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The Production of Biotin by Genetically Modified Micro-organisms

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Introduction

Micro-organisms have an enormous metabolic versatility. Many are capable of growing on a great variety of substrates and performing a wide range of synthesizing or transforming reactions. Specific molecules produced in this manner can frequently be overproduced as a function of a micro-organism's environment (Neijssel and Tempest, 1985). As a generalization, one can classify the molecules produced by micro-organisms as primary or secondary metabolites. Primary metabolites are those microbial products whose synthesis is an integral part of the normal growth process and which are made during optimal growth conditions. Examples would be amino acids or vitamins (Britz and Demain, 1985). In contrast, secondary metabolites are usually synthesized only under conditions where cell growth is minimal or in physiological conditions away from those most suitable for growth. Examples here would be antibiotics and pigments (Bunch and Harris, 1986).

Classical industrial microbiology has been very successful at developing commercially attractive processes which can provide the necessary quantities of valuable microbial metabolites (Vandamme, 1989). However, in some cases achieving metabolite overproduction is difficult due to tightly controlled metabolic regulation or the absence of key enzymes in specific microbes. In many of these cases genetic engineering has been used to provide a solution.

In the years since the advent of recombinant DNA technology this technique has been widely applied. Many organisms—bacteria, yeast, fungi, animal and plant cells—have been used as investigational tools or as host organisms for the production of potential and licensed pharmaceutical products or other commercially important biomolecules, such as vitamins and amino acids (Imanaka, 1986). Primrose (1986) has described the ways in which genetic engineering has been employed for the production of primary

Abbreviations: ACM, actithiazic acid; DAPA, 7,8-diamino pelargonic acid; DTB, dethiobiotin; KAPA, 7-keto-8-amino pelargonic acid; TVA, 5-(2-thienyl)-valeric acid.

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or secondary metabolites in micro-organisms. In particular, for systems where the basic biosynthetic pathway is relatively well understood, one can improve the efficiency of the existing biosynthetic reactions by the removal of rate-limiting steps. This being achieved by the cloning and optimized expression of the gene(s) responsible for the rate-limiting step(s).

The microbial production of biotin is a good example here. The metabolic reactions involved in the microbial synthesis of biotin are relatively well understood, but the amount of biotin made is quite low. In this context the application of genetic engineering to enable the development of a commercially viable biotin production process has been considered.

Discovery and nature of biotin

At the beginning of the century, Wildiers found that some strains of yeast required a growth factor, found only in yeast, which he called *bios*. Subsequent investigations confirmed this growth factor to be biotin, a substance first isolated from egg yolk in 1936 by Kögl and Tönnis. Independently, biotin was discovered in liver in 1931 and isolated in 1940 by György, who named it vitamin H. The first chemical synthesis was reported by Harris in 1943 (Bonjour, 1984).

Biotin, $C_{10}H_{16}N_2O_3S$ (molecular weight = 244·31), comprises two fused rings: an imidazole ring and a sulphur-containing ring, thiophane, substituted with valeric acid. Biotin is optically active and only the dextrorotatory isomer possesses vitamin activity (Florent, 1986). The structure of biotin is discussed by Bentley (1985). Biotin is found widely in nature: in micro-organisms, particularly yeasts; in a wide variety of vegetables; in liver; in egg yolk and in sewage sludge. Biotin is also synthesized by the human intestinal flora (György and Langer, 1968).

Many micro-organisms require biotin as a growth factor, and several of these have been used in assays of biotin (*see* p. 300). Biotin plays an important role in the intermediary metabolism of many organisms. The biotin-dependent carboxylases are important in lipogenesis, gluconeogenesis and amino acid metabolism (Gavin and Umbreit, 1965; Mizunaga, Kuraishi and Aida, 1975; Vagelos, 1975; Samols *et al.*, 1988; Knowles, 1989). Deficiency of biotin in humans is rare, probably due to this vitamin's widespread occurrence in foodstuff. Clinical aspects of biotin deficiency and its use in the treatment of inheritable diseases are discussed by Bonjour (1984), Nyhan (1985) and Dakshinamurti and Chauhan (1988). Biotin also plays a vital role in the production of bakers' yeast (Mizunaga, Kuraishi and Aida, 1975; Shimada, Kuraishi and Aida, 1978) and the microbial production of amino acids such as lysine, as a medium supplement (Ko and Chipley, 1984). The mechanism of action of biotin in glutamic acid production is related to the membrane permeability of glutamic acid producing bacteria such as *Brevibacterium* species (Takinami *et al.*, 1968). Biotin also finds use as a foodstuff and animal fodder supplement (Izumi and Ogata, 1975). The use of biotin as a cosmetic additive has been proposed (Gloeckler *et al.*, 1989),

and there is also much interest in developing sensitive assays based on biotin and avidin/streptavidin binding (Buckland, 1986).

Industrial production and consumption of biotin

In recent years the bulk price of biotin has varied between $US4000 and $US8000 per kilogram. The annual world market has been estimated to be in the range 10–20 tonnes per year with 90% of production spread between Roche, Sumitomo, Tanabe and E. Merck. Roughly speaking, a production process based on a recombinant micro-organism would need to produce well over 1 g l^{-1} biotin in order to become cost competitive with existing chemical manufacturing procedures (personal communication, Transgene Business Development Department).

Synthesis of biotin

CHEMICAL SYNTHESIS OF BIOTIN

On an industrial scale, biotin is prepared by a 14-step chemical synthesis developed by Hoffmann La Roche Inc. (Goldberg and Sternbach, 1949). Further details are given by György and Langer (1968) and Izumi and Ogata (1975).

MICROBIAL SYNTHESIS OF BIOTIN

Micro-organisms

Previous interest in the microbial production of biotin vitamers led to the screening of a variety of micro-organisms. Many hundreds of different species of bacteria, yeast and fungi were examined for their ability to excrete biotin vitamers into their cultivation medium (Ogata *et al.*, 1965; Iwahara *et al.*, 1966). The fungi and *Streptomyces* tested, generally accumulated larger amounts of biotin while, in contrast, most of the bacteria and yeast tested accumulated relatively small quantities of biotin. A common feature was that addition of pimelic acid to these cultures significantly enhanced the amounts of biotin vitamers excreted. An exception among the bacteria was the best producer of biotin vitamers, the Gram-positive bacterium, *Bacillus sphaericus*. Addition of pimelic acid to cultures of this micro-organism could enhance biotin vitamers accumulation by a factor of between 300 and 3000 (0·07 to 20–200 mg l^{-1}). In contrast, the biotin vitamers production of *Escherichia coli* was improved from 'trace' levels to 0·07 mg l^{-1}, an amount significantly less than that observed for *B. sphaericus* (Izumi and Ogata, 1975; Izumi, Tani and Yamada, 1980).

There have been other studies on biotin formation. For instance, Ogino, Fujimoto and Aoki (1974a, b) report a biotin production method based on an *n*-alkane-utilizing bacterium transforming a biotin analogue. Pearson, Mac-

Kenzie and Keenan (1986) have screened 129 different yeasts for their biotin and vitamer production. The best producers of biotin were species of *Rhodotorula* and *Sporobolomyces*, while the best producers of total vitamers were *Rhodotorula* and *Yarrowia*. The biotin vitamers formation by *Yarrowia lipolytica* has also been examined (Pearson, Fuller and McKee, 1990).

The above results help to explain the great research interest generated for investigating the synthesis of biotin, particularly in *B. sphaericus*. This work has been extensively reviewed by Izumi (1975), Izumi and Ogata (1975), Izumi, Tani and Yamada (1980), Tanaka, Izumi and Yamada (1988) and Izumi and Yamada (1989).

Biochemistry of the metabolic pathway in micro-organisms

A detailed history of the investigation and determination of the biotin biosynthetic pathway in micro-organisms, going back some 50 years, is given by Izumi and Ogata (1975). Most work concerning Gram-positive biotin-producing micro-organisms was carried out in Japan. Although there has been some overlap, a major contribution to the understanding of the biology of biotin synthesis has come from workers in the USA, including Eisenberg and Pai who have worked with *E. coli*.

A hypothetical pathway was established by the work of Okumura and co-workers in 1962 in varieties of the glutamic acid producing micro-organism, *Brevibacterium* (Izumi, Tani and Yamada, 1980). Using biotin auxotrophic mutants of *E. coli*, Rolfe and Eisenberg (1968) and Pai (1969) also verified the proposed pathway of Okumura and co-workers by means of cross-feeding tests, the identification of accumulated biotin vitamers and growth experiments with each vitamer. They confirmed the pathway of biotin biosynthesis from pimelic acid to biotin as shown in *Figure 1*. However, *Bacillus sphaericus* remains the only micro-organism in which the enzymes involved in biotin synthesis from pimelic acid have all been clearly identified (Izumi *et al.*, 1981; Izumi and Yamada, 1989). *Table 1* names the enzymes concerned and the metabolic intermediates involved with the conversion of pimelic acid to biotin by *Bacillus sphaericus*.

Although the biological aspects of the conversion of pimelic acid to

Table 1. Enzymes related to the metabolic intermediates involved in the conversion of exogenous pimelic acid to biotin in *Bacillus sphaericus*

Metabolic reaction*	Enzyme responsible
1. Pimelic acid → pimelyl-CoA	pimelyl-CoA-synthetase
2. Pimelyl-CoA → KAPA	KAPA synthase (7-keto-8-aminopelargonic acid synthetase)
3. KAPA → DAPA	DAPA aminotransferase (7,8-diaminopelargonic acid aminotransferase)
4. DAPA → DTB	DTB synthase (dethiobiotin synthetase or ureido ring synthetase)
5. DTB → biotin	biotin synthetase

* The metabolic intermediates in the biotin biosynthetic pathway from pimelyl-CoA to biotin (*see Figure 1*) shown here are: KAPA, 7-keto-8-amino pelargonic acid; DAPA, 7,8-diamino pelargonic acid; DTB, dethiobiotin. Individually or collectively they are referred to as vitamers, total vitamers or total biotin vitamers.

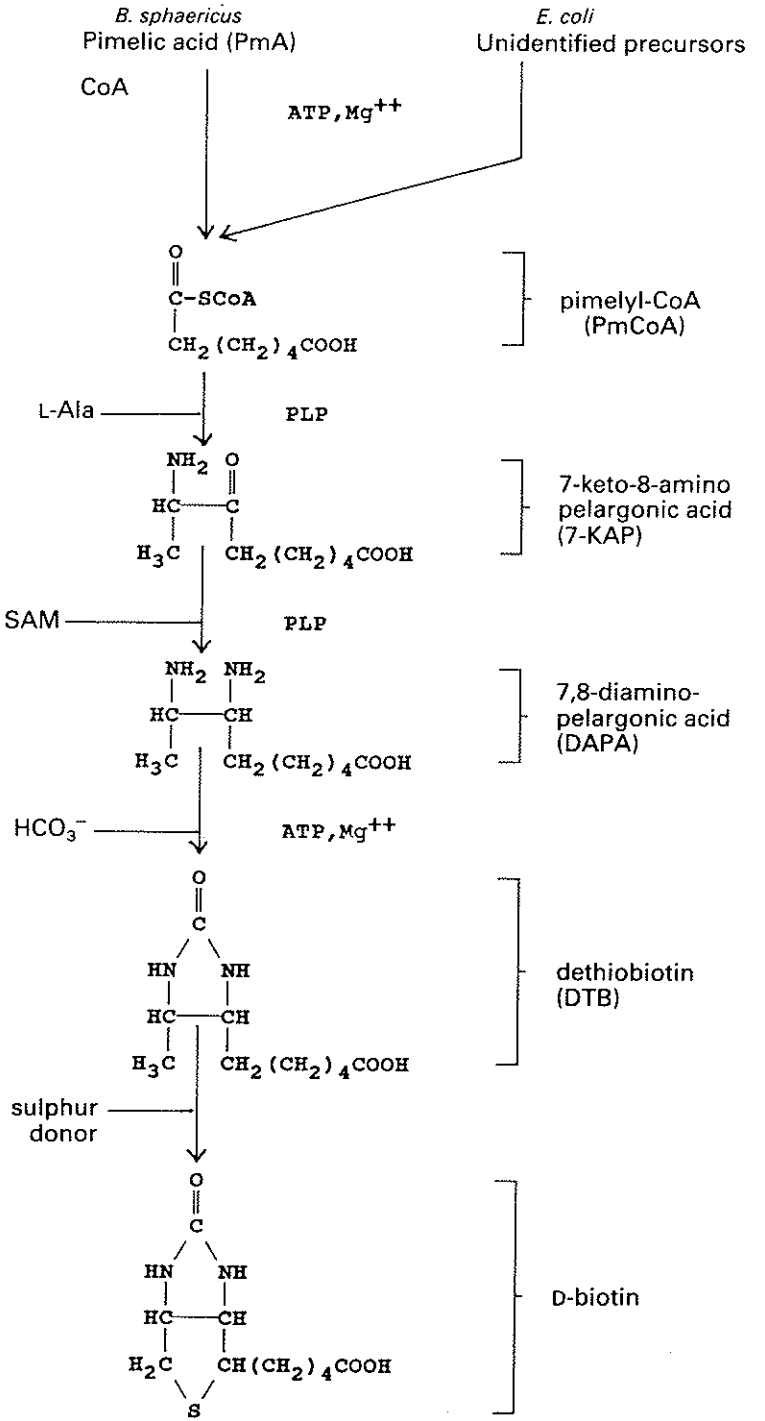

Figure 1. The pathway of biotin biosynthesis from exogenous pimelic acid in *Bacillus sphaericus* and *E. coli* (after Gloeckler *et al.*, 1990a, reproduced with permission). L-Ala, L-alanine; SAM, *S*-adenosyl L-methionine.

dethiobiotin (DTB) have been clearly documented, the actual mechanism of the last step of biotin biosynthesis remains to be elucidated precisely. The conversion of DTB into biotin requires the presence of a sulphur donor. This sulphur donor only appears to be active, and the reaction only catalysed, in actively growing cells; for this reason 'resting cells' (i.e. non-proliferating cells) cannot, or can only poorly, convert DTB into biotin (Izumi, 1975; Izumi and Ogata, 1975; Izumi, Tani and Yamada, 1980; Izumi *et al.*, 1981; Yamada *et al.*, 1983; Izumi and Yamada, 1989; Ohsawa *et al.*, 1989). Ohsawa *et al.* (1989) cite two major unsolved problems which are connected with this specific transformation. Both the precise nature of the sulphur donor and the mechanism of the sulphur introduction process, which leads to the formation of two carbon–sulphur bonds, are unknown. It has been shown that the sulphur atom in biotin can be derived from exogenously supplied cystine (Demoll and Shive, 1983) and much work continues on the nature of the sulphur donor, the mechanism of sulphur introduction and possible unknown intermediates between DTB and biotin (Salib *et al.*, 1979; Osakai *et al.*, 1986). It has been concluded by analogy with the isopenicillin-N synthesis that the formation of biotin could be linked to a carbon radical reaction (Parry, 1983).

Some authors have suggested that the reason *E. coli* is unable to make large amounts of biotin vitamers from exogenous pimelic acid (as a precursor) is due to a permeability block (Pai and McLaughlin, 1969). A toxic analogue of pimelic acid, ϵ-(2,4-dichloro-sulfanilido)-caproic acid, was reported not to inhibit the growth of *E. coli*, unlike other biotin prototrophic microbes (Woolley, 1950). However, a recent study of uptake of pimelic acid in *E. coli* showed that this molecule is taken up by passive diffusion (Ploux *et al.*, 1990).

Biotin has been shown to be degraded by fungi, yeasts and bacteria via β-oxidation of the side-chain of the molecule. This has been described in more detail by Izumi and Yamada (1989).

ANALYSIS OF BIOTIN AND ITS VITAMERS

There are three methods for the determination of biotin and its vitamers (7-keto-8-amino pelargonic acid (KAPA), 7,8-diamino pelargonic acid (DAPA) and DTB), microbiological, chemical and enzymatic techniques. These have been reviewed by György and Langer (1968) and Izumi and Yamada (1989). The microbiological assays have been the most commonly used methods. Biotin and total vitamers (measured as biotin+KAPA+DAPA+DTB) can be determined, being based on the growth of *Lactobacillus plantarum* ATCC 8014 or *Saccharomyces cerevisiae* ATCC 7754 (Snell, Eakin and Williams, 1940; Wright and Skeggs, 1944). Individual vitamers can be separated by thin layer chromatography and then assayed using *S. cerevisiae* ATCC 7754 (Sabatié *et al.*, 1991).

Synthesis of biotin by genetically engineered cells

The low levels of biotin synthesis reported from conventional studies of biotin formed in micro-organisms has prompted a commercial interest, based on the possibility of using genetic engineering to improve yields. In principle this might seem relatively straightforward, since the biosynthetic pathway and its enzymes are already well documented.

There are perhaps two main goals to achieve when employing such an approach. First of all, a well-characterized biochemical conversion giving a high product flux (flow) from any precursor (in the case of *B. sphaericus*, pimelic acid) to the final product (biotin). A high flux down a metabolic pathway might only be achieved when at least three criteria are satisfied:

1. that all of the amplified genes relevant to the pathway are expressed at a level relative to one another, to provide sufficient amounts of enzymes to overcome individual rate-limiting steps or regulatory controls;
2. that any precursors or enzyme co-factors are not limiting; and
3. that the relationship between the flux and growth of the micro-organism has been established.

Secondly, depending on (3) above, there must be the means of making this process highly productive by growing or using the producing microbe at very high cell densities.

The reality of achieving goals such as those above has to be tempered by the practical difficulties involved. Nevertheless, world-wide, several companies have been, or are, employing genetic engineering techniques as part of a programme for the development of an industrially viable biotin production process. Commercial confidentiality evidently places considerable constraints on the availability of new information about this interesting but difficult subject. However, although most recent information remains proprietary, a number of common strategies have been employed, and these are reviewed here.

STRATEGIES USED FOR THE PRODUCTION OF BIOTIN

Reference to the literature indicates that concerning the subject biotin, recombinant DNA methodology has been employed to a variety of ends: elucidation of the structure of the *E. coli bio* operon and its genes, studies on the regulation of biotin synthesis and, more recently, cloning of the *bio* genes from *E. coli* and *B. sphaericus*. Amplification of the *bio* genes and their respective enzymes has allowed the functioning of the biotin biosynthetic pathway to be studied, i.e. the activities of the enzymes and the accumulation pattern of biotin vitamers (the metabolic intermediates). Evidently, this requires the combined and simultaneous inputs of molecular biologists, biochemists and microbial physiologists. A better understanding of the operation of the pathway then enables fermentation technology to optimize and develop an efficient, industrially viable production process.

THE *bio* GENES

The historical development of man's knowledge of biotin biosynthesis has focused interest on the *bio* genes from *B. sphaericus* and *E. coli* and there are quite disparate reasons for the interest in cloning the *bio* genes from one or other of these species.

In the case of *B. sphaericus*, it has previously been stated that this is the only micro-organism in which the enzymes involved in biotin synthesis from pimelic acid have all been clearly identified (*see* p. 298). Another reason why the *bio* genes of *B. sphaericus* have attracted interest is because some strains of this micro-organism naturally produce much higher amounts of total biotin vitamers than wild-type *E. coli* strains (Izumi, Tani and Yamada, 1980). Examples of such strains being *B. sphaericus* IFO 3525 and NCIB 9370 (Gloeckler *et al.*, 1990a). Furthermore, the addition of pimelic acid to cultures of *B. sphaericus* can enhance the amount of vitamers excreted (Izumi, Tani and Yamada, 1980) and as much as 350 mg l^{-1} DTB has been obtained by using pimelic acid and actithiazic acid (ACM, an antibiotic isolated from a *Streptomyces* species). ACM addition results in the competitive inhibition of the conversion of DTB to biotin and can enhance the activity of DAPA aminotransferase and DTB synthetase (Eisenberg, 1973; Ogata, Izumi and Tani, 1973). Thus, some workers have argued that the *bio* genes of *B. sphaericus* have a high 'specific activity', i.e. the ability to produce large amounts of biotin vitamers from the precursor, pimelic acid (there could, equally, be other unknown reasons for this high productivity). However, until recently, little was known about the *bio* genes of this micro-organism.

In the case of *E. coli*, the first steps of the biosynthetic pathway to biotin, from unknown precursors to pimelyl-CoA, remain unclear. Even though a difference of about a thousandfold has been observed between the respective basal levels of vitamer concentrations in supernatants of cultures of wild-type and biotin-derepressed mutants of *E. coli* (Pai, 1972), the actual levels are quite low. Furthermore, the beneficial effect of pimelic acid addition on vitamers and biotin formation, as seen in *B. sphaericus*, had not been observed for *E. coli*, a phenomenon attributed to pimelic acid permeability (Pai and McLaughlin, 1969). In contrast to *B. sphaericus*, much academic work has been published on the molecular biology of biotin synthesis in *E. coli*. The *bio* genes of *E. coli* and their regulation have been described in great detail. For this reason many of the descriptions in this article with respect to the *bio* genes, concern *E. coli*.

In perspective, therefore, a major factor for the interest in cloning the *E. coli bio* genes was the existing body of knowledge concerning these genes. In contrast, the *B. sphaericus bio* genes had not been characterized but were reputed to encode enzymes that could produce higher amounts of biotin vitamers. Thus, cloning these genes might intrinsically result in the production of more biotin.

THE *bio* GENES OF *E. COLI*

Location and structure of the E. coli bio *genes*

The genes involved in the metabolism of biotin in *E. coli* are found in different loci within its chromosome.

A first locus consists of the *bio* operon which has been shown to be divided into five closely linked complementation groups, corresponding to genes *bioA*, *bioB*, *bioF*, *bioC* and *bioD* (del Campillo-Campbell *et al.*, 1967; Rolfe and Eisenberg, 1968; Rolfe, 1970; Cleary, 1971; Cleary and Campbell, 1972). A sixth gene (Open Reading Frame 1, encoding a protein of 17·1 kDa), of unknown function, is present downstream from *bioA* (Otsuka *et al.*, 1988). The *bio* operon is located in the above order between the bacteriophage λ attachment site and the *uvrB* gene loci at approximately 17 minutes on the *E. coli* genetic map of Bachmann (1987). The two divergent *bio* transcriptional units are controlled by a single operator, *o* (Ketner and Campbell, 1975), and thus constitute a single operon (Szybalski and Szybalski, 1982). *Figure 2* shows the gene order in the *bio* operon.

The *uvrB* locus, located adjacent to the *bioD* gene consists of a single gene, about 2 kb in size, and is transcribed in the same direction as *bioBFCD* from two tandem promoters located between *bioD* and *uvrB* upstream from the *Eco*R1 site in the *uvrB* gene (Pannekoek, Noordermeer and van de Putte, 1979; Pannekoek, Hille and Noordermeer, 1980; Sancar *et al.*, 1981, 1982; van den Berg *et al.*, 1981). The *uvrB* gene has no function in biotin biosynthesis but acts in some way to protect *E. coli* cells from ultraviolet radiation, as reported by Sancar *et al.* (1982).

A second locus has been identified to contain the gene, *bioH*, which is associated with the *malA* region (Schwartz, 1966; Eisenberg, 1985). The nucleotide sequence of the *E. coli bioH* gene has been published by O'Regan *et al.* (1989).

A number of workers have shown that there is a third genetic locus at 89 minutes on the *E. coli* chromosome map (Bachmann, 1987), having two genetic functions which regulate *bio* operon expression. These two functions are found in a single gene, designated *birA* or *bioR* (Campbell, del Campillo-Campbell and Chang, 1972; Pai, 1972; Barker and Campbell, 1980, 1981a, b).

A fourth genetic locus, the gene *bioP*, has been attributed to the transport (cellular uptake) of biotin (Eisenberg *et al.*, 1975).

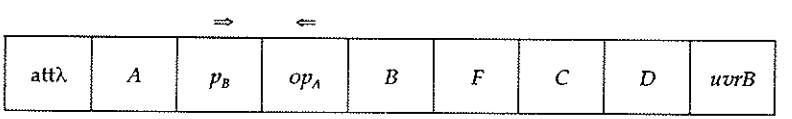

Figure 2. The *bio* operon of *E. coli*. The gene order in the *bio* operon is as shown. Transcription of the *bio* operon is divergent and originates *in vitro* (as shown by the arrows) from two partially overlapping head-to-head promoters, p_B and p_A, located between genes *bioA* and *bioB*, and is initiated by a single operator, *o*, as described by Szybalski and Szybalski (1982).

Physical map of the E. coli bio *operon*

The proximity of the *bio* operon to λ on the *E. coli* genome has facilitated the isolation of numerous *bio* transducing λ phages, many of which have been characterized genetically. These have been utilized to construct a physical map of the *E. coli bio* operon (Szybalski and Szybalski, 1982). The first heteroduplex mapping study of the *bio* operon was done by Hradecna and Szybalski (1969; errata, 1970). The *bio* enzyme sequences were predicted from the nucleotide sequence of the *bio* operon (Otsuka *et al.*, 1988).

Functions of the E. coli bio *genes*

Table 2 lists eight identified genes known to be concerned with the metabolism of biotin in *E. coli*, attributed to the enzymes of the biotin biosynthetic pathway (Izumi, 1975; Eisenberg, 1985; Bachmann, 1987; Otsuka *et al.*, 1988; Cronan, 1989; O'Regan *et al.*, 1989). The gene products of the *bioA, bioB, bioF* and *bioD* cistrons have been clearly linked to the pathway of bioconversion from pimelyl-CoA to biotin (Rolfe and Eisenberg, 1968; Cleary and Campbell, 1972).

There is no published information on the function of the product(s) of the *bioC* and *bioH* genes except that mutations in these genes result in the failure to excrete any of the known intermediates in the biotin pathway, and it has been proposed that these genes concern steps higher up in the metabolic pathway (Eisenberg, 1987). Gloeckler *et al.* (1990a) suggest that *bioC* and *bioH* are tentatively identified as encoding the enzymes involved in pimelyl-CoA synthesis.

Genetic (Barker and Campbell, 1981a, b), biochemical (Eisenberg, Prakash and Hsiung, 1982) and nucleotide sequence studies (Howard, Shaw and Otsuka, 1985) have shown that the *birA* gene encodes a bifunctional 33·5 kDa protein, BIRA, also called *E. coli* biotin-protein ligase (Cronan, 1989). Biotin-protein ligase acts both as the biotin operon repressor and as the enzyme, acetyl-CoA carboxylase biotin holoenzyme synthetase. The functions of the *birA/bioR* gene are discussed further in the next section.

Table 2. The *bio* genes of *E. coli*

Gene	Position on chromosome map (minutes)	Size of encoded protein (kDa)	Function encoded
bioA	17	47·4	DAPA aminotransferase
bioB	17	38·7	biotin synthetase
bioF	17	41·6	KAPA synthetase
bioC	17	28·3	? pimelyl-CoA-synthetase
bioD	17	23·9	DTB synthetase (ureido ring synthetase)
bioH	75	28·5	? pimelyl-CoA-synthetase
bioP	85	–	*bioP* is concerned with biotin permeation
bioR (birA)	89	33·5	biotin protein ligase, the transcriptional repressor of the *bio* operon (together with the co-repressor, biotinoyl-AMP)

The existence of the gene *bioP*, a gene concerned with the permeability of the cell membrane, was demonstrated by Eisenberg *et al.*, (1975) using, as a selection procedure, resistance to the biotin analogue, α-dehydrobiotin. This analogue had been shown previously to inhibit biotin uptake competitively (Prakash and Eisenberg, 1974). Mutants of *E. coli* designated *bioP* had a steady-state rate of biotin uptake reduced by over 90% when compared to a parent strain. The *bioP* gene has also been identified as *birB* (Campbell *et al.*, 1980). Piffeteau and Gaudry (1985) confirmed the results of Prakash and Eisenberg (1974), that biotin uptake is an active process. However, under steady-state conditions, they showed that the efflux of biotin was not energy dependent and was mainly mediated by a diffusion mechanism.

REGULATION OF THE *E. COLI bio* OPERON

Regulation of biotin synthesis in *E. coli* is effected by negative control at the transcriptional level rather than feedback inhibition (Eisenberg, 1985). This can be illustrated by the fact that biotin levels in cultures of *E. coli* are normally extremely low due to the low concentrations of biotin needed to cause repression (Pai and Lichstein, 1965b). Biotin synthesis is effectively repressed at biotin concentrations of 5–10 μg l^{-1} and only fully derepressed at concentrations of 0·1–0·2 μg l^{-1} (Pai and Lichstein, 1965b; Cohen *et al.*, 1978; Eisenberg, 1985).

Much understanding of the regulation of the *bio* operon has come from the study of biotin-deregulated mutants. Mutants of *E. coli* deregulated for biotin synthesis, with constitutive synthesis of the *bio* enzymes have been isolated. Campbell, del Campillo-Campbell and Chang (1972) reported a class of mutants designated *birA* isolated by screening for an elevated biotin requirement. Pai (1972, 1973, 1974) and Eisenberg *et al.* (1975) reported mutants designated *bioR*, obtained on the basis of their resistance to α-dehydrobiotin. The mode of action of α-dehydrobiotin has been discussed by Eisenberg (1975).

A variety of mutants designated *birA* and *bioR* have been analysed. The properties of the different categories of *birA* mutants can be summarized as resulting in

1. derepression of the *bio* operon;
2. being deficient in retention of biotin;
3. being deficient in biotin uptake;
4. exhibiting a temperature-sensitive deficient biotin holoenzyme synthetase function (growth at 28°C and no growth at 43°C); and
5. an elevated minimum biotin concentration necessary for growth (Campbell, del Campillo-Campbell and Chang, 1972; Barker and Campbell, 1980, 1981a; Campbell *et al.*, 1980).

Some of the *birA* mutants were designated *birB* (Campbell *et al.*, 1980) and map with *bioP* (*see* p. 304). The *bioR* mutants, which map in *birA*, have similar properties except that they are not noticeably affected with respect to minimal biotin growth requirement (Pai, 1972, 1974; Eisenberg *et al.*, 1975;

Pai and Yau, 1975; Barker and Campbell, 1980, 1981a; Campbell *et al.*, 1980; Howard, Shaw and Otsuka, 1985; Buoncristiani, Howard and Otsuka, 1986; Uchida and Otsuka, 1987).

Several workers have cloned and over-expressed the *birA* gene. Expression of a 2·2 kb region containing *birA* from a plasmid-borne promoter in *E. coli* resulted in complementation of *birA* mutations, stronger repression of the biotin operon, a 150-fold elevation of biotin holoenzyme synthetase activity but no repressor action on the gene *bioH* (Barker and Campbell, 1981b; Barker, Kuhn and Campbell, 1981; Howard, Shaw and Otsuka, 1985; Buoncristiani and Otsuka, 1988).

The regulatory region between genes *bioA* and *bioB* has been mapped by restriction analysis, cloned and sequenced (Otsuka, 1978; Otsuka and Abelson, 1978; Barker, Kuhn and Campbell, 1981). Cloning of the *bio* operator on a multicopy plasmid resulted in out-titration of the repressor and derepression of the *bio* operon (Barker, Kuhn and Campbell, 1981; Howard, Shaw and Otsuka, 1985; Buoncristiani, Howard and Otsuka, 1986; Otsuka, Uchida and Buoncristiani, 1987; Uchida and Otsuka, 1987).

The rate of biotin operon transcription is sensitive to both the intracellular concentration of biotin and to the supply of the *birA* gene product (BIRA, or biotin protein ligase; also called biotin holoenzyme synthetase by some authors) to which biotin must be attached in order to fulfil its essential metabolic role (Cronan, 1988, 1989). Biotin protein ligase activates biotin to form biotinyl-5′-adenylate and transfers the biotin moiety to biotin-accepting proteins. If the biotin-accepting proteins are completely biotinylated, the biotinyl-5′-adenylate (Biotinoyl-AMP) remains associated with BIRA and acts as a co-repressor (Prakash and Eisenberg, 1979). Because biotin modification is the final step in the utilization of biotin as an enzyme co-factor, it is therefore a logical control point for the synthesis of biotin (Barker and Campbell, 1981a; Buoncristiani, Howard and Otsuka, 1986). Regulation of the *E. coli* biotin operon is a well-understood example of transcriptional regulation and a model of *E. coli bio* operon transcriptional repression has been proposed by Cronan (1989).

A model of transcriptional overlap controlled by a single operator situated at the overlap region was proposed on the basis of genetic studies (Ketner and Campbell, 1975). A similar model suggesting that the *bioA* and *bioB* promoters lie *vis-à-vis* with the common operator in between and partially overlapping the promoters, was proposed on the basis of the nucleotide sequences of the *bio* regulator region (Otsuka, 1978; Otsuka and Abelson, 1978; Eisenberg, 1985).

Differential repression of enzymatic activity from the *bioA* and *bioBFCD* segments in a coupled transcription–translation system has been demonstrated in the presence of biotin (Prakash and Eisenberg, 1978), which suggested independent control of *bioA* and *bioBFCD* (Vrancic and Guha, 1973). Attenuation of transcription of the *bio* operon has been studied by Nath and Guha (1982) and Nath (1988). A transcription termination site in the *bioBFCD* segment has been demonstrated. From the nucleotide sequence of the promoter proximal region of the *bioB* gene, a 13-oligonucleotide

sequence, believed to be the transcription termination site, has been located (Nath, 1988).

THE *bio* GENES OF *BACILLUS SPHAERICUS*

The *bio* genes clusters or individual *bio* genes have been isolated and cloned into various expression vectors capable of replication in *B. sphaericus, Bacillus subtilis* and *E. coli* as reported on p. 310. No information on these genes was available prior to these publications.

REGULATION OF *bio* GENES EXPRESSION IN *BACILLUS SPHAERICUs*

For *B. sphaericus*, much less is known about the regulation of *bio* genes expression compared to that for *E. coli* (*see* pp. 305–307). Repression of biotin synthesis by biotin has been observed. The amount of biotin necessary to achieve repression of biotin synthesis in *B. sphaericus* has been observed to be in the range 50–100 $\mu g\ l^{-1}$ (Speck *et al.*, 1990). This amount is a factor between 10 and 50 times greater than that reported required to produce repression in *E. coli*, where negative, transcriptional control has been demonstrated (Speck *et al.*, 1991). Thus, it is not clear whether a similar mechanism of regulation is present. Derepressed strains of *B. sphaericus* are difficult to obtain by classical mutagenesis but have been isolated by using a gene fusion technique based on the use of a chromogenic marker, the *xylE* gene. This has previously been reported as a useful tool for the study of gene expression in species of *Bacilli* (Zukowski *et al.*, 1983).

Gloeckler *et al.* (1990a) recently reported the existence of a common 15 bp sequence in the 5′ non-coding region of two respective *bio* operons, typical of transcription attenuators. By analysing *cis*-acting mutations, it was found that this zone was subjected to punctual base changes or deletions which led to constitutive expression of the gene marker. These results suggested its role in a biotin regulation mechanism (Speck *et al.*, 1991).

CROSS-SPECIES CONSERVATION OF BIOTIN REGULATORY REGIONS

By way of comparison, biotin regulatory sequences in different Enterobacteriaceae, *E. coli, Salmonella typhimurium* and *Citrobacter freundii* have been examined. The position of the biotin regulatory region in *E. coli* has already been discussed. It lies between the *bioA* and *bioB* genes. Transcription proceeds to the left into *bioA* and, from the other strand, to the right into *bioBFCD*. In the segment identified as operator/*bioB* promoter, *C. freundii* and *S. typhimurium* DNA are identical and differ from *E. coli* only by 2 bp. The DNA to the right of this segment (identified as the *bioA* promoter of *E. coli*) has diverged in all three species, and only *E. coli* has a sequence resembling a consensus promoter (Shiuan and Campbell, 1988).

Biotin production: industrial process development strategies

A range of strategies have been employed in order to achieve optimal performance when using a recombinant DNA approach. Nevertheless, these strategies can be grouped into three major domains:

1. the cloning of part of or the whole *bio* operon from *E. coli* or *B. sphaericus*;
2. the use of *E. coli* or *B. sphaericus* host strains with modified genetic backgrounds, particularly with respect to the *bio* genes; and
3. varying the fermentation conditions to suit the expression of the *bio* genes and the host genetic background.

These different strategies are summarized in *Table 3*. Some of the approaches used are quite important to the general concept of biotin synthesis by recombinant micro-organisms, and merit further discussion.

ISOLATION, CLONING AND SEQUENCING OF THE *E. COLI bio* GENES

Different workers have isolated and cloned the entire *bio* operon, *bio-ABFCD*, the entire operon with a specific *bio* gene deleted or the individual *bio* genes into expression vectors, which when inserted into a suitable host are capable of amplifying the expression of the relevant genes (*see*, for instance, Hirono, Kojima and Kimura, 1986; Fisher, 1987). Presumably, the aim of cloning and over-expressing separate, or groups of *bio* genes has been to study their influence individually on the performance of the biotin biosynthetic pathway. Such an approach has been published by Ifuku and Yanagi (1989) who demonstrated the influence of individual *bio* gene deletions on biotin synthesis and showed that the whole *bio* operon was necessary for the best biotin production.

Cloning of the E. coli bioB *gene*

An obvious case for the cloning and over-expression of an individual *bio* gene is the gene *bioB*. This gene codes for biotin synthetase which catalyses the last step of the biotin biosynthetic pathway. Classically, DTB has been shown to accumulate in cultures of micro-organisms which have a high biotin vitamers synthetic capacity. Thus, over-expression of *bioB* is a clear candidate in its own right for attempting to improve the conversion of DTB to biotin. Cloning of *bioB* from *E. coli* has been reported by Fisher (1987), Hirono, Kojima and Kimura (1986) and MacKenzie Pearson and McKee (1989).

Cloning of the E. coli bio *operon*

The entire *bio* operon has been isolated from a genomic bank on restriction fragments of λ*bio* phage and cloned (DasGupta, Vrancic and Guha, 1977; Prakash and Eisenberg, 1977; Cohen *et al.*, 1978; DasGupta and Guha,

Table 3. Major features of published data concerning the cloning, expression and fermentation of the *bio* genes from *E. coli* or *B. sphaericus*

Feature	Reference
1. Cloning of the *bio* genes	
Utilization of part or all of the *bio* operon of *E. coli*, *bioABFCD* in an *E. coli* expression vector	Hirono, Kojima and Kimura (1986), Ifuku *et al.* (1986), Fisher (1987), Ifuku and Yanagi (1988, 1989), Haze, Ifuku and Kishimoto (1989)
Utilization of groups or single modified *E. coli bio* genes in expression vectors capable of replication in micro-organisms other than *E. coli*	MacKenzie Pearson and McKee (1989)
Utilization of part or all of the *bio* genes of *B. sphaericus*, *bioDAYB* and *bioXWF* in an *E. coli* or *B. sphaericus* expression vector	Ohsawa *et al.* (1987, 1989), Gloeckler, Speck and Lemoine (1988), Gloeckler *et al.* (1990a, b), Brown *et al.* (1991), Sabatié *et al.* (1991)
2. Modification of the host genetic background	
Utilization of *E. coli* mutants being deficient for one or more *bio* genes	Hirono, Kojima and Kimura (1986), Ifuku *et al.* (1986), Fisher (1987), Ohsawa *et al.* (1987, 1989), Gloeckler, Speck and Lemoine (1988), Ifuku and Yanagi (1988, 1989), Haze, Ifuku and Kishimoto (1989), Gloeckler *et al.* (1990a, b), Brown *et al.* (1991), Sabatié *et al.* (1991)
Utilization of *B. sphaericus* derepressed mutants	Speck *et al.* (1990)
Utilization of *E. coli* mutants which are *bioR* or *birA* (derepressed for biotin synthesis)	Hirono, Kojima and Kimura (1986), Ifuku *et al.* (1986), Fisher (1987), Ifuku and Yanagi (1988, 1989), Haze, Ifuku and Kishimoto (1989)
Utilization of *E. coli* mutants which have a modified, temperature-sensitive, $birA^{TS}$ gene	Fisher (1987)
3. Fermentation and gene expression	
Use of the leftward promoter from phage Lambda (P_L) to express one or more *bio* genes	Hirono, Kojima and Kimura (1986), Ifuku *et al.* (1986), Fisher (1987), Ifuku and Yanagi (1988, 1989), Haze, Ifuku and Kishimoto (1989)
Use of the *trp* promoter from *E. coli* to express one or more *bio* genes	Brown *et al.* (1991), Sabatié *et al.* (1991)
Use of the *lac* promoter from *E. coli* to express one or more *bio* genes	Brown *et al.* (1991), Sabatié *et al.* (1991)
Use of the *tet* promoter from *E. coli* to express one or more *bio* genes	Gloeckler, Speck and Lemoine (1988), Gloeckler *et al.* (1990a, b)
Use of a modified ribosome binding site to improve the expression of the *bioB* gene in species of bacilli	Ohsawa *et al.* (1987, 1989)
Utilization of a temperature-sensitive copy number runaway replicon	Fisher (1987)
Selection and utilization of low-acetate-forming strains of *E. coli* for biotin synthesis	Haze, Ifuku and Kishimoto (1989)
Supplementing the fermentation medium with amino acids to improve biotin production	Fisher (1987), Haze, Ifuku and Kishimoto (1989), J. Sabatié (personal communication)
Major analysis of producing strains' fermentation physiology in order to improve biotin yields	Brown *et al.* (1991), Sabatié *et al.* (1991)

1978). A restriction map of the entire operon has been published by de Wet *et al.* (1980).

Some workers (Hirono, Kojima and Kimura, 1986; Fisher, 1987) have isolated the *bio* operon and its genes from a plasmid, pLC2523, which is deposited with the American Type Culture Collection (ATCC 53237), part of this plasmid consists of the *bio* operon and the *uvrB* gene located upstream of *bioD*. Fisher (1987) reports the deletion of the adjacent *uvrB* gene. Three RNA molecules are transcribed from the *uvrB* locus, one of which could interact with RNA polymerase A. Thus, if *uvrB* was amplified with the *bio* operon, such an interaction could be lethal for an *E. coli* cell. Elsewhere (Ifuku *et al.*, 1986; Ifuku and Yanagi, 1988, 1989; Haze, Ifuku and Kishimoto, 1989) the *bio* operon has also been cloned, the DNA having been isolated from a wild-type strain of *E. coli*, W3110. The amounts of biotin synthesized, reported by these authors, ranges from 1·3 $\mu g\ l^{-1}$ (using a strain with a $bio^{-}bioR^{+}$ genetic background, giving a twentysixfold increase (Cohen *et al.*, 1978)) to values in the range 5–105 $mg\ l^{-1}$. However, such elevated concentrations were achieved by the alteration of other parameters, as discussed in the following sections.

Cloning of the E. coli bio *genes and their use in other micro-organisms*

MacKenzie Pearson and McKee (1989) describe the cloning of the *E. coli bio* operon from the phage Charon 4A. The construction of a series of plasmids is described, these being capable of replication in yeasts, fungi and lactobacilli, and which contain one or more of the *bio* genes. Plasmids containing the *bio* genes are also described whose codon sequence has been modified in favour of the yeast, *S. cerevisiae*. The aim of the work has been to provide genetic material (*E. coli bio* genes in plasmids absent of other *E. coli* DNA) which may be introduced into micro-organisms such as *S. cerevisiae* in order to improve their ability to synthesize biotin.

ISOLATION, CLONING AND SEQUENCING OF THE *B. SPHAERICUS bio* GENES

Cloning of the B. sphaericus bioB *gene*

The cloning and sequencing of the biotin synthetase gene (*bioB*) from *B. sphaericus* T-178–367, an ACM- and TVA-resistant mutant of *B. sphaericus* IFO 3525, and its expression in *E. coli* and strains of *B. sphaericus* and *B. subtilis* has been reported by Ohsawa *et al.* (1987, 1989). ACM (actithiazic acid) and TVA (5-(2-thienyl)-valeric acid) are metabolic analogues. The action of ACM has already been described (*see* p. 302). TVA has been shown to augment the production of KAPA and decrease the amounts of other vitamers and biotin because it inhibits DAPA aminotransferase (Izumi *et al.*, 1977, 1978). The *bioB* gene catalyses the conversion of DTB to biotin (*see Table 4*). The synthesis of biotin by these transformed cells was shown to be strain-dependent, and excreted biotin accumulated to 15–16 $mg\ l^{-1}$ in the

culture medium. The factor of improvement obtained for biotin production by cells transformed with *bioB* over cells without *bioB* amplification for strains of *B. subtilis* (with a modified ribosome-binding site) and *E. coli* was up to a factor of 1600, and for strains of *B. sphaericus*, a factor of between 40 and 100.

Cloning of the B. sphaericus bio *genes*

The *bio* genes of *B. sphaericus* IFO 3525 have been isolated using 8·8 kb of genetic information from a genomic bank made from this organism (Gloeckler, Speck and Lemoine, 1988; Gloeckler *et al.*, 1990a, b). The *bio* genes were borne on two recombinant plasmids with inserts of 4·3 kb and 4·5 kb which had been isolated following phenotypic complementation with various *E. coli bio* mutants. The *bioD* and *bioA* genes were identified within the 4·3 kb insert and shown to be closely linked to a gene, *bioY* (coding for a protein with a presently unknown function) and to *bioB*. These genes were clustered in the order *bioDAYB*. The 4·5 kb fragment contained genetic information for three different proteins, the products of *bioX, bioW* and *bioF*. Complementation studies demonstrated that the third open reading frame of this cluster was *bioF*. A combination of *bioW* and *bioF* allowed an efficient complementation of *E. coli bioC* and *bioH* mutants, provided that pimelic acid was added to the biotin-depleted growth medium, suggesting that *bioW* encodes pimelyl-CoA synthetase. However, *bioW* was not thought to encode a pimelic acid transport function. No function was identified for the product of *bioX*. The gene order of this cluster was *bioXWF*. *Table 4* identifies and compares the known functions of the *E. coli* and *B. sphaericus bio* genes.

The problem of pimelic acid permeation in *E. coli* has already been discussed as either not taking place (Pai and McLauchlin, 1969) or being by passive diffusion (Ploux *et al.*, 1990), refer to p. 300 for further details. However, in the case of Gloeckler *et al.* (1990a) exogenous pimelic acid was efficiently converted to KAPA, being linked to the level of expression of the *bioXWF* gene cluster. One explanation for the opposing data may be that the strain of *E. coli* used (C268) by Gloeckler and co-workers was more permeable to pimelic acid than that used by Pai and McLauchlin.

Table 4. A comparison of the *bio* genes of *B. sphaericus* and *E. coli* and their encoded functions

B. sphaericus gene	*E. coli* gene	Function encoded
bioX	?	unknown
bioW	*bioC? bioH?*	? pimelyl-CoA-synthetase
bioF	*bioF*	KAPA synthetase
bioD	*bioD*	DTB synthetase (ureido ring synthetase)
bioA	*bioA*	DAPA aminotransferase
bioY	–	unknown
bioB	*bioB*	biotin synthetase
–	*bioP*	biotin permeation
–	*bioR (birA)*	biotin protein ligase, the transcriptional repressor of the *bio* operon (together with the co-repressor, biotinoyl-AMP)

STRUCTURAL SEQUENCE HOMOLOGIES BETWEEN THE BIOTIN BIOSYNTHETIC ENZYMES OF *B. SPHAERICUS* AND *E. COLI*

Sequence comparisons between the *E. coli* (Otsuka *et al.*, 1988) and the *B. sphaericus* (Ohsawa *et al.*, 1989; O'Regan *et al.*, 1989; Gloeckler *et al.*, 1990a) genes have been made. Amino acid sequence analysis indicated broad similarities between the corresponding *bioA, bioB, bioD* and *bioF* gene products of 65, 55, 52 and 65%, respectively (with conservative replacements considered). However, no significant homology was found between the *E. coli bioC* and *bioH* genes and the *B. sphaericus bioX, bioW* or *bioY* genes. Reciprocally, no homology was found between the putative BIOX, BIOW or BIOY proteins and the gene product of Open Reading Frame 1, located downstream from the *E. coli bioA* gene (Otsuka *et al.*, 1988). The total absence of similarity between the *bioW* or *bioH E. coli* gene products (O'Regan *et al.*, 1989) could underline essential differences in the cellular function of these proteins (Gloeckler *et al.*, 1990a).

Ohsawa *et al.* (1989) raised a question as to whether the *bioB* gene of strain T-178-367, an ACM- and TVA-resistant mutant, was in some way different and superior to that of strain IFO 3525. However, no difference was found between the nucleotide sequences of the *bioB* genes from the wild-type *B. sphaericus*, IFO 3525, and the mutated strain, T-178-367, derived from it.

INFLUENCE OF THE HOST GENETIC BACKGROUND

Deregulated strains of E. coli (bioR)

It has been mentioned previously that the use of biotin deregulated strains (*bioR*) can significantly increase biotin levels produced over those found in wild-type strains (*see* p. 305). Mutants of *E. coli* which are *bioR* and which have been transformed with a plasmid containing the *bio* operon, can produce substantially higher amounts of biotin in terms of that excreted into the culture medium. These data are summarized in *Table 5*.

Table 5. Influence of the use of biotin deregulated strains on the excretion of biotin in culture media

E. coli strain	Relevant strain genotype	Biotin excreted per litre	Reference
Wild type*	*bio*$^+$*bioR*$^+$*birA*$^+$	<0·015 μg	Pai (1974)
P48*	*bioR*	16 μg	Pai (1972)
S942*	*birA*	7–22 μg	Campbell, del Campillo-Campbell and Chang (1972)
FM6*,†	*bio*$^+$*bioR*$^+$*birA*$^+$	<0·39 μg	Fisher (1987)
FM6/pKA5*,†	*bio*$^+$*bioR*$^+$*birA*$^+$	4·2 μg	Fisher (1987)
BM4062*,†	*bio*$^-$*bioRbirA*^{TS}	<0·39 μg	Fisher (1987)
BM4062/pKA5*,†,‡	*bio*$^-$*bioRbirA*^{TS}	0·5–1·2 mg	Fisher (1987)

* Erlenmeyer flask cultures at 37°C.
† Precultures grown at 30°C.
‡ Plasmid pKA5 is a temperature-sensitive runaway replicon containing the *bio* operon, *bioABFCD*, with a temperature-sensitive origin of replication (Fisher, 1987).

Strains of E. coli *having a temperature-sensitive* birA *function*

Table 5 describes the use of mutant strains having a temperature-sensitive *birA* gene (*birA*^{TS}). Strains of *E. coli* possessing a *birA*^{TS} mutation are viable at 28°C, but cannot grow at 43°C. The dual function of the *birA* gene product (biotin-protein ligase) as the *bio* operon transcriptional repressor and its involvement in the transfer of biotin to acetyl-CoA carboxylase has already been described (p. 306). Acetyl-CoA carboxylase catalyses a critical step in fatty acid synthesis, which is essential for viability. For cell growth, the complete elimination of *birA* activity at the initiation of a culture would probably be lethal. Therefore, at the start of a culture, sufficient *birA* activity should be present to support growth of the cells. Towards the end of the growth phase, and during a possible biotin production phase, *birA* activity could be substantially diminished or eliminated in order to derepress the *bio* operon. By employing a *birA*^{TS} gene, regulation of the *birA* function has been achieved by controlling the temperature of the fermentation process. As the temperature of the system was increased from 30°C at the beginning, to 40°C towards the end of the fermentation, the *birA* function of the cell was decreased (Fisher, 1987).

Low-acetate-synthesizing E. coli *strains*

Escherichia coli has been shown to produce acetate under certain growth conditions (Mori *et al.*, 1979; Andersen and von Meyenburg, 1980; Meyer, Leist and Fiechter, 1984). Acetate formation can lead to growth inhibition and, in consequence, a reduced product yield. Meyer *et al.* (1984) and Brown, Meyer and Fiechter (1985) demonstrated this for the production of recombinant α-interferon by *E. coli*. The metabolism of acetate formation in *E. coli* has been attributed to a mechanism similar to the Crabtree effect in yeast (Andersen and von Meyenburg, 1980; Rinas, Kracke-Helm and Schügerl, 1989). This can be clarified and explained by way of analogy: there is a somewhat better-known situation in some species of yeast, e.g. *S. cerevisiae*, which is manifested as the Pasteur/Crabtree effect. In glucose-sensitive yeasts, such as *S. cerevisiae* (Fiechter, Fuhrmann and Käppeli, 1981), one can observe the synthesis of an inhibitor (ethanol) as a consequence of the organisms' limited inability to oxidatively decarboxylate pyruvate above a certain critical glycolytic carbon flux and, hence, growth rate (Fiechter, Fuhrmann and Käppeli, 1981; Sonnleitner and Käppeli, 1986; Marquet *et al.*, 1987). For *E. coli*, one way of overcoming the problem of acetate formation is to produce fluoroacetate-resistant strains. Fluoroacetate is a metabolic analogue of acetate. Under these circumstances an excess flux of pyruvate is balanced by the diversion of carbon flux to lactate excretion rather than to acetate, and hence a higher growth rate can be obtained (El-Mansi and Holms, 1989).

Haze, Ifuku and Kishimoto (1989) have published information regarding an improvement in the operation of the last biosynthetic step, DTB → biotin (catalysed by the enzyme biotin synthetase, which is encoded by the gene,

bioB). It has already been mentioned that the conversion of DTB into biotin requires the presence of an unidentified sulphur donor (*see* p. 300). Haze, Ifuku and Kishimoto (1989) demonstrate that the use of low-acetate-forming *bioR* mutants of *E. coli*, transformed with a plasmid containing the *E. coli bio* operon, have a significantly improved ability to convert DTB into biotin (*Table 6*). This improved conversion ability is claimed to result from prolonged growth made possible by using low-acetate-forming *E. coli bioR* strains. One interpretation of this is that because the cells have a higher growth rate and a much longer period of active growth (than normal acetate producers), the unidentified sulphur donor will be produced during a much longer period, therefore allowing better DTB → biotin conversion. It is not clear if there is a *minimum* growth rate necessary for good DTB → biotin conversion. However, this might be a relevant factor *together* with continuing, balanced, nutrient-unlimited exponential growth.

In the case of Haze, Ifuku and Kishimoto (1989), low-acetate-synthesizing mutants were obtained from mutation experiments using N-methyl-N′-nitro-N-nitrosoguanidine, followed by selection for growth in the presence of sodium fluoroacetate (Harford and Weitzman, 1980). Individual mutants were then selected for their ability to produce, at most, 20% of the amount of acetate produced by a wild-type *E. coli* strain (calculated on a weight basis by the yield, $Y_{P/X}$). These mutants were low-acetate-forming in conditions of high or low aeration.

INFLUENCE OF FERMENTATION CONDITIONS ON THE PRODUCTION OF VITAMERS AND BIOTIN BY GENETICALLY MODIFIED *E. COLI* STRAINS

Data published with respect to the physiological performance of genetically engineered biotin producers *in vivo* culture conditions focus on the use of the *bio* genes from *E. coli* or *B. sphaericus*, transformed into *E. coli*. Virtually no data are available for the *bio* genes transformed into *B. sphaericus* (but *see* p. 310). Reasons cited for employing *E. coli* rather than *B. sphaericus* include a greater understanding of its microbial physiology and the ease of its cultivation to high cell densities (Sabatié *et al.*, 1991). Optimization of *B. sphaericus* for growth to high cell densities, although considered possible, has been seen as requiring a much longer development time, due to this

Table 6. Influence of low-acetate-forming *E. coli* strains on DTB and biotin synthesis

E. coli strains	Final biomass ($g\ l^{-1}$)	Final acetate ($g\ l^{-1}$)	Acetate yield, $Y_{P/X}$	DTB ($mg\ l^{-1}$)	Biotin ($mg\ l^{-1}$)
DRK332	31	16	0·52	2	2
DRK322/pXBA312	25	15	0·6	23	21
DRK3323*	73	5	0·07	2	5
DRK3323/pXBA312*	62	4	0·06	10	52
DRK322/pXBA312*,†	27	17	0·63	83	40
DRK3323/pXBA312*,†	65	6	0·09	15	105

All strains were grown in bioreactors

* Strain DRK3323 is a low-acetate-synthesizing *bioR* mutant obtained from strain DRK322 (*bioR*). Plasmid pXBA312 contains the *bio* operon, *bioABFCD*.

† In these experiments the culture medium was supplemented with L-alanine.

micro-organism's complex nutritional demands (Brown *et al.*, 1991).

Numerous aspects of a recombinant strain's fermentation physiology have been shown to influence biotin biosynthesis (Fisher, 1987; Ifuku and Yanagi, 1989; Brown *et al.*, 1991; Sabatié *et al.*, 1991).

Striking gains in productivity, notably due to changing the cultivation method (i.e. tubes, Erlenmeyer (baffled) flasks or fermenter cultures) have been demonstrated (Brown *et al.*, 1991). Values of roughly 1 mg l^{-1} biotin with yields ($Y_{P/X}$, mg biotin per g dry weight cells) between 0·03 (Brown *et al.*, 1991) and 0·4 (estimated from Fisher, 1987) have been reported for small-scale cultures, e.g. tubes or Erlenmeyer flasks. Variation of yield with small cultures has been highlighted as one reason for performing optimization with fermenters rather than flask or tube cultures (Fisher, 1987; Brown *et al.*, 1991; Sabatié *et al.*, 1991). *Table 7* illustrates the gains in biotin yields achieved with successive changes of cultivation method.

Fisher (1987) proposed the use of a thermoinducible high-copy-number plasmid, giving between about 40 and 200 plasmid copies upon temperature induction, as aiding biotin production.

The amino acids, L-alanine (required for the step, pimelyl-CoA → KAPA), L-methionine (required for *S*-adenosyl-L-methionine for the step, KAPA → DAPA) and L-cysteine (as a source of sulphur for L-methionine and for the sulphur donor for the last step, DTB → biotin) have been shown to have a stimulatory effect on the biotin biosynthetic pathway (Izumi and Ogata, 1975). Refer to *Figure 1* for the pathway. The benefit of the addition of L-alanine to the culture medium was illustrated by Haze, Ifuku and Kishimoto (1989). An improvement in biotin production is suggested (*see Table 6*). Fisher (1987) also included the above three amino acids in the cultivation medium, but it is not clear whether biotin formation was improved as a result. The addition of these amino acids to cultures of *E. coli* transformed with the *B. sphaericus bio* genes did not apparently cause any improvement in biotin production (J. Sabatié, personal communication). The use of an *E. coli metJ* strain (methionine overproducer) did not cause any improvement in biotin production, but did increase the total vitamers formed (Sabatié *et al.*, 1991).

Brown *et al.* (1991) and Sabatié *et al.* (1991) showed, while working to obtain a reproducible, standard, substrate-fed, batch culture, that aspects of the strain storage, preculture preparation, culture type, medium components

Table 7. Progressive improvements in vitamer and biotin production using *E. coli* C268/pTG3410 (*bioDAYB* and *bioXWF* genes of *B. sphaericus*): influence of cultivation method (after Brown *et al.*, 1991, reproduced with permission)

Cultivation method	Cell dry weight (g l^{-1})	Vitamers (mg l^{-1})	Biotin (mg l^{-1})	Factor of improvement for biotin
5 ml tube culture, 30°C	3·7	30	1	1
2 l batch fermenter, 30°C	18	74	10	10
2 l batch with substrate-fed fermenter, 30°C	21	250	16	16
2 l batch fermenter, 37°C	18	200	30	30
20 l fed-batch fermenter, 30°C, shifted to 37°C at start of fed-batch feeding	50	350	45	45

and physical conditions needed to be considered. They played a major role in the formation of biomass, plasmid stability and the flux of intermediate compounds from the precursor pimelic acid (in the case of the *B. sphaericus bio* genes) to biotin. Equally, the use of a standardized batch fermentation demonstrated that the host-strain gene-cluster orientation and control of gene expression also influenced growth and the productive metabolic flux to biotin.

Table 8 summarizes the highest published biotin-producing results obtained in batch or fed-batch fermentations. It is very difficult to quantitatively compare the results from different workers. Neither the genetic background of the strain nor the expression system is the same. It was previously mentioned that some workers considered the *B. sphaericus bio* genes to be more active, based on the observation that this micro-organism could synthesize significantly more biotin vitamers than *E. coli*, particularly from the precursor pimelic acid (*see* pp. 297 and 298). Notwithstanding the difficulty of making a direct comparison, as mentioned above, *Table 8* shows that yield constants ($Y_{P/X}$) of 1·62 mg biotin/g cells (for *E. coli bio* genes) and 1·7 mg biotin/g cells (*B. sphaericus bio* genes) have been calculated. It is at least possible that, subject to a comparative kinetic analysis of each enzyme, there may be no difference between the *bio* genes of these two micro-organisms.

Analysis of biotin vitamers and biotin formation: potential for improvements

An analysis of the overall performance of the biosynthetic capability of the *B. sphaericus bio* genes, *bioXWF* (catalyses exogenous pimelic acid → KAPA) and *bioDAYB* (catalyses KAPA → biotin) in *E. coli* can be made from data published by Ohsawa *et al.* (1989), Gloeckler *et al.* (1990a), Brown *et al.* (1991) and Sabatié *et al.* (1991). *Figure 3* shows data from a substrate-fed, batch culture of *E. coli* C268/pTG3410. These data show a close relationship between growth and total vitamers formation. Total vitamers continued to accumulate when the absorbance of the culture no longer increased. Not all of the vitamers showed identical production kinetics. Although KAPA formation generally followed the total vitamers curve, biotin and DTB formation were more disparate. The DAPA assay was too unreliable to give usable results. The individual vitamer having the highest amount formed was KAPA, indicating that, in terms of metabolic flux, there is a bottleneck between this intermediate and DAPA. There was also a considerable amount of DTB present, suggesting some degree of bottleneck between this intermediate and biotin. Analysis of vitamers formation kinetics in fed-batch culture revealed that biomass, total vitamers and biotin formation did not follow the relationship observed for the standard batch. However, the reason for this seemed to be associated with a culture viability problem.

One is able to draw conclusions and point to at least six parameters that require investigation when producing biotin from exogenous pimelic acid via the above system. These are shown schematically in *Figure 4*.

Table 8. Production levels of vitamers and biotin in strains of *E. coli* transformed with plasmids containing the *E. coli* or the *B. sphaericus bio* genes and grown in fermenters

Strain	Relevant genotype	Genes* used	Cell density ($g\ l^{-1}$)	Total vitamers ($mg\ l^{-1}$)	Biotin ($mg\ l^{-1}$)	$Y_{P/X}$	Reference
BM4062/pKA5	*bio*$^{-}$*bioRbirA*^{TS}	*bioABFCD*	11·4‡	NS	30	≥2·62‡	Fisher (1987)
DKR3323/pXBA312	*bioR*	*bioABFCD*	65	15§	105	1·62	Haze, Ifuku and Kishimoto (1989)
C268/pTG3410‖,**	Δ*bioAbioR*$^{+}$*birA*$^{+}$	*bioDAYB bioXWF*	18	200	30	1·7	Sabatié *et al.* (1991)
C268/pTG3410¶,**	Δ*bioAbioR*$^{+}$*birA*$^{+}$	*bioDAYB bioXWF*	45	350	45	1	Sabatié *et al.* (1991)

* Genes used: *bioABFCD* = *E. coli*: *bioDAYB* and *bioXWF* = *B. sphaericus*.
† $Y_{P/X}$ = mg biotin measured in medium (produced) per g dry weight cells.
‡ Values estimated from available data.
§ This value for DTB only.
‖ Batch fermentation employing a glycerol substrate feed.
¶ Fed-batch fermentation.
** In these fermentations the medium contained the precursor pimelic acid.

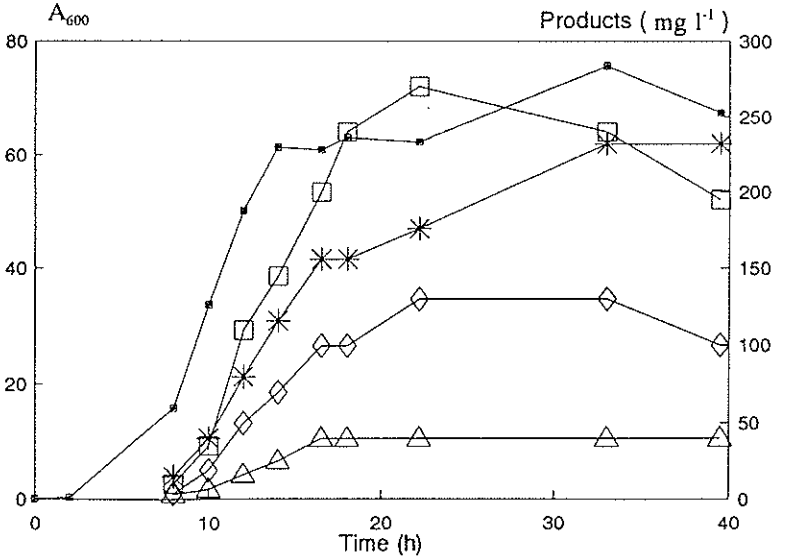

Figure 3. Time-course for the production of biotin and vitamers by *E. coli* C268/pTG3410 using *B. sphaericus bio* genes, *bioDAYB* and *bioXWF* (after Sabatié *et al.*, 1991, reproduced with permission). Time-course of growth for the recombinant strain in 2 l standard substrate-fed, batch culture and kinetics of biotin and vitamers synthesis. A_{600} (■); biotin × 10 (mg l^{-1})(★); total vitamers (mg l^{-1}) (□); KAPA (mg l^{-1}) (◇); DTB (mg l^{-1}) (△). Culture conditions: 37°C; pH 7; P_{O_2}, 30% of saturation; induction at A_{600} 0·3; glycerol feed (4 g h^{-1}) starting at A_{600} 25–28.

Conclusions and perspectives

The application of genetic engineering technology to achieving an improvement in biotin production by micro-organisms is a commercially competitive subject. A common theme amongst all of the teams who have publications or patents in this field is that the highest biotin yields have always been attained by a combination of genetic engineering, the use of host micro-organisms having modified genetic backgrounds and an optimization of fermentation conditions. Nevertheless, existing biotin yields remain well below those considered necessary for a commercial process.

It is possible that micro-organisms other than *E. coli* or *B. sphaericus* may attract attention for biotin production. This can be illustrated by the recent publication of Komatsubara *et al.* (1990). These authors have cloned the *E. coli bio* operon into an actithiazic acid (ACM) resistant strain of *Serratia marcescens* and achieved a biotin yield of 190 mg l^{-1}.

Although information availability remains constrained by confidentiality, it seems likely that significant gains in biotin production levels over those reported here already have been or will be achieved during the next 12–18 months.

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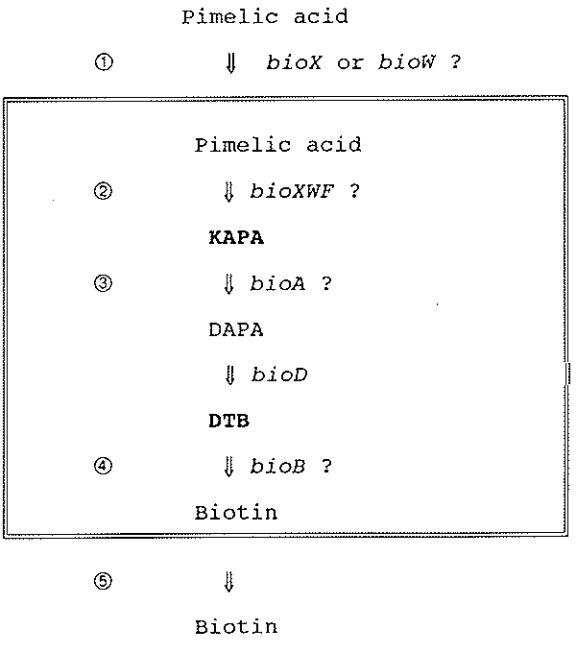

Figure 4. Schematic representation of the production of biotin by *E. coli* transformed with the *bio* genes of *B. sphaericus*. Problem points for the biotransformation of exogenous pimelic acid into biotin. The *interior* of the continuous double-lined box represents the *interior* of an *E. coli* cell expressing *bioXWF* and *bioDAYB*. The precursor, pimelic acid, has been added to the culture medium outside the cell. The ionization state of pimelic acid has been ignored for the purposes of convenience in this figure. The numbers refer to points described below as being question marks in the biotransformation of exogenous pimelic acid to biotin by *E. coli* C268/pTG3410 (see Sabatié *et al.*, 1991). (1) Transport of pimelic acid into the cell: role of *bioW* or *bioX*. Under the conditions used, the precursor pimelic acid was not limiting for vitamers production. A higher concentration resulted in the formation of more vitamers but no extra biotin. (2) Conversion of pimelic acid into KAPA: role of *bioW* and *bioX*, relationship to *bioF*. (3) Conversion of KAPA into DAPA: the first bottleneck in the pathway. Significant amounts of KAPA accumulate in the culture medium. Expression level of *bioA*? (4) Conversion of DTB into biotin: the second bottleneck in the pathway. Significant amounts of DTB also accumulate in the culture medium. Expression of *bioB* was high, was biotin synthetase insoluble? This step seems to be the most difficult. It has been noted previously (*see* p. 300) that a co-factor molecule (a sulphur donor, as yet unidentified) is crucial for the operation of this step. Data from several authors suggest that *E. coli* only synthesizes this co-factor during growth. What is the relationship to growth rate? (5) Export of biotin out of the cell. (6) Fermentation conditions (optimization of gene expression, culture medium, production of high biomass and influence of the host strain).

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