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MINI-REVIEW

Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems

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Abstract During the proteomics period, the growth in the use of recombinant proteins has increased greatly in the recent years. Bacterial systems remain most attractive due to low cost, high productivity, and rapid use. However, the rational choice of the adequate promoter system and host for a specific protein of interest remains difficult. This review gives an overview of the most commonly used systems: As hosts, *Bacillus brevis*, *Bacillus megaterium*, *Bacillus subtilis*, *Caulobacter crescentus*, other strains, and, most importantly, *Escherichia coli* BL21 and *E. coli* K12 and their derivatives are presented. On the promoter side, the main features of the L-arabinose inducible *araBAD* promoter (P_{BAD}), the *lac* promoter, the L-rhamnose inducible $rhaP_{BAD}$ promoter, the T7 RNA polymerase promoter, the *trc* and *tac* promoter, the lambda phage promoter p_L, and the anhydrotetracycline-inducible *tetA* promoter/operator are summarized.

Introduction

The production of recombinant proteins in a highly purified and well-characterized form has become a major task for the protein chemist within the pharmaceutical industry (Schmidt 2004). Bacterial expression systems for heterologous protein production are attractive because of their ability to grow rapidly and at high density on inexpensive substrates, their often well-characterized genetics and the availability of an increasingly large number of cloning vectors and mutant host strains. To produce high levels of protein, it is often useful to clone the gene downstream of a well-characterized, regulated promoter.

In general it is difficult to decide which host and promoter system is the best for heterologous protein production. It depends often on the target protein itself. This review describes a variety of bacterial host and promoter systems widely used for heterologous protein production. Nevertheless many bacterial systems are not able to modify proteins posttranslationally such as glycosylation. If the posttranslational modification is essential for bioactivity, bacterial expression systems should not be used for heterologous protein production. Alternative hosts such as yeasts, filamentous fungi, or insect and mammalian cell cultures are available for this application (reviewed in Schmidt 2004).

Escherichia coli as host

The gram-negative bacterium *E. coli* is the most commonly used organism for heterologous protein production. One of the reasons seems to be that this organism is very well-known and established in each laboratory. So it is no surprise that *E. coli* systems are also most commonly used for industrial and pharmaceutical protein production. Large-scale production systems are established. A disadvantage for therapeutic use of produced recombinant proteins in *E. coli* is the accumulation of lipopolysaccharide (LPS), generally referred as endotoxins, which are pyrogenic in humans and other mammals. Proteins for this application must be purified in a second step to become endotoxin-free (Petsch and Anspach 2000).

In general, overexpressed recombinant proteins accumulate either in the cytoplasm or periplasmic space. Most

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Table 1 Some *E. coli* strains most frequently used for heterologous protein production and their key features

E. coli strain	Derivation	Key features
AD494	K-12	*trxB* mutant; facilitates cytoplasmic disulfide bond formation
BL21	B834	Deficient in *lon* and *ompT* proteases
BL21 trxB	BL21	*trxB* mutant; facilitates cytoplasmic disulfide bond formation; deficient in *lon* and *ompT* proteases
BL21 CodonPlus-RIL	BL21	Enhances the expression of eukaryotic proteins that contain codons rarely used in *E. coli*: AGG, AGA, AUA, CUA; deficient in *lon* and *ompT* proteases.
BL21 CodonPlus-RP	BL21	Enhances the expression of eukaryotic proteins that contain codons rarely used in *E. coli*: AGG, AGA, CCC; deficient in *lon* and *ompT* proteases.
BLR	BL21	*recA* mutant; stabilizes tandem repeats; deficient in *lon* and *ompT* proteases
B834	B strain	Met auxotroph; ^{35}S-met labeling
C41	BL21	Mutant designed for expression of membrane proteins
C43	BL21	Double mutant designed for expression of membrane proteins
HMS174	K-12	*recA* mutant; Rif resistance
JM 83	K-12	Usable for secretion of recombinant proteins into the periplasm
Origami	K-12	*trxB/gor* mutant; greatly facilitates cytoplasmic disulfide bond formation
Origami B	BL21	*trxB/gor* mutant; greatly facilitates cytoplasmic disulfide bond formation; deficient in *Ion* and *ompT* proteases
Rosetta	BL21	Enhances the expression of eukaryotic proteins that contain codons rarely used in *E. coli*: AUA, AGG, AGA, CGG, CUA, CCC, and GGA; deficient in *lon* and *ompT* proteases
Rosetta-gami	BL21	Enhances the expression of eukaryotic proteins that contain codons rarely used in *E. coli*: AUA, AGG, AGA, CGG, CUA, CCC, and GGA; deficient in *Ion* and *ompT* proteases; *trxB/gor* mutant; greatly facilitates cytoplasmic disulfide bond formation

Most strains are also available as DE3 and DE3 pLysS strains. Strains are commercially available from different manufacturers

frequently, the cytoplasm is the first choice for heterologous protein production because the higher yield seems to be more attractive. Also, remarkable yields of secreted proteins are well-documented (reviewed in Georgiou and Segatori 2005). For routine protein expression, *E. coli* BL21 and K12 and their derivatives are most frequently used (Table 1). In contrast to K12 strains, BL derivates are *lon* (Phillips et al. 1984) and *ompT* protease deficient. Overexpression of a gene in a foreign host resulted often in lots of unwanted problems (Table 2a). Most problems are the result of the difference between the codon usage of the *E. coli* and the overexpressed protein, e.g., eukaryotic

Table 2a Some problems of heterologous protein production in *E. coli* and possible solutions

Symptom	Possible problem	A collection of solutions
Cell death or no colonies	Toxic protein, high basal expression	More stringent control over basal expression Tightly controlled promoter system Weaker promoter Lowering temperature Lowering inducer concentration
Insoluble disulfide protein (inclusion bodies)	Reduction of disulfide bonds	Minimize reduction in cytoplasm Accumulation in the periplasm
Insoluble protein (inclusion bodies)	Too much expression	Attenuate expression by: weaker promoter, lowering temperature, lowering inducer concentration, decrease plasmid copy number, fusion of a hydrophilic affinity tag
No activity	Misfolded protein, affinity tag can decrease activity	Minimize reduction in cytoplasm Accumulation in the periplasm Attenuate expression Change affinity tag
No protein, truncated protein	*E. coli* codon usage (codon bias)	Supply rare tRNAs Stronger promoter Increase plasmid copy number Lower temperature Tightly controlled promoter system

Nevertheless, another bacterial host than *E. coli* could also solve the problem

Table 2b Comparison of rare codons in *E. coli*

Organism	AGG arginine	AGA arginine	CUA leucine	AUA isoleucine	CCC proline	GGA glycine
Bacterial hosts						
Escherichia coli B	2.1	2.4	3.4	5.0	2.4	8.2
E. coli K12	1.2	2.1	3.9	4.3	5.5	7.9
Anabaena sp.	2.6	8.3	14.0	8.3	13.0	12.4
Bacillus megaterium	2.7	9.1	10.9	10.3	2.9	26.2
Bacillus subtilis	3.9	10.5	4.8	9.3	3.3	21.8
Caulobactert crescentus CB 15	2.2	0.8	1.4	0.6	18.7	4.3
Methylobacterium extorquens	1.2	0.6	0.8	0.3	18.7	3.7
Staphylococcus carnosus	0.4	7.9	4.6	9.8	0.8	16.7
Streptomyces lividans	4.0	1.1	0.6	0.8	22.7	6.5
Some organisms						
Arabidopsis thaliana	10.9	18.9	9.9	12.6	5.3	24.2
Caenorhabditis elegans	4.0	15.4	7.9	9.4	4.4	31.6
Clostridium tetani E88	5.0	25.5	11.2	67.4	1.8	34.6
Drosophila melanogaster	6.3	5.2	8.2	9.5	18.0	17.7
Homo sapiens	11.9	12.0	7.2	7.4	19.9	16.5
H. sapiens Mitochondriom	0.4	0.4	70.4	44.5	33.9	18.9
Plasmodium falciparum	3.3	17.0	5.3	40.8	3.0	20.0
Pichia pastoris	6.6	20.2	10.9	11.7	6.7	19.1
Picrophilus torridus DSM9790	22.9	16.2	7.8	30.3	2.6	18.1
Saccharomyces cerevisiae	9.3	21.3	13.4	17.8	6.8	10.9

The codon usages of bacteria used for heterologous protein expression and some organisms are listed (for *Bacilus brevis* not available). Codon frequencies are expressed as codons used per 1,000 codons encountered. The arginine codons AGG and AGA are recognized by the same tRNA and should therefore be combined. Codon frequency of more than 15 codons/1,000 codons may cause problems for high-level expression in *E. coli*. A complete summary of codon usages can be found at http://www.kazusa.or.jp/codon/

proteins. Rare codons could especially be a problem (Table 2b). It is well-known that amino acids are encoded by more than one codon, and each organism carries its own bias in the usage of the 61 available amino acid codons. In each cell, the tRNA population closely reflects the codon bias of the mRNA population (Dong et al. 1996). If the mRNA of heterologous target genes is intended to be overexpressed in *E. coli*, differences in the codon usage can impede translation due to the demand for one or more tRNAs that may be rare or lacking in the expression host (Kane 1995; Goldman et al. 1995). Insufficient tRNA pools can lead to translational stalling, premature translation termination, translation frameshift, and amino acid misincorporation (Kurland and Gallant 1996).

Table 3 Incubation temperature and time after induction to change codon bias in *E. coli* enhancing protein concentration or increasing soluble protein

Incubation temperature (°C)	Incubation time (h)
8	24–72
15	16–24
20	12–16
25	6–12
30	5–6
37	3–4

Yield of soluble protein could be also increased by lowering the temperature of the preculture, e.g., 30 or 20 °C

Theoretically, modification of culture conditions, e.g., lowering the temperature (Table 3) or changing media composition might shift the codon usage bias enough to alleviate some codon-usage based expression problems. However, it was reported that the levels of most tRNA isoacceptors corresponding to rare codons remain unchanged at different growth rates (Dong et al. 1996). Translation problems similar to those caused by codon usage bias can also be created by high-level expression of proteins having an abundant amino acid. In this case, expression may be improved by supplying the limiting amino acid in the culture medium (Kane 1995). Reduction of the inducer concentration seems to be possible also, but mostly unsuccessful. To enhance expression of eukaryotic proteins or proteins that contain codons rarely used in *E. coli*, many *E. coli* strains were engineered to meet this problem (Table 1). These strains supply additional tRNAs under control of their native promoters.

Independent on host and promoter system, low, middle, and high copy plasmids could be used to reduce expression problems due to an inadequate expression level. For example, the most frequently used vectors based on the plasmid ColE1, which aids in the isolation of large amounts

Table 4 Some *E. coli* promoter systems that are in use for heterologous protein production and their characteristics

Expression system based on	Induction (range of inductor)	Level of expression	Key features	Original reference
lac promoter	Addition of IPTG 0.2 mM (0.05–2.0 mM)	Low level up to middle	Weak, regulated suitable for gene products at very low intracellular level Comparatively expensive induction	Gronenborn (1976)
trc and *tac* promoter	Addition of IPTG 0.2 mM (0.05–2.0 mM)	Moderately high	High-level, but lower than T7 system Regulated expression still possible Comparatively expensive induction High basal level	Brosius et al. (1985)
T7 RNA polymerase	Addition of IPTG 0.2 mM (0.05–2.0 mM)	Very high	Utilizes T7 RNA polymerase High-level inducible over expression T7*lac* system for tight control of induction needed for more toxic clones Relative expensive induction Basal level depends on used strain (pLys)	Studier and Moffatt (1986)
Phage promoter p_L	Shifting the temperature from 30 to 42 °C (45 °C)	Moderately high	Temperature-sensitive host required Less likelihood of "leaky" uninduced expression Basal level, high basal level by temperatures below 30 °C No inducer	Elvin et al. (1990)
tetA promoter/operator	Anhydrotetracycline 200µg/l	Variable from middle to high level	Tight regulation Independent on metabolic state Independent on *E. coli* strain Relative inexpensive inducer Low basal level	Skerra (1994)
araBAD promoter (P_{BAD})	Addition of L-arabinose 0.2 % (0.001–1.0 %)	Variable from low to high level	Can fine-tune expression levels in a dose-dependent manner Tight regulation possible Low basal level Inexpensive inducer	Guzman et al. (1995)
rhaP$_{BAD}$ promoter	L-rhamnose 0.2 %	Variable from low to high level	Tight regulation Low basal activity Relative expensive inducer	Haldimann et al. (1998)

of recombinant protein, may be replaced by low copy number (l cn) vectors when high copy number vectors cannot be used because large amounts of the recombinant protein are toxic. The vectors pWKS29, pWKS30, pWKS129, pWKS130 and its derivates carry the pSC101 replicon that produces six to eight plasmid copies per cell (Wang and Kushner 1991) and could be a useful tool to express proteins in *E. coli* like membrane proteins and kinases. Many different vectors are described but not further discussed in this review.

Nevertheless, lots of proteins could not be expressed in *E. coli*. For this case, bacterial hosts like *Bacillus brevis*, *Bacillus megaterium*, *Bacillus subtilis*, or *Caulobacter crescentus* could be an interesting alternative described in this review.

Another disadvantage of overexpression in the cytoplasm is that a lot of proteins form inclusion bodies. Strategies for resolution could be lowering temperature, amino acids substitution, coexpression of chaperones, hydrophilic large fusion partner, adding of sorbitol, glycyl betain, sucrose, raffinose in the growth medium, changing culture conditions, e.g., pH, or changing the bacterial strain (reviewed in Hockney 1994; Makrides 1996). Alternatively, inclusion bodies can be solubilized and refolded to get functional and active products (reviewed in Singh and Panda 2005).

BL21 (DE3) derivatives were especially designed for the overexpression of membrane proteins (Miroux and Walker 1996). The mutant C41 (DE3) and double mutant C43 (DE3) could improve expression level of membrane proteins, e.g., b subunit of F_1F_o ATP synthase (Arechaga et al. 2000).

Periplasmic expression (e.g., proteins with disulfide bonds)

Export of proteins from bacterial cytoplasm is widely employed (Georgiou and Segatori 2005). It could be used

for simplifying downstream and N-terminal processing. It generally facilitates correct folding. Proteins with disulfide bonds should be especially accumulated in the periplasm because the cytoplasm is too reducing. This oxidation process is catalyzed by disulfide binding proteins DsbA, DsbB, DsbC, DsbD, and peptidyl-prolyl isomerases SurA, RotA, Fk1B, and FkpA (Joly and Swartz 1994; Shokri et al. 2003). To secrete recombinant proteins into the periplasmic space, a fusion with a leader peptide at the N terminus is necessary. Generally, types I and II mechanisms are commonly used for protein secretion in *E. coli* K-12 or B strains. The following signal peptides are in use for secretion of heterologously expressed proteins: Lpp, LamB, LTB, MalE, OmpA, OmpC, OmpF, OmpT, PelB, PhoA, PhoE, SpA, and Tat signal peptides (reviewed in Choi and Lee 2004; Mergulhao et al. 2005).

In contrast to the signal peptides of the *sec*-system, the Tat signal peptides of the twin-arginine translocation pathway transported folded proteins across the inner membrane. This could be an advantage because chaperons, who could be responsible of correct folding, are mainly located in the cytoplasm (reviewed in Choi and Lee 2004).

Proteins located in the periplasm can be secreted into the culture medium with an osmotic shock or cell wall permeabilization. Cell lysis is not necessary, so that cytoplasmic proteins cannot contaminate the purification process (Shokri et al. 2003). Most problems for heterologous protein production in the periplasmic space are incomplete translocation across the inner membrane (Baneyx 1999), proteolytic degradation (Huang et al. 2001), and insufficient capacity of the export machinery (Mergulhao and Monteiro 2004; Rosenberg 1998). When this capacity is overwhelmed, the excess of expressed recombinant protein is likely to accumulate in inclusion bodies. For optimization of the expression level, a careful balance of the promoter strength and gene copy number is necessary.

Alternatively, proteins with disulfide bonds can be overexpressed in the cytoplasm by thioreductase-deficient (*trxB*) and glutathione reductase (*gor*) deficient strains (Bessette et al. 1999; Ritz et al. 2001; see Table 1).

E. coli promoter systems

Many promoter systems of *E. coli* are described as tools for protein expression, but only a few of them are commonly used (Table 4). A useful promoter must be strong, has a low basal expression level (i.e., it is tightly regulated), must be easily transferable to other *E. coli* strains, and the induction must be simple and cost-effective, and should be independent on the commonly used ingredients of culturing media.

lac, *tac*, and *trc* promoter systems

The *E. coli* lactose utilization is one of the well-known regulation mechanism. Many promoters were constructed from *lac*-derived regulatory element (Polisky et al. 1976). The *lac* promoter is rather weak and rarely used for high-level production of recombinant proteins. But the leakiness may be an advantage for the production of membrane proteins or other gene products that are toxic to the cells. The synthetic *tac* (De Boer et al. 1983) and *trc* promoter (Brosius et al. 1985), which consists of the −35 region of the *trp* promoter and the −10 region of the *lac* promoter, only differ by 1 bp in the length of spacer domain. The *tac* promoter is at least five times more efficient than the *lac*UV5 promoter (Amann et al. 1983). *tac* and *trc* promoters are strong and allow the accumulation of up to 15–30 % of total cell protein. Induction of all these promoters including *lac* promoter could be achieved by adding non-hydrolysable lactose analog isopropyl-β-D-1-thiolgalactopyranoside (IPTG) (Table 4). The strength of the *tac* and *trc* promoter can be a problem to express successfully recombinant proteins, which are toxic to the cells. Especially, over expression of membrane proteins with the strong *trc* promoter can result into degradation (Quick and Wright 2002). All three promoter systems are regulated by catabolite repression and the metabolic state, which is represented by the cyclic AMP level. To reduce these problems, the T7 RNA polymerase system was developed.

T7 RNA polymerase system

One of the most widely used expression systems is the T7 RNA polymerase system (Studier and Moffatt 1986). The T7 RNA polymerase elongates chains about five times faster than *E. coli* RNA polymerase. The two polymerases recognize completely different promoters and can completely be used selectively. Thousands of homologous and heterologous proteins were successfully expressed to high levels in *E. coli* BL21 (DE3). The gene of the T7 polymerase is in strain BL21 (DE3) chromosomal located and under the control of a *lac* promoter derivate L8-UV5 *lac* (Grossman et al. 1998; Pan and Malcolm 2000). The L8-UV5 *lac* promoter contains point mutations that distinguish it from the wild-type *lac* promoter. Two point mutations are in the −10 region, which increase promoter strength and decrease its dependence on cyclic AMP, and its receptor protein called CAP. A third point mutation creates a stronger promoter that is less sensitive to glucose (Grossman et al. 1998). This allows strong induction of T7 RNA polymerase with IPTG (Table 4) even in the presence of glucose. Nevertheless, there is basal expression of T7

RNA polymerase for induction (Dubendorff and Studier 1991; Pan and Malcolm 2000), which can lead to problems if the produced proteins are toxic for the host cells (Studier 1991). One way to reduce basal level is to work with host strains containing pLysS or pLysE vectors. These vectors express the T7 lysozyme, a natural inhibitor of T7 RNA polymerase (Moffatt and Studier 1987). The basal level of BL21 compared to BL21 (pLysS) is nearly ten times lower but a residual background is still present which can also result in the described problems. A further problem is that T7 lysozyme is a bifunctional protein. It cuts a specific bond in the petidoglycan layer of the *E. coli* cell wall. This seems to be the reason why growth rates with strains containing pLysS or pLysE decreased. Another aspect is that T7 lysozyme can reduce expression following induction, resulting in markedly lower yields (Studier 1991). The reduction of basal T7 RNA polymerase level can also be achieved by adding 0.5–1.0 % glucose into the medium (Grossman et al. 1998). This effect of catabolite repression is much stronger for BL21 (DE3) than for BL21 (DE3) pLys (Pan and Malcolm 2000).

Phage promoter p_L

Another approach that is widely used for protein overexpression is to place a gene under the control of a regulated phage promoter p_L, which has moderately high expression level. The genes must be cloned downstream of a tightly regulated phage promoter p_L that is regulated by the cI repressor. The temperature-sensitive cI857 repressor allows control of gene expression by changing the growth temperature instead of induction by a chemical inducer. At 30 °C, the cI857 repressor is functional and it turns off expression, but at 42 °C, the repressor is inactivated so that gene expression is induced (Elvin et al. 1990; Love et al. 1996). Functional protein could be also purified by a shift up to 45 °C (Armarego et al. 1989). It is interesting to note that the phage promoter p_L is constitutive at low temperatures because cI857 repressor becomes fully active at 29 °C and higher (Lowman and Bina 1990). This constitutive system can be used for the production of proteolytically susceptible proteins at low temperatures (Menart et al. 2003).

tetA promoter/operator system

The *tetA* promoter/operator is useful for the tight regulation, high-level synthesis of foreign gene product in *E. coli* (Skerra 1994). It is regulated by the tetR repressor, which is not coded by an *E. coli* gene. The system is independent on the used *E. coli* strain, e.g., B or K12 derivates. Cells are induced by low concentration of anhydrotetracycline (Table 4). Anhydrotetracycline binds the promoter nearly 35-fold higher than tetracycline and its antibiotic activity is 100-fold lower (Degenkolb et al. 1991). Concentrations starting from 50 ng/ml cause full induction and have no effect on the growth of *E. coli* (Lutz and Bujard 1997). Lots of proteins were successfully expressed with this system, e.g., F_{ab} fragments or toxins (Skerra 1994). The maximum of induction is ca. 100-fold over uninduced level (Korpela et al. 1998). In contrast to other systems, the basal level is very low and independent on the *E. coli* strain and the metabolic state.

L-arabinose inducible P_{BAD} promoter

The promoter *araBAD* (P_{BAD}) of the arabinose operon is a useful alternative for heterologous protein production in *E. coli*. When a gene is cloned downstream of the P_{BAD} promoter, its expression is controlled by the AraC activator. Expression is induced to high levels on media containing arabinose. Moreover, expression is tightly shut off on media containing glucose but lacking arabinose. In general, genes cloned under the control of the *araC*-P_{BAD} promoter system are efficiently repressed. However, the levels of expression of P_{BAD} controlled genes may not be zero in the repressed state (Guzman et al. 1995). However, *araC*-P_{BAD} promoter system allows high-level expression, tightly regulated protein expression, and very inexpensive induction with L-arabinose. In bacterial strains, which are deleted for *ara* genes, expression of the cloned gene reaches maximal induction upon adding 0.001 % L-arabinose. In strains that can ferment, 1 % is necessary for full induction (Mayer 1995). L-arabinose acts as inducer with the activator AraC in the positive control of the arabinose regulon (Haldimann et al. 1998). It was proven to function under high cell density fermentation, but the protein quality was shown to be lower than in low densities (DeLisa et al. 1999).

L-rhamnose inducible *rhaP*$_{BAD}$ promoter

The rhamnose-inducible *rhaP*$_{BAD}$ promoter is also an interesting tool for tightly regulated heterologous protein production in *E. coli*. The regulon of the operon is described in detail (Egan and Schleif 1993). In principle, L-rhamnose acts as an inducer with the activator RhaR for synthesis of RhaS, which in turn acts as an activator in the positive control of the rhamnose regulon (Haldimann et al. 1998). The L-rhamnose regulons are also regulated by catabolite repression. High cell density fermentation for production L-*N*-carbamoylase using the rhaBAD promoter is reported (Wilms et al. 2001).

Table 5 Some pharmaceutically and industrially relevant proteins that were successfully expressed in different Bacilli strains

Recombinant protein	Bacillus strain	Yield (mg/l)	Reference
α-amylase (*Bacillus amyloliquefaciens*)	*B. subtilis*	1,000–3,000	Palva (1982)
α-amylase (*Bacillus stearothermophilus*)	*B. brevis*	3,000	Udaka and Yamagata (1993)
α-amylase (human)	*B. brevis*	60	Konishi et al. (1990)
Cellulase	*B. brevis*	100	Kashima and Udaka (2004)
Cholera toxin B	*B. brevis*	1400	Ichikawa et al. (1993)
Dextransucrase (*Leuconostoc mesenteroides*)	*B. megaterium*	n.d. (362 U/g)	Malten et al. (2005)
Gelatin	*B. brevis*	500	Kajino et al. (2000)
Epidermal growth factor (human)	*B. brevis*	240	Yamagata et al. (1989)
Epidermal growth factor (human)	*B. subtilis*	7	Lam et al. (1998)
Epidermal growth factor (mouse)	*B. brevis*	50	Wang et al. (1993)
Mouse/human chimeric Fab′	*B. brevis*	100	Inoue et al. (1997)
Interferon-α2 (human)	*B. subtilis*	0.5–1.0	Palva et al. (1983)
Interleukin-2 (human)	*B. brevis*	120	Takimura et al. (1997)
Interleukin-6 (human)	*B. brevis*	200	Shiga et al. (2000)
Lipase A	*B. subtilis*	600	Lesuisse et al. (1993)
Penicillin G acylase	*B. subtilis*	n.d.	Yang et al. (2001)
Pepsinogen (swine)	*B. brevis*	11	Udaka and Yamagata (1993)
PHA depolymerase A (*Paucimonas lemoignei*)	*B. subtilis*	1.9	Braaz et al. (2002)
Proinsulin	*B. subtilis*	1000	Olmos-Soto and Contreras-Flores (2003)
Protein disulfide isomerase	*B. brevis*	1100	Kajino et al. (1999)
ScFv	*B. subtilis*	10–15	Wu et al. (2002)
Staphylokinase	*B. subtilis*	337	Ye et al. (1999)
Streptavidin	*B. subtilis*	35–50	Wu and Wong (2002)
Thioredoxin (*Aliciclobacillus acidocaldarius*)	*B. subtilis*	500	Anna et al. (2003)
Toxin A (*Clostridium difficile*)	*B. megaterium*	n.d.	Burger et al. (2003)

Other bacterial hosts than *E. coli*

Other bacterial hosts become more and more attractive for heterologous protein production. One reason is that increasing genomics knowledge enables to compare the codon usage of host and original organism. A host with a similar codon usage is optimal. *E. coli* may especially cause problems for high-level expression. Strains like BL21 CodonPlus-RIL, BL21 CodonPlus-RP, Rosetta, and others (see Table 1) are attempts to reduce these problems, but nevertheless with limited success. The codons AGG, AGA, CUA, AUA, CCC, CGA are very rarely used in *E. coli* (Table 2b). Codon frequency of more than 15 codons/1000 codons may cause problems for high-level expression in *E. coli* (Table 2b). Other bacterial hosts, like Bacilli and others (Table 2b), have another usage and can reduce this problem for high level expression. In comparison to *E. coli*, other bacterial hosts have more advantages in the field of protein production described in this review.

Bacilli as hosts

After *E. coli*, gram-positive Bacilli strains seem to be the most popular organism for heterologous protein production. Many pharmaceutically relevant proteins were successfully expressed in different strains (Table 5). In contrast to the well known *E. coli*, Bacilli strains have the general advantage that the outer membrane has no lipopolysaccharides. These LPS are well-known as endotoxins, which are pyrogenic in humans or other mammals. Furthermore the Bacilli strains are attractive hosts because they have a naturally high secretion capacity, and they export proteins directly into the extracellular medium. In contrast to *E. coli*, little is known about disulfide bond formation and isomerization (Westers et al. 2004).

B. megaterium

Heterologous gene expression in *B. megaterium* seems to be an interesting alternative system in contrast to *E. coli*. It has a number of favorable features as an expression host, including low protease activity, structural and segregational stability of plasmids, and the ability to grow on a wide variety of substrates. Highly efficient expression of homologous and heterologous genes was reported in the 1980s (Meinhardt et al. 1989) and was becoming popular in the 1990s (Rygus and Hillen 1991). Plasmids with the promoter of the xylose-operon are most frequently used for inducing high-level expression of heterologous genes (Rygus et al. 1991). Genes were 130- to 350-fold induced by using 0.5 % xylose. Induction was strongly inhibited by adding glucose because it bounds to XylR and thereby

acted as an anti-inducer (Kim et al. 1996). In contrast to *E. coli*, the transformation seems to be more difficult. Polyethylene glycol-mediated protoplasting is necessary for efficient transformation (Meinhardt et al. 1989; Vorobjeva et al. 1980). Toxins and other difficult to produce proteins were successfully made with *B. megaterium* as host. In some cases, recombinant protein made up 30 % of total soluble protein (England et al. 1997). Its protein secretion capacity makes it a suitable host for the production of exoenzymes such as various amylases, penicillin amidase, steroid hydrolases, or dextransucrase (Vary 1994; Malten et al. 2005). Signal peptides guide these exoenzymes to the secretion machinery of the Sec pathway in the cell membrane. Before translocation through the cell membrane, these proteins remain unfolded in the cell and fold outside the cytoplasm after proteolytic removal of the signal peptide (Tjalsma et al. 2000).

B. subtilis

B. subtilis is a well-studied prokaryote and much more used for heterologous protein production (reviewed in Li et al. 2004) than *B. megaterium*. Over the years, a number of expression systems for heterologous protein production were constructed (Henner 1990; Le Grice 1990). Its ability to secrete proteins directly into the medium is one of the greatest advantages. In the past, the major limitation of its application was the secretion of high levels of proteases into the culture medium, which have the potential to degrade other secreted proteins (Ulmanen et al. 1985; Nakamura et al. 1985). A double protease-deficient strain improves the stability of secreted proteins (Kawamura and Doi 1984, Stahl and Ferrari 1984). The use of a strong vegetative promoter and the manipulation of the medium compositions could improve the situation (Wong et al. 1986), but the development of an induction system based on the regulatory region and the SP of *sacB* makes it much more attractive (Wong et al. 1986). Highest expression level was achieved by using the *sacU*^{h} mutant strain WB30. This improved expression–secretion system was described in 1991 (Wu et al. 1991). The strain WB600 was deficient of six extracellular proteases and showed 0.32 % of the wild-type intracellular protease activity. To increase the production level, an expression cassette carrying *sacY*, a *sacB*-specific regulatory gene, was constructed. This gene was placed under the control of a strong, constitutively expressed promoter, P43. The *sacB* transcript stability correlated with the amount of produced recombinant protein (Daguer et al. 2005). Furthermore, development resulted in the strain WB800, which was eight proteases deficient (Murashima et al. 2002). All known extracellular proteases are deficient in the strain WB800 (Margot and Karamata 1996). This system could be optimized for culture and fermentation condition reached up to a level of 3,000 mg recombinant protein per liter (Table 5). Many different proteins are produced with this system, e.g., functional monomeric streptavidin (Wu and Wong 2005). The coexpression of EngB endoglucanase and mini-CbpA1 resulting in the in vivo synthesis of minicellulosomes (Cho et al. 2004) seems to be a remarkable application. Nevertheless, many promoter systems for *B. subtilis* are described in detail and are used, e.g., the vegetative *vegI* promoter (Lam et al. 1998), the xylose-inducible promoter (Kim et al. 1996), or the tetracycline inducible promoter (Geissendörfer and Hillen 1990). In contrast to *E. coli*, the transformation seems to be more difficult. Polyethylene glycol-mediated protoplasting is necessary for efficient transformation (Puyet et al. 1987). Nevertheless, *B. subtilis* seems to be an interesting species for industrial and pharmaceutical protein production (Westers et al. 2004).

B. brevis

B. brevis is not so well-studied as *B. subtilis*, but it is also an interesting host for heterologous protein production. Heterolog expressed proteins are secreted directly into the culture medium where they are accumulated at high levels in a relatively pure state. The secreted proteins are usually correctly folded, soluble, and biologically active. *B. brevis* has a very low level of extracellular protease activity, so that secreted proteins are usually stable and not significantly degraded. Protein expression could be enhanced by modification of the signal sequence (Sagiya et al. 1994) and isolation of a protease-deficient mutant (Kajino et al. 1997). Efficient secretion of heterologous proteins can be enhanced by fusion with a protein disulfide isomerase at the N terminus (Kajino et al. 1999). In contrast to *E. coli*, the transformation efficiency is not so high. *B. brevis* 47-5Q shows higher transformability than other *B. brevis* strains using the Tris–polyethylene glycol method or electroporation (Udaka and Yamagata 1993). High copy number plasmids (McKenzie et al. 1986) and low copy number plasmids (Horinouchi and Weisblum 1982) are in use for efficient protein production. The produced yield of secreted recombinant proteins is most frequently between 10 mg and 3 g per liter (Table 5).

Caulobacter crescentus

The gram-negative bacterium *Caulobacter crescentus* is generally found in freshwater environment. It is mainly in mind because of its distinctive life cycle. However, it secretes large amounts of the hydrophilic protein RsaA using the efficient type I secretion mechanism (Awram and Smit 1998). If the RsaA secretion signal is C-terminally fused to the protein of interest, it will be secreted into the

medium (Bingle et al. 2000). The C-terminal secretion signal appears to mediate the export of a wide variety of nonnative proteins through a large hydrophilic channel that traverses the membrane. A *lac* promoter system was used for the first attempts of heterologous protein production. Small and high copy vectors were designed later (Umelo-Njaka et al. 2001a). The hybrid proteins formed macroscopic aggregates in the culture fluids, which could be recovered through a nylon mesh in a highly purified form by coarse filtration of the culture. The system has the limitation that it is most efficient for small- and medium-size proteins up to 450 amino acids. It is now not so frequently used, but it seems to be an interesting tool for low-cost protein production. Overexpression of vaccine candidate proteins is reported (Umelo-Njaka et al. 2001b).

Other systems

In principle, all bacteria can be used for heterologous protein production. But the unknown information about their regulation and mechanism, as well as the fact that there are no commercial vector and promoter systems are the reasons that these systems are not so frequently used.

One of them is the gram-positive soil bacteria *Streptomyces* (Brawner 1994). Soluble forms of the human T-cell receptor CD was produced with *Streptomyces*.

Methylotrophic bacteria are attractive hosts for recombinant protein production because of the low cost of single substrates that sustain them. Expression level depends on the strain and could reach 10 % of total cell protein (FitzGerald and Lidstrom 2003). Enterocin P, a strong anti-listerial pediocin-like bacteriocin from *Enterococcus faecium* P13, was produced by *Methylobacterium extorquens* (Gutierrez et al. 2005).

The cyanobacterium *Anabaena* sp. could be an alternative to *E. coli* for the production recombinant proteins highly enriched with stable isotopes used for structural studies by nuclear magnetic resonance spectroscopy (Desplancq et al. 2005). Proteins were overexpressed using the endogenous promoter of the nitrate assimilation. Standard proteins were overexpressed upon induction with $NaNO_3$, yielding up to 250 mg/l of culture. When the cyanobacteria were grown in the presence of inexpensive ^{15}N-, ^{13}C-labeled mineral salts, and $^{2}H_2O$, the expressed polypeptides were highly enriched in stable isotopes. Furthermore, the tight repression of the *nir* promoter upon induction allowed the production of the toxic oncoprotein E6.

The nonpathogenic gram-positive bacterium *Staphylococcus carnosus* is able to secrete large amounts of proteins to the culture supernatant. The proteins can thus be isolated in soluble and relatively pure state. Laborious solubilization, renaturation, and purification procedures, which often strongly reduce the product recovery in *E. coli*, are dispensable in *S. carnosus*. The almost complete absence of proteolytic activities and the high genetic stability are furthermore advantages of the *S. carnosus* system (Hansson et al. 2002).

In recent years, *Pseudomonas fluorescens* (Schneider et al. 2005; Landry et al. 2003) and *Ralstonia eutropha* (Barnard et al. 2004) were used to produce remarkably high yields of recombinant proteins.

Nevertheless, these system are rarely in use, but might become relative more important in the near future.

Conclusion

During the last decades many bacterial hosts where optimized for heterologous protein production. Principally, all bacteria could be used for heterologous protein production. The genomics era offers new information about the bacteria hosts that are frequently and rarely used. Rarely used codons or endotoxins could be a reason to change from an *E. coli* system to another host. Another attractive reason for pharmaceutical industry to work with another host or new promoter system could be the patent situation. However, *E. coli* is still the most commonly used host for industrial production of pharmaceutical proteins, but it seems to be only a matter of time when FDA-approved pharmaceutical proteins are produced by other bacterial hosts.

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