that biotin synthase plays an important flux control role [43], and could therefore be a limiting factor in an industrial process for biotin production. In order to both improve biotin production and to examine the effect of biotin synthase expression on biotin production, the *lacZ*

Table 3 Production of dethiobiotin and biotin by cells of *E. coli* JM109 with various *bio* operon plasmids in shake flasks

Plasmid	Dethiobiotin (mg L^{-1}) (Cells grown on NYB medium)	Biotin (mg L^{-1}) (Cells grown on NYB medium)	Biotin (mg L^{-1}) (Cells grown on NYB medium plus 100 μg ml^{-1} dethiobiotin)
pBO3	0.5	0.03	0.02
pBO30	1.8	0.50 ± 0.23 (0.74)	0.60 ± 0.10
pBO30A-15	2.76	0.48 ± 0.10 (0.74)	0.62 ± 0.05
pBO30A-15/9	2.63	1.39 ± 0.48 (1.84)	1.61 ± 0.52
pBO30A-15/9ΔORF1	2.1	1.0 (1.84)	1.1
pBO30ΔC		bg	0.46
pBO30ΔA		0.04	0.34
pBO30ΔD		0.02	0.42

The production of dethiobiotin and biotin by cells grown on NYB medium was determined to measure synthesis *de novo* through the biotin biosynthetic pathway (columns 2 and 3), and the production of biotin by cells grown on NYB medium supplemented with 100 μg ml^{-1} dethiobiotin was determined (column 4) to measure biotin synthesis from dethiobiotin. The average value of two determinations (which were always nearly identical) is shown, except where a standard deviation value is shown, in which case the number of determinations was five. Dethiobiotin was measured by TLC, and biotin by the microbiological method and TLC (values in brackets). 'bg' indicates that the values were not significantly above background levels, which were about 0.02 mg L^{-1}. All other biotin values determined by the microbiological method have background levels subtracted. For comparison, wild-type strains of *E. coli* produce about 10–15 ng L^{-1} biotin, and de-repressed strains about 5–15 μg L^{-1} [6,30]. For all plasmids, the host cells grew to a final OD_{650} value of between 3 and 4.

Table 4 *bio* gene expression measured by experiments with mini-cells

Plasmid	Gene expression relative to CAT					
	BioC	BioF	BioA	BioD	BioB	ORF1
pBO30	0.3	0.19	0.08	1.56	0.29	0.29
pBO30A-15	0.25	0.27	0.28	1.36	0.23	0.33
pBO30A-15/9	nd	0.20	0.25	1.31	0.61	0.32

Mini-cell experiments were carried out as described in the Methods section.
The bands on the resulting autoradiographs were quantified by scanning densitometry, and the results normalised with respect to the intensity of the CAT band and the number of methionine residues in each protein. The results are expressed relative to the expression of CAT, which would have a value of 1.

ribosomal binding site (RBS) upstream from *bioB* in pBO30 was replaced by an RBS that resulted in improved expression of biotin synthase, as measured by mini-cell experiments (Table 4). Mixed oligonucleotides that conformed to the empirical rules for good RBS function (a Shine–Dalgarno sequence complementary to the 3′ end of the *E. coli* 16S rRNA situated nine base pairs from the *bioB* start codon, as many A and T bases as possible and at least one stop codon [16,41]) were evaluated in a *bioB-lacZ* screening system [8], translation being measured by determining the β-galactosidase activity of the fusion protein with a chromogenic substrate. The results showed that one RBS sequence (Figure 3) gave a 5.9-fold increase in biotin synthase expression in this test system. This sequence was introduced into pBO30A-15 to form pBO30A-15/9 (Figure 2b). Biotin production from pBO30A-15/9 was about three times higher than from the plasmids with the *lacZ* ribosomal binding site (pBO30 and pBO30A-15, Table 3). Biotin production from all the plasmids tested in *E. coli* in shake flasks was growth phase dependent: the highest rate was always observed during the late exponential phase and early stationary phase. The rate in later stationary phase declined.

The effect of bio gene deletions on biotin production in E. coli cells transformed with the bio plasmids

Derivatives of pBO30 (pBO30AΔA, pBO30ΔC and pBO30ΔD) and pBO30A-15/9 (pBO30A-15/9ΔORF1) were made with deletions in the individual *bio* genes to investigate the influence of over-expression of each of these genes on biotin production (Table 3). *bioC*, *bioA* and *bioD* were all essential for the increased production of biotin from cells containing pBO30. Deletion of *ORF1* in pBO30A-15/9ΔORF1 did not affect biotin production, indicating that the product of the putative *ORF1* gene is not involved in biotin synthesis.

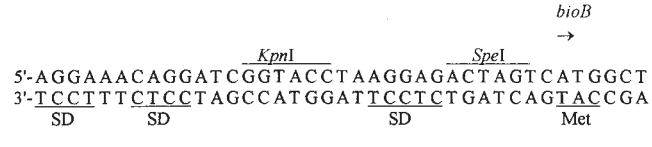

Figure 3 The DNA sequence of the improved RBS for *bioB*.

Dethiobiotin production in E. coli cells transformed with the bio plasmids

Dethiobiotin production was greater than biotin production from cells containing the plasmids pBO3, pBO30, pBO30A-15, pBO30A-15/9, and pBO30A-15/9ΔORF1 (Table 3), confirming biotin synthase as an important flux control point. Cells containing the plasmids with improved *bioA* expression, namely pBO30A-15, pBO30A-15/9 and pBO30A-15/9ΔORF1 produced more dethiobiotin than those containing pBO30. This modification may therefore be important for future biotin production, if the increased dethiobiotin can be converted to biotin.

Biotin gene expression in E. coli cells transformed with the bio plasmids studied by mini-cell experiments and 2-D PAGE

Expression of the *bio* genes was measured by mini-cells (Figure 4a and Table 4) and 2-D PAGE (Figure 4b and c). The results of the mini-cell experiments were used to monitor the cloning work and to quantify gene expression. Expression of the *bioA* gene was increased four-fold in pBO30A-15 compared with pBO30 (Figure 4a, Table 4). However, there was no increase in biotin production, although dethiobiotin production did improve (Table 3). Also clearly visible in Figure 4a are the deletion of *bioA* (pBO30ΔA), the expression of *bioA* (pBO30) and *bioF* (pBO30, pBO30A-15 and pBO30A-15/9) as double bands with different molecular weights, and the increase in molecular weight of BioD from pBO30A-15 and pBO30A-15/9, due to the lengthening of the 3′ end of *bioD* by 11 codons (see above). Measurement of protein expression by mini-cell and 2-D PAGE analysis showed that *bioB* expression was about 2.5- to 3-fold higher in pBO30A-15/9 than in pBO30A-15. The 5.9-fold increase in expression of the *bioB-lacZ* test system was therefore not reached, probably due to differences between the two plasmids. The increase of biotin production from pBO30A-15 to pBO30A-15/9 was also about 2.5- to 3-fold, correlating well with the increase in *bioB* expression.

2-D PAGE is a powerful technique that is being used extensively in proteome research [44]. The biotin pathway enzymes from wild-type *E. coli* are difficult to detect and identify by 2-D PAGE. To learn more about the expression and nature of the *bio* enzymes in our system with the cloned pathway, experiments using 2-D PAGE were carried out with both silver staining of the gels and radioactive labelling of the cell proteins with [^{35}S]methionine. Spots corresponding to the cloned gene products were identified from their predicted molecular weights, and by comparison of a gel from an extract of *E. coli* JM109 pBO30A-15/9 with a gel of a control extract from the host strain, *E. coli* JM109, without the plasmid carrying the *bio* genes (Figure 4b and c). Spots were identified for all the cloned gene products, including BioC and ORF1. The molecular weights of the proteins agreed with those predicted from their DNA sequences [29]. Multiple spots were observed for BioB (three spots), BioC (two spots) and BioD (two spots). The pI values of the proteins also agreed with those predicted from their sequences, with the exception of BioC (7.45 and 7.75 instead of 8.8–10.0) and BioD (5.43 and 5.66 instead of 8.8–10.1).

Fermentation of Escherichia coli XL1 Blue pBO30A-15/9 and Agrobacterium/Rhizobium HK4 pBO47

Scale-up of shake flask experiments to fermenters usually results in increased productivity. This was also the case for biotin production from cells containing our plasmid constructs. An *E. coli* strain transformed with pBO30A-15/9 reached 35 mg L^{-1} biotin in one day in a 2-L fed-batch fermenter with an end OD_{650} of 25. Biotin production ceased during the stationary phase. The specific production rate reached 0.28 mg L^{-1} h^{-1} $(OD_{650} = 1)^{-1}$ in the exponential phase (Figure 5a).

Agrobacterium/Rhizobium HK4 is a biotin auxotroph. When transformed with a plasmid analogous to pBO30-A15/9 (pBO47), it produced 50 mg L^{-1} biotin in 7 days and 110 mg L^{-1} after 20 days (Figure 5b). Cells were grown to an OD_{650} of 70 in a fed-batch process at 30°C on an l-glutamine/betaine minimal medium. The plasmid was stabilised with tetracycline (2 mg ml^{-1}) and 1 mg ml^{-1} was fed each 2–3 days. No plasmid loss was observed during the whole fermentation. The cell-count remained constant at 3×10^{10} colony forming units ml^{-1}, which is equivalent to an OD_{650} of 40. Glucose was limiting during the whole fermentation. After the exponential growth phase (OD_{650} of 12), a glucose-betaine feed (360 g L^{-1} glucose plus 103 g L^{-1} betaine in water) was introduced slowly (1.5 ml h^{-1}) to maintain long-term limited (maintenance) growth. The culture grew with a doubling rate of 5.6 h in the exponential phase, and 300 h in the maintenance phase. The specific biotin production rate reached 0.042 mg L^{-1} h^{-1} $(OD_{650} = 1)^{-1}$ in the maintenance phase. Between days 13 and 20, biotin production continued under zero growth conditions with a specific rate of 0.0054 mg L^{-1} h^{-1} $(OD_{650} = 1)^{-1}$. DAPA was supplied to the cells in three feeds (at the start of fermentation, after 200 h and after 415 h), since they are unable to synthesise precursors such as pimeloyl CoA for biotin synthesis. The cells converted the DAPA to dethiobiotin (Figure 5b) and then to biotin. The dethiobiotin formed after each DAPA feed was completely metabolized, which would facilitate down-stream processing in a commercial process (it is not easy to separate biotin from dethiobiotin on a large scale). The third DAPA feed was 100 mg L^{-1}, and the resulting dethiobiotin peak was proportionally smaller. The analytical yields of biotin, calculated from the amounts of DAPA fed were 38%, 25% and 39% respectively for the three feeds. We have determined (unpublished results) that *Agrobacterium/Rhizobium* HK4 co-metabolises dethiobiotin, and this could account for these losses. A future aim will be to control the substrate feed so as to avoid dethiobiotin accumulation.

These results confirm that the enzymes of biotin synthesis were expressed from pBO47 and were functional in *Agrobacterium/Rhizobium* HK4. Our studies on biotin synthase in *E. coli* have shown that it requires a number of accessory proteins to function *in vitro* [3]. The *E. coli* enzyme functions in the *Agrobacterium/Rhizobium* strain, indicating that the enzyme is able to interact successfully with the host accessory proteins *in vivo*.

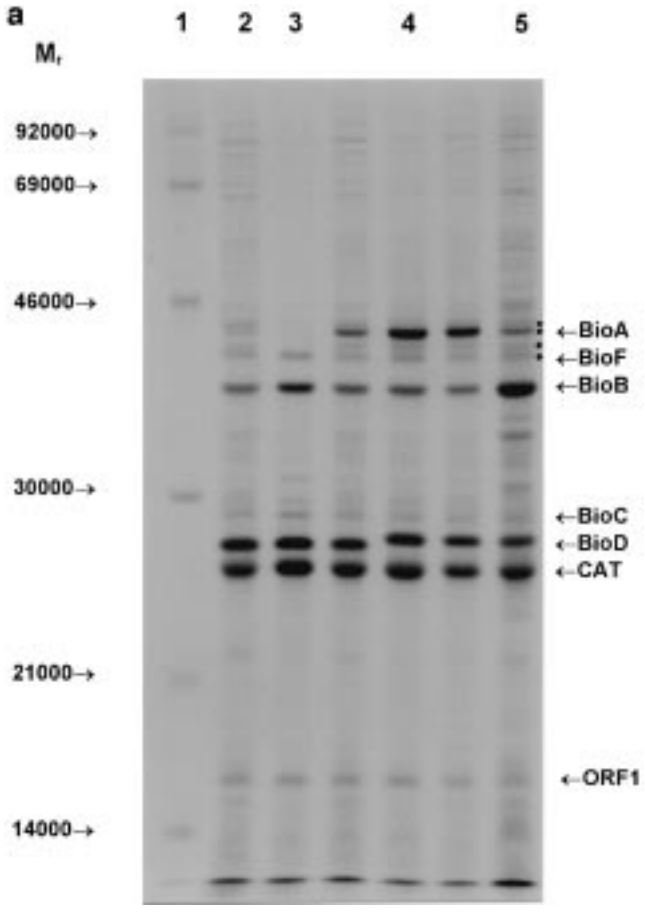

Figure 4 Mini-cell and 2-D PAGE measurement of *bio* gene expression. (a) Mini-cell expression. Lane 1, molecular weight markers; lane 2, pBO30; lane 3, pBO30ΔA; lane 4, pBO30A-15; lane 5, pBO30A-15/9. (b) Silver-stained 2-D PAGE of *E. coli* JM109 pBO30A-15/9. Cells were grown aerobically in NYB plus chloramphenicol (20 μg ml^{-1}) and IPTG (0.5 mM) to $A_{600} = 2$. The first dimension is isoelectric focusing at 15°C to equilibrium (105 000 Vh) using nonlinear immobilised pH gradient strips with a gradient ranging from pH 3.5 to 10.0. The second dimension is a 9–16% T gradient SDS-gel. The *bio* proteins are indicated by B (BioB); F (BioF); C (BioC); D (BioD); A (BioA) and the *ORF1* protein by ORF1. The identities of the other labelled proteins were described previously [32]. (c) Silver-stained 2-D PAGE of *E. coli* JM109 (control strain).

Discussion

An earlier study of homologous *bio* gene expression in *Escherichia coli* [23] showed that strains carrying the *bio* operon under the control of the natural promoters on a high copy number plasmid produced more biotin (1 mg L^{-1}, cells with an OD_{650} of 5) than a wild-type strain (100 ng L^{-1}). The introduction of stronger promoters and improved ribosomal binding sites resulted in increased activities of the biotin biosynthetic enzymes, but these did not correlate with the far greater increase in the amount of the biotin enzymes themselves. More importantly from a commercial standpoint, there was no increase in biotin production. Over-expression of *bioC* under the control of a strong promoter led to poor cell growth. For all the plasmids tested, dethiobiotin production was less than that of biotin. The authors concluded that the biotin synthase step is not an important control point in the biotin biosynthetic pathway.

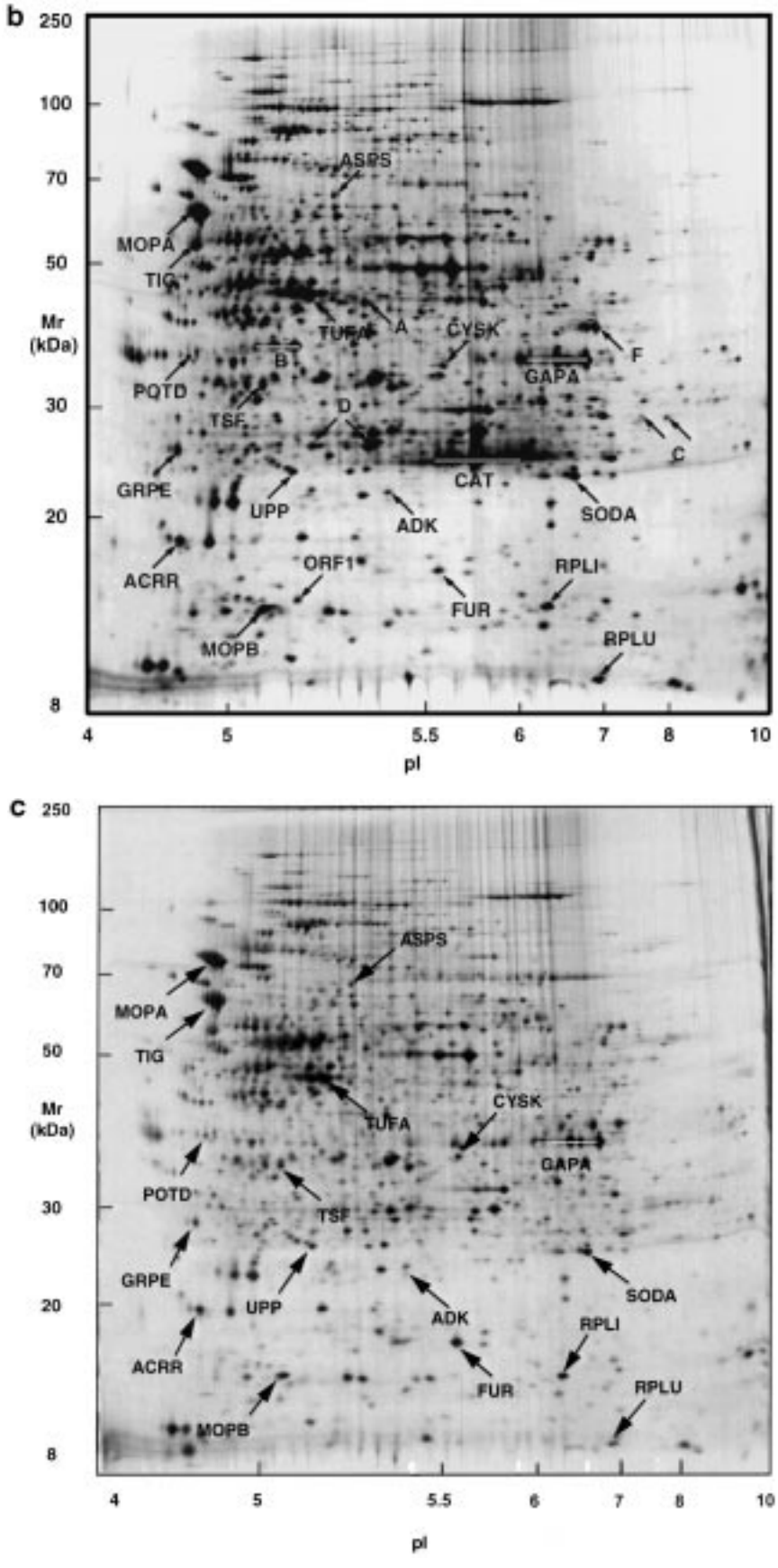

Figure 4 Continued.

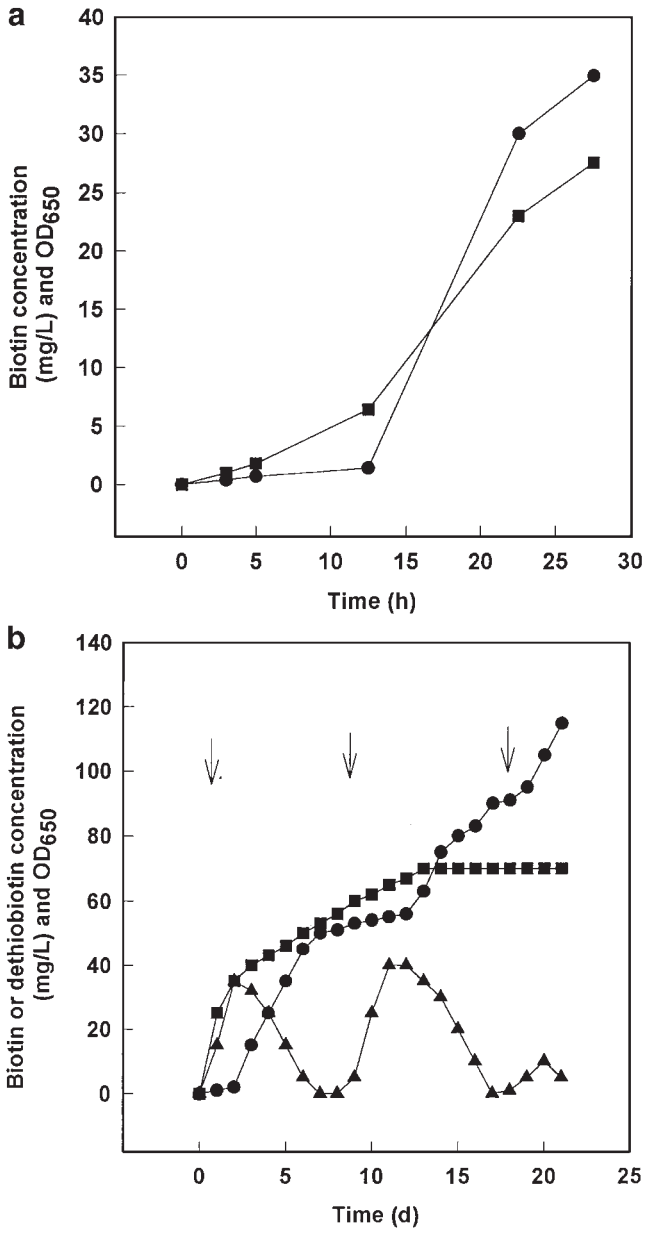

Figure 5 Biotin production by fed-batch fermentation. (a) *E. coli* XLI-Blue pBO30A-15/9; (b) *Agrobacterium/Rhizobium* HK4 pBO47. ■, $OD_{650\ nm}$; ●, biotin; ▲, dethiobiotin. DAPA was introduced into the fermenter at the start of fermentation (200 μg ml^{-1}), and after 200 h (200 μg ml^{-1}) and 415 h (100 μg ml^{-1}), as indicated by the arrows.

Our results lead us to different conclusions. Over-expression of the wild-type *bio* operon resulted in increased biotin production compared to wild-type strains. However, production could be further improved by reorganising the *bio* operon into one transcriptional unit, the use of the strong artificial *tac* promoter, and the introduction of an improved RBS in front of *bioB*. We have shown a direct correlation between *bioB* expression and biotin production, which increased about 2.5- to 3-fold between pBO30A-15 and pBO30A-15/9. Since dethiobiotin production was higher than biotin production from our plasmids, and feeding high levels of dethiobiotin to the cells did not result in large increases in biotin production, we conclude that the biotin synthase step is a major flux control point that will be important for any commercial process. This does not however discount the importance of steps earlier in the pathway. Our conclusions are compatible with flux control theory, which states that the control of flux through a metabolic pathway is a function of all the enzymes in that pathway, and is not only dependent on one key enzyme [43].

Experiments involving mini-cells and 2-D PAGE were used to study expression of the *bio* genes during the modification of the *bio* operon. The reason for the appearance of BioA and BioF as double bands with different molecular weights is not clear, but could be due to the use of a secondary RBS or a stop codon being missed. 2-D PAGE analysis of pBO30A-15/9 revealed multiple spots for some of the over-expressed biotin biosynthetic enzymes. This may have resulted from errors in translation under pressure of over-expression [31], and could be responsible for reduced activity of the enzymes. We estimate that in pBO30A-15/9 the biotin enzymes constitute about 3–5% of total cellular protein. This value is much lower than those possible for protein over-expression, and is also much lower than those reported for the biotin enzymes [23]. This lower level of expression was in our case advantageous. We could detect only low levels of *bioC* expression from our plasmids, whereas the higher levels of expression reported by [23] caused growth inhibition. The higher levels of *bioA*, *bioB*, *bioD* and *bioF* expression (up to 25% of total cellular protein) previously reported [23] appear to have been largely redundant, possibly due to protein insolubility, incomplete or innaccurate folding, or lack of sufficient cofactors or accessory proteins [3]. Strong over-expression of *bioB* also leads to growth inhibition in *E. coli* [20] and *Serratia marcescens* [34].

In *Serratia marcescens* only over-expression of the *bioB* and *bioC* genes is essential for increased biotin production [35]. Our results showed that over-expression of the *bioA*, *bioC* and *bioD* genes in *E. coli* were essential for increased biotin production. Cells without over-expressed *bioB* have very low levels of biotin synthase activity *in vivo* (unpublished results), so it follows that this gene is also essential for increased biotin production. A plasmid with a *bioF* deletion was not tested, and although the *bioH* gene was not cloned and over-expressed, levels of biotin production of up to 2 million times that of the wild-type were observed. Whether a further increase in biotin production will require cloning of *bioH*, or some other enzyme before pimeloyl CoA in the pathway, is an open question, and steps in this direction have already been taken [7], although the initial experiments to over-express *bioH* have had negative results in terms of biotin production in *E. coli* [22].

Using the strategies outlined in the introduction, a number of groups have been able to produce amounts of biotin of up to 600 mg L^{-1} by fermentation [24]. However these fermentations often require considerable time and large amounts of glucose, which increase costs, and complex media and high cell densities, which pose problems for down-stream processing, which will be a crucial part of any commercial process for biotin production. One related and specific problem is the inability of many strains to produce biotin under limiting growth conditions or in the stationary phase. *Agrobacterium/Rhizobium* HK4 and related strains

can actively metabolize 4-butyrobetaine to carnitine under limiting growth conditions, and this reaction is used in a multi-ton industrial process. The rationale for introducing the *bio* operon into this strain was the hope that it would also be able to produce biotin under limiting or zero growth conditions, on a defined growth medium, and we have shown this to be so. The challenge for a future biotechnological process for biotin production remains to find a strain that grows on a simple and defined medium and that has high productivity in terms of time and substrate use. The work described in this paper shows stable biotin production in non-growing cells in a defined medium, which is a major step toward this goal.

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