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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/69, 15/70, 15/74, C12P 7/62

(11) International Publication Number:
WO 95/21260

(21) International Application Number:
PCT/US95/01471

(22) International Filing Date:
2 February 1995 (02.02.95)

(30) Priority Data:
08/195,481 3 February 1994 (03.02.94) US

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(54) Title: HIGH EXPRESSION NUCLEIC ACID VECTORS

(57) Abstract

Vector constructs comprising (a) a negatively regulated promoter operably linked to the desired nucleic acid sequence, (b) a runaway replicon, providing multiple copies of the vector construct upon heat induction, and (c) a stabilization locus. The vector constructs will typically be a plasmid, but viral vectors, cosmids, and other nucleic acid vector constructs are also included within the scope of the present invention. In a preferred embodiment, the vector construct further comprises a consensus Shine-Dalgarno sequence (preferably a lac Shine-Dalgarno sequence), operably linked to the desired nucleic acid sequence, thereby providing for increased translation of the desired nucleic acid sequence; the consensus Shine-Dalgarno sequence may either replace the native Shine-Dalgarno sequence or it may be in addition to such native sequence. Also, methods of producing a desired product encoded by, or resulting from, the desired nucleic acid sequence, bacterial host cells transformed with such vector constructs, and desired products produced according to the methods of the present invention.
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Description

HIGH EXPRESSION NUCLEIC ACID VECTORS

Cross-Reference to Related Applications

This application is a continuation-in-part of pending application U.S. Serial No. 08/035,433, filed March 24, 1993, which is a continuation-in-part of pending application U.S. Serial No. 07/890,925, filed May, 29 1992 and a continuation-in-part of pending application U.S. Serial No. 07/767,008, filed September, 27, 1991, all of which are incorporated herein by reference.

Technical Field

The present invention relates generally to nucleic acid vectors for the expression of a desired product arising from a desired nucleic acid sequence, including downstream products made by enzymes encoded by the desired nucleic acid sequence.

Background of the Invention

Numerous nucleic acid vectors suitable for the cloning, sub-cloning and expression of nucleic acid sequences, or molecules, such as genes, are well known in the fields of genetic engineering and biotechnology. One such group of vectors are used largely for cloning and sub-cloning, such as pBR322, considered to be one of the workhorses in these fields, which contains numerous restriction endonuclease recognition sites suitable for the insertion of a desirable nucleic acid sequence (Maniatis et al., Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982; this reference, and all other references cited herein, are hereby expressly incorporated by reference in their entirety).

Another group of such vectors include multicopy expression vectors able to produce large amounts of a desired product encoded by, or resulting from, the desired nucleic acid sequence, such as a gene. Desirable products include mRNA, proteins such as enzymes, and products produced by the action of an encoded enzyme. Typically, such expression vectors act by providing a regulable promoter that is induced to produce large amounts of mRNA encoded by the desired nucleic acid sequence in the presence of an inducer molecule. One example of such a system is the lac promoter, which can be derepressed by the addition of a chemical inducer, such as isopropyl-β-D-
thiogalactoside (IPTG), resulting in increased production from the desired nucleic acid sequence (Jacob and Monod, *J. Mol. Biol.* 3: 318-356, 1961; Maniatis et al., *supra*). Such vectors permit close control of the production of the desired gene product and provide an ample amount of mRNA corresponding to the desired nucleic acid sequence, but the addition of the inducer molecule can be very expensive. In an alternative approach, runaway replicon expression vectors produce large amounts of the operon itself by providing large quantities of the nucleic acid vector, as with pOU71 (Larsen, et al., *Gene* 28: 45-54, 1984) or pRA89 and pRA90 (Benzon Pharma A/S, Helseholmen 1, P.O. Box 1185, DK-2650 Hvidovre, Denmark). Such a vector may not need the addition of an inducer molecule (the vector copy number (*i.e.*, the gene dosage) can be increased by an upshift in temperature, for example), but the vector may not provide a large quantity of mRNA, and may not provide as rapid a production of the desired product.

However, when such vectors are grown (in a suitable host cell and growth medium), the vectors require a selective marker gene in order for the vector to be maintained in its host cell over a number of generations. Such selective marker genes include, for example, the chloramphenicol resistance gene (*cat*) or the ampicillin resistance gene (*bla*), which genes in turn require the addition of a selective pressure agent (such as an antibiotic) in the growth medium.

The expense of adding a chemical inducer and/or a selective pressure agent can be seen, for example, in the production of poly-\(\beta\)-hydroxyalkanoates (PHAs), which are a heterogeneous family of biodegradable aliphatic polyesters that are typically formed of monomers from 4-10 carbons. PHAs can be, for example, "random" copolymers wherein the copolymer comprises poly-\(\beta\)-hydroxybutyrate (PHB) and poly-\(\beta\)-hydroxyvalerate (PHV) dispersed randomly in the polymer backbone, or "semi-random," or blocked, copolymers wherein the copolymer comprises long or short chains of one particular PHA, for example PHB, that is separated by long or short chains of other PHAs, for example, randomly dispersed PHB and PHV. PHAs are synthesized by the action of three enzymes: \(\beta\)-ketothiolase, acetoacetyl-CoA reductase, and PHB synthase (Oeding and Schlegel, *Biochem. J.* 134:239, 1973; Senior and Dawes, *Biochem. J.* 134:225, 1973). \(\beta\)-ketothiolase condenses two acetyl-CoA molecules to acetoacetyl-CoA. Acetoacetyl-CoA reductase reduces this compound to \(\beta\)-hydroxybutyryl-CoA. PHB synthase typically polymerizes \(\beta\)-hydroxybutyryl-CoA into PHB, although other PHAs are produced under
particular conditions. PHB synthase, β-ketothiolase, and acetoacetyl-CoA reductase, respectively, are encoded in the phb operon, consists of three genes designated phbC, phbA, and phbB.

Clones that carry the phb operon on a multicopy plasmid can produce PHAs to levels as high as 80% (Janes et al., *Novel Biodegradable Microbial Polymers*, 175, 1990) and the cost of producing about 22,000 pounds of PHAs in a 100,000 liter fermentor would be about $22,000 if no antibiotic were necessary. But, the addition of the commonly used antibiotic chloramphenicol (at a concentration of 25 μg/ml) increases the cost by more than $14,000, and the addition of IPTG (at a concentration of 1 mM (0.238g/L)) increases the cost by more than $749,000.

Further information with respect to the production of PHAs, including PHB, can be found the following United States patent applications: U.S. Serial No. 07/362,514, filed June 7, 1989; U.S. Serial No. 07/528,549, filed May 25, 1990; U.S. Serial No. 07/705,806, filed May 24, 1991; U.S. Serial No. 07/767,008, filed September, 27, 1991; U.S. Serial No. 07/890,925, filed May 29, 1992; and, U.S. Serial No. 08/035,433 filed March 24, 1993. As noted above, each of these references are expressly incorporated herein in its entirety.

One advantage in the expression of a desired product arising from a desired nucleic acid sequence would be a nucleic acid vector capable of being induced to rapidly produce large amounts of the desired gene product without the addition of an inducer molecule and capable of being maintained in a host cell without the addition of a selective pressure agent to the growth medium. These and other advantages provided by the present invention will become apparent in the following summary and description.

**Summary of the Invention**

The present invention provides nucleic acid vector constructs capable of regulating the transcription and/or translation of a desired nucleic acid sequence, such as a gene or an operon. The present invention also provides methods of using such constructs to produce a desired product from the desired nucleic acid sequence, host cells transformed with such constructs, and desired products from the desired nucleic acid sequence.

Thus, in a first aspect the present invention provides a runaway replicon nucleic acid vector construct, comprising a) a promoter that is negatively regulated by a repressor molecule, b) an operator region capable of
binding the repressor molecule, c) a desired nucleic acid sequence, wherein the promoter is operably linked to the operator region and the desired nucleic acid sequence, and d) a stabilization locus.

In preferred embodiments, the promoter comprises a -35 region of a trp promoter operably linked to a -10 region of a lac promoter, and an operator region of the lac promoter, such as a tac promoter; the runaway replicon includes a λ pR promoter operably linked to a repA gene; and/or the stabilization locus is parB. Further preferably, the vector construct further comprises a consensus Shine-Dalgarno sequence operably linked to the desired nucleic acid sequence. One example of such a Shine-Dalgarno sequence is a lac Shine-Dalgarno sequence.

In a further aspect, the present invention provides methods of inducing a vector construct comprising, a) introducing into a host cell a runaway replicon vector construct, comprising i) a promoter that is negatively regulated by a repressor molecule, ii) an operator region capable of binding the repressor molecule, iii) a desired nucleic acid sequence, wherein the promoter is operably linked to the operator region and to the desired nucleic acid sequence, and iv) a stabilization locus; and b) increasing the temperature of the host cell, thereby expressing the desired nucleic acid sequence. In preferred embodiments, the methods further comprise culturing the host cell on an appropriate medium, then increasing the temperature of the host cell, and then further culturing the host cell for a time sufficient to produce a desired product from the desired nucleic acid sequence. In further preferred embodiments, the methods comprise the step of isolating the desired product from the host cell.

As above, in preferred embodiments the promoter of the vector construct comprises a -35 region of a trp promoter operably linked to a -10 region of a lac promoter, and an operator region of the lac promoter, such as a tac promoter; the runaway replicon includes a λ pR promoter operably linked to a repA gene; and/or the stabilization locus is parB. Further preferably, the vector construct further comprises a consensus Shine-Dalgarno sequence operably linked to the desired nucleic acid sequence. Further preferably, the host cell is an Enterobacteriaceae host cell, more preferably an E. coli or Klebsiella, particularly Klebsiella aerogenes.

In a further embodiment, the method is performed in a host cell containing an operable lacI9 gene, and/or during step (b), the methods include determining whether the culture of the host cells is growing rapidly or slowly; and then increasing the temperature early in a log phase of a growth cycle of the
culture when the culture is fast-growing, or increasing the temperature late in a
log phase of a growth cycle of the culture when the culture is slow-growing.
Preferably, the temperature is increased to at least 33°C when the vectors are
maintained in *Klebsiella* and at least 36°C when the vectors are maintained in
*E. coli*.

In a preferred embodiment, the methods further comprise growing
the host cell for multiple generations and not adding a selective pressure agent,
typically an antibiotic, to the medium.

In another aspect, the present invention provides an
Enterobacteriaceae host cell containing a vector construct as described above.
Preferably, the Enterobacteriaceae is *E. coli* or *Klebsiella*, particularly *Klebsiella
eaerogenes*.

These and other aspects of the present invention will become
evident upon reference to the following detailed description, examples and
attached drawings.

**Brief Description of the Drawings**

Figure 1 depicts the nucleotide sequence of a series of vector
constructs having certain transcriptional and/or translational fusions to the *phb*
operon. The nucleotide sequence denoted "a" comprises a *phb* promoter
operably linked to a putative *phbC* Shine-Dalgarno sequence (denoted "SD" in
the figure) (Seq. ID No. ______). This nucleotide sequence is present in the
plasmids pJM9131 and pJM9117. The nucleotide sequence denoted "b"
comprises a *tac* promoter and a *phbC* Shine-Dalgarno sequence wherein there is
an approximately 72 base pair leader sequence prior to the structural gene (Seq.
ID No. ______). This nucleotide sequence is present in the pJM9229 and
pJM9236 vector constructs. The nucleotide sequence denoted "c" comprises a
*tac* promoter and a putative *phbC* Shine-Dalgarno sequence wherein there is an
approximately 355 base pair leader prior to the structural gene (Seq. ID
No. ______). This nucleotide sequence is present in the pJM9232 and pJM9238
vector constructs. The nucleotide sequence denoted "d" comprises a *tac*
promoter and two *lac* Shine-Dalgarno sequences (Seq. ID No. ______). This
nucleotide sequence is present in the pJM9375 vector construct. The nucleotide
sequence denoted "e" comprises a *tac* promoter, two *lac* Shine-Dalgarno
sequences and a *phbC* Shine-Dalgarno sequence (Seq. ID No. ______). This
nucleotide sequence is present in the pJM9376 vector construct.
Figure 2 depicts a map of the vector construct pJM8801 (formerly known as p4A), which contains the phb operon, and the construction of the vector construct pJM9002, which is an 8.10 kb plasmid produced by cleaving the Eco RI/Hind III phb operon-containing fragment from p4A and ligating the fragment into the same sites of pBluescript SK+. Figure 2 also depicts the construction of the pJM9131 vector construct, which is an 8.55 kb plasmid derived from pJM8801, but a kanamycin gene block is located at the Eco RI site, and ampicillin resistance has been removed by Dra I digestion. Figure 2 further depicts the construction of the pJM9226 vector construct, which is a 3.10 kb plasmid comprising the tac promoter and a lac Shine-Dalgarno sequence ligated into the 3 kb Hind III-Bam HI fragment of pJM9002.

Figure 3 depicts a map of the vector construct pJM8703, which is an 8.50 kb plasmid comprising the phb operon cloned into pTZ18U from United States Biochemicals. The transcription pathway starts at the Kpn I site and ends at the Eco RI site. Figure 3 also depicts the construction of the pTZ18U-4c vector construct, which is a 7.00 kb plasmid produced by digesting pJM8703 with Sph I and Bam HI, then deleting from the Bam HI to base 835 of the published sequence followed by religation. Figure 3 further depicts the construction of the pSP72/PHB vector construct, which is a 8.50 kb plasmid comprising the Eco RI/Pst I fragment from pJM8703 ligated into the same sites of pSP72 (Promega). Figure 3 also depicts the construction of the pJM8905 vector construct, which is an 8.49 kb plasmid comprising a Pst I partial of Eco RI-digested pJM8703 and ligated into pSP72, which was in turn digested with Xho I and Eco RI to provide a phb operon-containing fragment that was ligated into pGEM7Zf+. Figure 3 further depicts the construction of the pJM9117 vector construct, which is a 10.13 kb plasmid that contains the phb operon from pGEM7-PHBr cloned into the Bam HI site of pRA89, which is a 5.13 kb plasmid that is inducible above 41°, and typically has a basal copy number of 1. The pJM9117 vector construct was formerly known as pRA89/PHB/Fo.

Figure 4 depicts the construction of the pJM9227 vector construct, which is a 7.20 kb plasmid comprising the Bst BI-Bam HI fragment from pJM8905 inserted into the Bam HI site of pJM9226 to provide a vector construct having a tac::phb fusion. Figure 4 also depicts construction of the pJM9228 vector construct, which is an 8.50 kb plasmid comprised of a kanamycin gene block inserted into the Eco RI site downstream of the phb operon of pJM9227 (i.e., pJM9228 is similar to pJM9227 with kanamycin resistance). Figure 4 further depicts the construction of the pJM9229 vector construct, which is a 7.80
kb plasmid comprising a 0.71 kb deletion in the _bla_ gene of pJM9228 (i.e., the ampicillin resistance of pJM9228 was deleted).

Figure 5 depicts the construction of the pJM9230 vector construct, which is a 7.50 kb plasmid that includes the _phb_ operon-containing _Hind_ III- _Eco_ RI fragment from pTZ18U-4c ligated into the _Bam_ HI site of pJM9226. Figure 5 also depicts the construction of the pJM9231 vector construct, which comprises an 8.80 kb plasmid including a kanamycin gene block inserted into the _Spe_ I site downstream of the _phb_ operon in pJM9230 (i.e., pJM9231 is similar to pJM9230 with kanamycin resistance). Figure 5 also depicts the construction of the pJM9232 vector construct, which is an 8.10 kb plasmid, including a 0.71 kb deletion in the _bla_ gene of pJM9231 (i.e., the ampicillin resistance of pJM9231 was deleted).

Figure 6 depicts the construction of the pJM9233 vector construct, which is a 9.10 kb plasmid including the _Hind_ III- _Eco_ RI fragment from pJM9227 (which contains the _tac::phb_ fusion), ligated into the filled-in _Bam_ HI site of pRA89, and the construction of the pJM9234 vector construct, which is a 9.10 kb plasmid similar to the pJM9233 vector construct, except that the _phb_ operon is in the reverse orientation.

Figure 7 depicts the construction of the pJM9235 vector construct, which is a 9.40 kb plasmid that is similar to the pJM9233 vector construct, except that the insert was placed in the pRA90 vector construct, which is a 5.37 kb plasmid that is inducible at 41°C, has a basal copy number of 1 and is resistant to chloramphenicol at 30 μg/ml. Figure 7 also depicts the construction of the pJM9236 vector construct, which is a 9.40 kb plasmid that is similar to the pJM9235 vector construct, except that the _phb_ operon is in the reverse orientation.

Figure 8 depicts the construction of the pJM9237 vector construct, which is a 9.70 kb plasmid comprising the _Hind_ III-_Spe_ I fragment from pJM9230 vector construct (which contains the _tac-leader-phb_ operon fusion) ligated into the filled-in _Bam_ HI site of pRA90, and the pJM9238 vector construct, which is a 9.70 kb plasmid similar to the pJM9237 vector construct except that the _phb_ operon is in the reverse orientation.

Figure 9 depicts the pMS421 vector construct, which is a 5.5 kb plasmid containing the _lac_ I gene.

Figure 10 depicts a series of graphs indicating the synthesis of _phb_ operon gene products in the _E. coli_ strains HMS174 pJM9131 (panel a), HMS174 pJM9232 pMS421 (panel b), HMS174 pJM9117 (panel c), and
HMS174 pJM9238 (panel d), as a function of percentage of total protein over time.

Figure 11 depicts the construction of the pJM9275 and pJM9376 vector constructs, which are 7.20 kb plasmids constructed by Exo III deletion from the Bst BI site in pJM9230 to potentially remove all or part of the native phb Shine-Dalgarno sequence, while inserting a consensus Shine-Dalgarno sequence.

Figure 12 depicts a series of graphs indicating the production of PHB in the E. coli strains HMS174 pJM9238 (panel a), HMS174 pJM9117 (panel b), HMS174 pJM9231 pMS421 (panel c), and HMS174 pJM9232 pMS421 (panel d), as a function of time and glucose consumption.

Figure 13 depicts a pair of graphs comparing PHB yield as a percentage of dry weight in clones containing runaway replicon vector constructs.

Figure 14 depicts a pair of graphs comparing plasmid copy number in clones containing a runaway replicon vector construct.

Figure 15 depicts a graph indicating a comparison of PHB production in clones containing a multicopy tac:phb vector construct.

Figure 16 depicts a graph indicating PHB production in E. coli strain HMS174 pJM9238 at different incubation temperatures.

Figure 17 depicts a graph indicating PHB yield in E. coli strain HMS174 pJM9238 as a function of the optical density of a culture at the time of induction.

Figure 18 depicts a graph indicating PHB yield in E. coli strain HMS174 pJM9238 with and without chloramphenicol.

Figure 19 depicts a graph indicating a comparison of PHB production in clones containing transcriptional and translational fusions (pJM9375 and pJM9376) versus a vector construct having only a transcriptional modification (pJM9232).

Figure 20 depicts a pair of graphs as follows: Panel a depicts the stability of plasmid pJM9238 Klebsiella strain KC2671 over approximately 120 generations when grown in media without chloramphenicol (or other antibiotics). Panel b depicts PHB production in KC2671 pJM9238 at 31°C and 33°C.

Figure 21 depicts a pair of graphs as follows: Panel a depicts the production of PHB in mg/ml in KC2671 pJM9238 over time. The graph also indicates the total dry cell weight of the Klebsiella host cells including the PHB.
Panel b depicts a comparison of PHB yield in KC2671 for plasmids pJM9131 and pJM9238.

**Detailed Description of the Invention**

The present invention provides nucleic acid vector constructs suitable for introduction into an appropriate prokaryotic host wherein the vector constructs provide for stable, regulatable overproduction of a desired product encoded by, or resulting from, a desired nucleic acid sequence (typically a gene), but without the addition of a chemical inducer or a selective pressure agent to a growth medium suitable for host cells containing the inventive nucleic acid vector constructs.

Thus, in a first aspect the present invention provides a vector construct comprising (a) a negatively regulated promoter operably linked to the desired nucleic acid sequence, (b) a runaway replicon, providing multiple copies of the vector construct upon heat induction, and (c) a stabilization locus. The vector construct will typically be a plasmid, but viral vectors, cosmids, and other nucleic acid vector constructs are also included within the scope of the present invention. In a preferred embodiment, the vector construct further comprises a consensus Shine-Dalgarno sequence (preferably a lac Shine-Dalgarno sequence), operably linked to the desired nucleic acid sequence, thereby providing for increased translation of the desired nucleic acid sequence; the consensus Shine-Dalgarno sequence may either replace the native Shine-Dalgarno sequence or it may be in addition to such native sequence. Representative embodiments of suitable promoters and Shine-Dalgarno sequences are depicted in Figure 1 (Seq. ID Nos.  to ). The present invention also provides methods of producing a desired product encoded by, or resulting from, the desired nucleic acid sequence, bacterial host cells transformed with such vector constructs, and desired products produced according to the methods of the present invention.

The negatively regulated promoters of the present invention are strongly expressed and tightly regulated promoters, and are operably linked to the desired nucleic acid sequence. Such promoters can be controllably "turned on" and "turned off" by titrating out the effects of a repressor, or de-inducer, in the cell. When "turned on," such promoters permit substantially uninterrupted transcription of the desired nucleic acid sequence operably linked thereto (and are not repressed by substances found in the host cell). When "turned off," such promoters do not permit any substantial transcription of the gene. Methods for determining whether and when such promoters are "on" and "off," as well as the
detection of desired products from the operably linked desired nucleic acid sequence, are well known in the art in light of the present specification.

The -35 region of such a negatively regulated promoter typically comprises an approximately 6- to 12-base sequence centered around the -35 nucleotide (plus or minus two or three nucleotides, measured from the transcription initiation site). For example, the -35 region of the trp promoter includes the nucleotide sequence TTGACA (Darnell, et al., *Mol. Cell Biol.*, 270-85, 1986; Seq. ID No. ______). The -10 region, also known as a Pribnow box, typically comprises an about 6-base sequence centered around the -10 nucleotide (plus or minus two or three nucleotides, also measured from the transcription initiation site). Also for example, the lac -10 region includes the nucleotide sequence TATAAT (Darnell, *supra*; Seq. ID No. ______).

In a preferred embodiment, the negatively regulated promoter comprises a -35 region of a trp promoter operably linked to a -10 region of a lac promoter, the promoter operably linked to (and typically overlapping) an operator region of a lac promoter, such promoter being further operably linked to the desired nucleic acid sequence, thereby providing multiple copies of mRNA encoded by the desired nucleic acid sequence. Examples of such a promoter are the tac promoter (Russell and Bennett, *Gene* 20:231, 1982) and the trc promoter (Borel et al., *FEBS* 324:162, 1993). Representative examples of a tac promoter are found in Figure 1 (Seq. ID Nos. ______ to ______).

Other negatively regulated promoters are also suitable for use in the present invention, provided that such promoters are repressed when present in lower numbers in a cell than the given promoter's repressor molecule, and that an increase in the number of operators (i.e., repressor binding sites) effectively titrates out the effects of the repressor molecules, thereby inducing transcription of the desired gene. One example of such a promoter is an unaltered trp promoter (Yansura and Henner, *Meth. Enz.* 185: 54-61, 1990). A person having ordinary skill in the art in light of the present specification would be able to utilize other promoters in the vector constructs, methods and other aspects of the invention. Such a person, in light of the present specification, would also be able of the make nucleotide substitutions or other changes within either the -35 region or the -10 region of a suitable promoter, such as those discussed above, to provide slightly different but fully functional regions that would come within the scope of the present invention. Whether a promoter is operable can be readily determined by a person of ordinary skill in the art in light of the present specification, by screening for the presence or absence of a desired product.
arising from the desired nucleic acid sequence (for example, by examining cells under a light microscope for the presence of PHA and/or PHB), or for the presence or absence of mRNA produced from the desired nucleic acid sequence (for example by hybridization assay).

In a promoter useful in the present invention, initiation of transcription may be repressed by binding a repressor, such as the lacI gene product, to the operator, which is located between the promoter and the phb operon. In the presence of a chemical inducer such as isopropyl-β-D-thiogalactoside (IPTG), the repressor is converted to an inactive form, and transcription from the promoter is initiated. Other suitable inducers will be apparent to a person having ordinary skill in the art, in light of the present specification. Such inducers may include glucose-β-galactoside (lactose), glucose-α-galactoside (melibiose), and other lactose analogues such as methyl-β-galactoside and methyl-β-thiogalactoside (Jacob and Monod, J. Mol. Biol. 3: 318-356, 1961).

The runaway replicons of the present invention can be controllably induced and, upon induction, significantly increase the copy number of the vector construct in the cell. Preferably, the runaway replicon is controlled by temperature. Further preferably, the runaway replicon includes the repA gene, which encodes a protein that is required for the initiation of plasmid replication, under the control of the λ pR promoter (Nordstrom and Uhlin, Biotechnology 10:661, 1992). The λ cI857 gene encodes a heat-sensitive repressor that actively inhibits transcription from the λ pR promoter at low temperature, but that is inactive at high temperatures. Therefore, the incorporation of the λ cI857 gene in a host cell permits repression of the λ pR promoter at a low temperature. Thus, at low temperatures, such as 30°C, the vector construct copy number is low, while at high temperatures, such as 42°C, synthesis of repA mRNA increases, and the vector construct copy number is high.

The vector constructs of the present invention further comprise a stabilization locus. Suitable stabilization loci include parB (Gerdes, K., Bio/Technology 6:1402-1405, 1988), ccd, which appears to operate by a mechanism that involves post-segregational mortality of cells that lose a plasmid carrying the ccd locus (Gerdes, supra), the pemK/pemI system (Tsuchimoto, S. et al., J. Bact. 170:1461-1466, 1988), which also appears to involve mortality of plasmid-free segregants, and the plasmid maintenance system found in the F factor and encoded by the sopA, sopB, and sopC genes (Ogura and Hiraga, Cell 32:351-360, 1983). The provision of such a stabilization locus promotes stability
of a desired plasmid in a cell. Determination of other suitable stabilization loci, in light of the present specification, that would be suitable for use with the vector constructs, methods and other aspects of the present invention is within the skill of the art.

Thus, the vector constructs of the present invention comprise a novel expression system in which the copy number and the transcription of the desired nucleic acid sequence are both efficiently controlled by temperature, even when IPTG (or other derepressor or inducer) is not present. Referring, for example, to the preferred embodiment wherein the promoter includes a lac operator region, at low temperatures the copy number of the vector construct is lower than the number of lacI repressor proteins present in the cell (such number is typically about 5-10 proteins per cell (Muller-Hill et al., Proc. Natl. Acad. Sci. 59:1259, 1968)). Such a number of lacI repressor molecules is sufficient to substantially repress transcription of the phb operon at low temperatures. At higher temperatures (which induce expression of the repA gene and therefore increase the copy number of the vector construct), the number of vector constructs rapidly surpasses the number of lacI repressor molecules. This results in non-repressed phb operons, and provides the highly advantageous, and unexpected, result that the phb operons are then expressed without the addition of an inducer such as IPTG. However, in a less preferred embodiment, addition of an inducer may be utilized for induction of expression.

Further, the vector constructs of the present invention provide the highly advantageous, and unexpected, result that the system is stable without the addition of antibiotics or other selective pressure agents to retain the vector construct. It is believed that the vector constructs of the present invention are stable without the provision of a selective pressure agent in the growth medium even without a stabilization locus, and thus a preferred embodiment of the present invention comprises a vector construct and method able to provide the above-discussed superior and unexpected results, comprising a negatively regulated promoter operably linked to a desired nucleic acid sequence, and comprising a runaway replicon, but without a stabilization locus.

In another preferred embodiment, the present invention provides vector constructs in which the native Shine-Dalgarno sequence (e.g., the native phbC Shine-Dalgarno sequence) is supplemented by or replaced with a consensus Shine-Dalgarno sequence, preferably the lac Shine-Dalgarno sequence. A Shine-Dalgarno sequence is a sequence located about 10 bases to the 5' side of the start codon (typically AUG) of an mRNA sequence (Zubay,
Biochemistry, 944-45, 1983). In a preferred embodiment, the consensus Shine-Dalgarno sequence comprises AGGA, although other suitable Shine-Dalgarno sequences could be easily utilized by a person having ordinary skill in the art in light of the present specification. Determination of the effectiveness of a Shine-Dalgarno sequence is also well within the skill of the art in light of the present specification, for example by screening for mRNA copy number.

The provision of a consensus Shine-Dalgarno sequence increases the translation of the desired nucleic acid sequence, production of the desired nucleic acid sequence product, and therefore production of downstream products (such as PHAs).

In still a further aspect, the present invention provides methods of producing large amounts of a desired product encoded by, or resulting from, the desired nucleic acid sequence, such as mRNA, proteins such as enzymes, or a product produced by the action of an encoded enzyme (such as PHAs), utilizing the vector constructs described above. In one embodiment, such methods include elevating the temperature of a culture at a certain time point in order to maximize production. When a culture is slow growing (such as on minimal media), the temperature is preferably elevated at a later time in the log phase of the growth curve. For a fast growing culture, the temperature is preferably elevated earlier in the log phase of the growth curve. Whether a culture is slow growing or fast growing will depend upon such factors as growth media, strain background, temperature, and aeration. In light of the present specification, determination of whether a culture is slow growing or fast growing and the preferred time at which to induce the culture involves routine experimentation well within the ordinary skill in the art.

In still another aspect, the present invention provides prokaryotic host cells transformed by the vector constructs described above. Various prokaryotic host cells may be utilized within the context of the present invention. Generally, preferred prokaryotic host cells should have a well-characterized genetic system, including known cloning vectors and methods of genetic manipulation. They should also preferably grow well in minimal medium, ideally to a high cell density, without any special requirements (physical or physiological). Representative examples of such host cells include members of the Bacillaceae, Nocardiaceae, Streptomycetaceae, Pseudomonadaceae, Corynebacteria, and Enterobacteriaceae. Preferred host cells in the Family Enterobacteriaceae include Escherichia, Citrobacter, Klebsiella, Enterobacter, and Serratia, as well as Zymomonas and Flavobacterium, which are within the
Enterobacteriaceae but of uncertain affiliation. Particularly preferred host cells include *E. coli*, *Klebsiella oxytoca*, and *Klebsiella aerogenes*. Preferred host cells in the Family Pseudomonaceae include *P. aeruginosa*.

With respect to *Klebsiella*, and particularly *K. aerogenes*, the present invention provides an advantageous and unexpected result that the overproduction may be induced by a temperature increase to generally about 32°C to about 35°C, typically about 32.5°C to about 34°C, and preferably to about 33°C. This result is unexpected because it was previously believed (prior to the instant of invention) that the induction of *repA* required a temperature increase to at least 36°C, and preferably to 42°C or more. Further unexpectedly, it has been discovered a temperature increase typically above 33°C, and generally above 34°C, results in smaller cell size and decreased yields.

The above-described prokaryotes may be readily obtained from a variety of commercial sources including, for example, the American Type Culture Collection (ATCC) (Rockville, Maryland). Alternatively, many of the above-described bacteria may be isolated from sources that are known by those of skill in the art to contain such prokaryotes, based upon techniques that are known in the art (*See* Bergy's *Shorter Manual of Determinative Bacteriology*, Williams & Wilkins (pub.), John G. Holt (ed.), 8th edition, 1977).

Once the host cell has been cultured under conditions and for a time sufficient to generate large amounts of the desired product, the desired product is preferably isolated from the host cell. Isolation may be accomplished by a variety of methods. For example, the host cells may be lysed, and desired product assayed via hybridization or immunological methods, or where the desired product is PHA, the product may be agglomerated, essentially as described in U.S. Application Serial No. 07/528,549. Alternatively, lysozyme plasmids may be introduced into the host cell to aid isolation of the desired product, as described in U.S. Application Serial No. 07/890,925.
EXAMPLES

Summary of the Examples

Generally, Examples 1-10, 12 and 13 are directed toward the construction of desired nucleic acid vectors. Examples 11 and 14-19 are directed toward assays for the effectiveness of various aspects of the present invention.

Example 1 is directed toward the construction of plasmid pJM9002 by inserting the phb operon-containing gene fragment from plasmid pJM8801 (previously designated p4A) into pBluescript SK⁺.

Example 2 is directed toward the construction of plasmid pTZ18U-4c by deleting a segment containing the phb genes from plasmid pJM8703, which is also known as pTZ-18U-PHB.

Example 3 is directed toward the construction of plasmid pJM8905 by transferring a phb operon-containing fragment from pJM8703 into pSP72 to create pSP72/PHB, followed by excision of the phb fragment from pSP72/PHB and inserting it into pGEM-7Zf⁺.

Example 4 is directed toward the construction of plasmid pJM9131 by the insertion of kanamycin resistance into, and the deletion of ampicillin resistance from, pJM8801.

Example 5 is directed toward the construction of plasmid pJM9117 by the insertion of the phb operon-containing fragment from pJM8703 into pRA89.

Example 6 is directed toward the construction of plasmid pJM9226 by the deletion of the phb operon-containing fragment from pJM9002 and the insertion of the tac promoter into pJM9002.

Example 7 is directed to the creation of tac::phb fusion plasmids pJM9227-pJM9229 by inserting the phb operon-containing fragment from pJM8905 into the tac promoter-containing pJM9226. pJM9227 has only ampicillin resistance, pJM9228 has both ampicillin resistance and kanamycin resistance, and pJM9229 has only kanamycin resistance.

Example 8 is directed toward the construction of tac::phb fusion plasmids pJM9230-pJM9232 by the insertion of the phb operon-containing fragment from pJM8703 into pJM9226. pJM9230 has only ampicillin resistance, pJM9231 has both ampicillin and kanamycin resistance, and pJM9232 has only kanamycin resistance. pJM9230-pJM9232 differ from pJM9227-pJM9229 in that pJM9230-pJM9232 have a phbC leader of approximately 355-bases that
contains a cis-acting element, while pJM9227-pJM9229 have a phbC leader of approximately 72 base pairs without such an element.

Example 9 is directed to the construction of runaway replicon tac::phb fusion plasmids pJM9233-pJM9236 by the insertion into the runaway replicon vectors pRA89 and pRA90 the tac::phb fusion from pJM9227. Thus, pJM9233-pJM9236 have both a tac promoter and a heat inducible promoter (λ pR). These plasmids differ from each other in the orientation and precise placement of the phb gene fragment within the vector.

Example 10 is directed to the construction of runaway replicon tac::phb fusion plasmids pJM9237 and pJM9238, which have an approximately 355-base leader.

Example 11 is directed to a graphic analysis of phb operon gene products, which analysis indicates that the phbC gene product (PHB synthase) is subject to post-translational regulation, and therefore is not overproduced by the plasmids constructed pursuant to Examples 1-10.

Examples 12 and 13 are directed to the construction of plasmids pJM9375 and pJM9376, which were created by the addition of a consensus Shine-Dalgarno sequence operably linked to the phbC gene. In pJM9375, the consensus (lac) Shine-Dalgarno sequence replaced the native phbC Shine-Dalgarno sequence. In pJM9376, the consensus (lac) Shine-Dalgarno sequence was added to the native phbC Shine-Dalgarno sequence.

Example 14 is directed to a graphic comparison of PHB production in native versus tac promoter clones.

Example 15 is directed to a graphic comparison of PHB production in the approximately 72 base pair leader and 355 base pair leader phbC tac::phb fusion constructs.

Example 16 is directed to the optimization of PHB production at different temperatures using the heat-inducible plasmid pJM9238.

Example 17 is directed to the determination of the optimal cell density during the cell growth cycle for initiation of PHB production using the plasmid pJM9238.

Example 18 is directed to a comparison of PHB production using the plasmid pJM9238 with or without chloramphenicol.

Example 19 is directed to the quantitation of PHB production in plasmids pJM9375 and pJM9376, each of which contain a tac promoter and a consensus (lac) Shine-Dalgarno sequence.
Example 20 is directed to the determination of the stability of PHB-producing plasmids in *Klebsiella*.

Example 21 is directed to the production of PHB in *Klebsiella* at varying temperatures.

Example 22 is directed to the production of PHB in *Klebsiella* using plasmid pJM9238 based on fed-batch fermentation. Example 22 is also directed to a comparison of PHB production using plasmid pJM9238 versus plasmid pJM9131.

**Example 1. Construction of plasmid pJM9002.**

The *phb* operon fragment was cloned into pBluescript SK⁺ (Stratagene) as follows. In separate tubes, plasmid pJM8801 (previously designated p4A in U.S. Application Serial No. 07/890,925) and the vector pBluescript SK⁺ were digested with the restriction endonucleases *Eco* R I and *Hind* III (Gibco BRL) as described (Maniatis et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1982.). The released fragment containing the *phb* genes from plasmid pJM8801 was ligated into the *Eco* R I-*Hind* III digested pBluescript SK⁺ fragment using T4 DNA Ligase (Gibco BRL) as described in Gibco Focus Technical Bulletin 5224-1. The resulting recombinant plasmid was designated pJM9002 (Figure 2).

**Example 2. Construction of plasmid pTZ18U-4c**

The plasmid pTZ18U-4c was constructed as follows. Plasmid pTZ-18U-PHB (deposited with American Type Culture Collection and assigned ATCC Deposit No. 299006, currently designated as pJM8703 (Figure 3), was digested with *Sph* I (which yields a 3' overhang) and *Bam* HI (which creates a 5' overhang). The resulting linearized fragment containing the *phb* genes was deleted from the *Bam* HI end using the procedure of Henikoff (Henikoff, S., Gene 28, 351, 1984) to approximately base 835 in the *phb* operon sequence previously disclosed (U.S. Application Serial No. 07/705,806). The fragment was then religated using T4 DNA Ligase and the resulting circularized plasmid was designated pTZ18U-4c (Figure 3).
Example 3. Construction of plasmid pJM8905

Plasmid pJM8905 was constructed as follows. The vector pJM8703, discussed above with respect to Figure 3, was linearized by digestion with Eco RI. The linearized plasmid DNA was then partially digested with Pst I as follows. From a 100μl digestion reaction, performed as described (Maniatis, supra), 10 μl aliquots were removed every 2.0 minutes to microplate wells containing 2 μl of 150 mM EDTA on ice. A total of 12 time-points were taken. Three microliters of 6X loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 40% (w/v) sucrose) were added to each well. Samples were separated on a 1% SeaKem GTG Agarose gel at 75 v for 30 minutes and visualized by ethidium bromide staining. The 11, 13, and 15 minute timepoints were selected because they contained the approximately 3.5 kb Pst I-EcoR I phb fragment, which was excised from the gel. To each gel slice was added plasmid pSP72 (Promega) that had been digested with Pst I and EcoR I. The mixture was then purified using GENECLEAN® (BIO101) according to the manufacturer's protocol. The eluted DNA was ligated using T4 DNA Ligase (Gibco BRL) as described above, and transformed into E. coli strain DH5α (endA1 hsdR17 (rk+), mk+) supE44 thi-1 recA1 gyrA (NalR) relA1 Δ(lacZYA-argF)U169 (φ 80dlacI(lacZ)M15)). The resulting plasmid was pSP72 containing the Pst I-EcoR I phb fragment in the multiple cloning site. This plasmid, designated pSP72/PHB (Figure 3), was next digested with EcoR I and Xho I. This released the phb fragment, which was then ligated into the same restriction sites of Xho I - EcoR I digested pGEM-7Zf+ (Promega). The resulting plasmid was designated pJM8905 (Figure 3).

Example 4. Construction of plasmid pJM9131

The Kanamycin Resistance GENBLOCK® (Eco RI) (Pharmacia) restriction fragment was ligated into the Eco RI site of plasmid pJM8801 (p4A) (Janes et al., supra). The plasmid was then digested with Dra I and ligated to delete a restriction fragment within the bla gene. The resulting plasmid was designated pJM9131 (Figure 2).
Example 5. Construction of runaway replicon vector construct pJM9117

The phb fragment was cloned into pRA89 (Benzon Pharma A/S, Helseholmen 1, P.O. Box 1185, DK-2650 Hvidovre, Denmark; Figure 3) as follows. Plasmid p8703 (pTZ-18U-PHB) was digested with Eco RI. The resulting DNA fragment was partially digested with Pst I and the approximately 5 kb fragment containing the entire phb fragment was ligated using T4 DNA Ligase (Gibco BRL) into plasmid pSP73 (Promega) that had been digested with Eco RI and Pst I. The recombinant plasmid was transformed into E. coli strain DH5α. This plasmid was purified by the alkaline-lysis method (Maniatis et al., supra). The plasmid was then digested with Xho I and EcoR I to release the DNA fragment containing the phb operon. This fragment was ligated using T4 DNA Ligase into plasmid pGEM-7Zf+ (Promega) that had been digested with Xho I and EcoR I. The resulting plasmid was designated pGEM7f-PHB reverse. Plasmid pGEM7f-PHB reverse was digested with BamH I. The released phb fragment was ligated using T4 DNA Ligase into the Bam HI site of Bam HI-digested pRA89, which had been treated with Calf Intestinal Alkaline Phosphatase (Boehringer Mannheim). The resulting plasmid, designated pJM9117 (Figure 3), was introduced into E. coli strain HMS174 recA1 hsdR Rif’ by electroporation as previously described (see U.S. Application Serial No. 08/035,433).

Example 6. Construction of the tac promoter plasmid pJM9226

The tac promoter was cloned into the plasmid pBluescript SK+ (Stratagene) as follows. The vector pJM9002 (described above) was digested with the restriction endonucleases BamH I and Hind III (Gibco BRL) as described (Maniatis et al., supra). This released the phb fragment from the pBluescript plasmid. The tac promoter GENBLOCK® (Hind III/BamH I) (Pharmacia) restriction fragment was ligated into the Hind III-BamH I-digested pBluescript fragment using T4 DNA Ligase (Gibco BRL) as described in Gibco BRL Focus Technical Bulletin 5224-1.

The recombinant plasmid was introduced into E. coli strain DH5α by electroporation (Miller, Bacterial Electroporation, Molecular Biology Reports No. 5, Bio-Rad Laboratories, Richmond, CA, 1988) using the GENE PULSER® (Bio-Rad). Electroporation was performed as follows. An isolated
colony of *E. coli* strain DH5α was inoculated into a 13 x 100 mm S/P diSPo culture tube (Baxter) containing 3 ml of Luria-Bertani (LB) medium (Maniatis et al., *supra*). This culture was grown overnight in a Lab-Line Incubator-Shaker (Lab-Line Instruments, Inc.) at 37°C, 225 rpm. The next morning 1 ml of the culture was inoculated in 50 ml of LB medium in a baffled 250 ml Erlenmeyer flask (Wheaton). Growth of the culture was followed by withdrawing aliquots at regular time intervals and measuring the optical density at 600 nanometers using a Shimadzu UV 160 U Spectrophotometer. The culture was incubated at 37°C, 225 rpm, until the optical density at 600 nanometers reached approximately 0.5. At this time, the culture was placed on ice for 10 minutes. It was then transferred to a sterile 50 ml Falcon 2098 Blue Max conical centrifuge tube and centrifuged in a Varifuge RF (Heraeus Instruments) at 3,000 g for 10 minutes. The supernatant was aseptically removed and 40 ml of sterile ice-cold 10% glycerol in deionized water was added to the pellet. The pellet was resuspended by vortexing, followed by pelleting as above. The supernatant was aseptically aspirated, 40 ml of sterile ice-cold 10% glycerol was again added and the pellet was resuspended. The bacteria were again pelleted by centrifugation as before and the supernatant was aseptically aspirated. Ten ml of sterile ice-cold 10% glycerol was added, the pellet was resuspended, and centrifuged as described above. The supernatant was aseptically aspirated and the pellet was resuspended in a final volume of approximately 200 µl. Forty microliter aliquots of this suspension were used for electroporation. One microliter of plasmid DNA was added to 40 µl of the cell suspension and this mixture was added to a 0.2 cm electrode gap GENE PULSER®/*E. coli* Pulser Cuvette (Bio-Rad Laboratories). The mixture was subjected to a pulse of 2.5 KV, at 200 Ohms and 25 µfarads using a GENE PULSER® (Bio-Rad Laboratories). The bacterial suspension was then transferred to a sterile 13 x 100 mm culture tube containing 3 ml of LB broth. The culture was incubated for 1 hour in a Lab-Line Incubator-Shaker (Lab-Line Instruments, Inc.) at 37°C, 225 rpm. Transformants were selected by spreading the cells on LB agar plates containing 200 µg/ml ampicillin (Sigma). The plates were incubated at 37°C in a Fisher IsoTemp Oven Model 350D until colonies were visible. Transformants were purified by picking well-isolated colonies and streaking for single colony isolation on LB agar plates containing 200 µg/ml ampicillin. Purified clones were inoculated into 3 ml of LB media containing 200 µg/ml ampicillin in 13 x100 mm culture tubes (Baxter) and incubated overnight at 37°C in a Lab-Line Incubator-Shaker (Lab-Line Instruments, Inc.) at 200 rpm.
The plasmid DNA was purified by a modification of the alkaline lysis method as follows: A single colony was inoculated into a 13 x 100 mm culture tube containing 3 ml of LB broth + 200 µg/ml ampicillin and grown overnight. The culture was pipetted into two sterile 1.5 ml microfuge tubes (West Coast Scientific, Inc.) and pelleted in an Eppendorf Centrifuge 5415C microcentrifuge for two minutes. The supernatant was decanted. Each pellet was resuspended in 1 ml of ice-cold SET butter (20% sucrose, 50 mM EDTA, 50 mM Tris, pH 8.0) and centrifuged two minutes as before. The supernatant was withdrawn with a pipette. The cells were resuspended in a total of 150 µl of SET buffer and placed on ice. Five microliters of RNase (Boehringer Mannheim) (10 µg/ml, boiled for 2 minutes) was added and the tube was vortexed. Three hundred-fifty microliters of 0.2 N NaOH/1.0% SDS was added and the tube was inverted several times. The tube was incubated on ice for 20 minutes. Two hundred-fifty microliters of ice-cold 3 M sodium acetate, pH 5.2, was added and the tube was inverted gently several times and incubated on ice for 20 minutes. The tube was centrifuged for 5 minutes and the supernatant was decanted into a sterile 1.5 ml microfuge tube. Three hundred-fifty microliters of phenol (equilibrated with 0.1 M Tris buffer) (United States Biochemical Corp.) was added, the tube was vortexed, then 350 µl of chloroform was added and the tube was vortexed. The tube was centrifuged for 5 minutes in an Eppendorf Centrifuge 5415C microcentrifuge at room temperature. The top aqueous phase was transferred to a sterile microfuge tube (West Coast Scientific, Inc.). An equal volume (approximately 650 µl) of isopropanol was added, the tube was inverted and incubated at room temperature for 30 minutes. The tube was then centrifuged for 15 minutes in a microfuge at room temperature. The supernatant was removed, 500 µl of ice-cold 70% ethanol was added, and the tube was centrifuged in an Eppendorf Centrifuge 5415C microcentrifuge for 5 minutes at room temperature. The supernatant was removed and the pellet was dried. The dry pellet was resuspended in 10 µl of sterile water.

To confirm the presence of the tac promoter fragment in the vector construct, the DNA was digested with BamH I (Gibco BRL) and Hind III (Gibco BRL). The restriction fragments were separated by gel electrophoresis on a 1.0% SeaPlaque (FMC BioProducts) agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM EDTA, pH 8.0) using a BRL Horizon Model 58 Horizontal Gel Electrophoresis System and a BRL Model 200 Power Supply. The DNA was stained by placing the gel in a 0.5 µg/ml solution of ethidium bromide for 20 minutes, followed by destaining the gel in water for 30 minutes. The DNA was
visualized using a UV transilluminator (Fotodyne). This plasmid, designated pJM9226 (Figure 2), was subsequently used to construct tac promoter fusions to the phb operon. The construction of these fusions is described below.

Example 7. Construction of the multicopy tac::phb fusion plasmids pJM9227 and pJM9229 containing a phbC leader of approximately 72 base pairs

Plasmid pJM8905 (Figures 3 and 4) was digested with BstB I (New England Biolabs), which cleaves 31 bp upstream of the phbC structural gene. T4 DNA Polymerase (Gibco BRL) was used to remove the single stranded DNA from the ends. Phosphorylated BamH I linkers (New England Biolabs) were ligated to the blunt ends using T4 DNA Ligase (Gibco BRL). The resulting DNA was digested with BamH. This released a BamH I fragment that contained the phb structural genes but did not contain the promoter/regulatory region. The DNA restriction fragments were separated by preparative gel electrophoresis on a 1.0% SeaPlaque (FMC BioProducts) agarose gel in TAE buffer using a BRL Horizon Model 58 Horizontal Gel Electrophoresis System and a BRL Model 200 Power Supply. The DNA was visualized by staining with ethidium bromide. The restriction fragment containing the phb genes was excised from the gel and purified using GENECLEAN® (BIO101) according to the manufacturer's protocol. This fragment was then ligated into the BamH I site of plasmid pJM9226 (Figures 2 and 4). The recombinant plasmid was then introduced into E. coli strain DH5α by electroporation and the transformants were spread on LB agar plates containing 100 μg/ml ampicillin and 1.0% glucose. The plates were incubated at 37₀C in a Fisher IsoTemp Oven Model 350D until colonies were visible. White (PHB⁺) colonies were picked and streaked for single isolates. Purified clones were inoculated into 3 ml of LB media containing 200 μg/ml ampicillin and grown to saturation. Plasmid DNA was prepared from the cultures as described above and digested with BamH I and Hind III to confirm the tac::phb fusion construct. The resulting plasmid was designated pJM9227 (Figure 4).

This pJM9227 vector construct contains the tac promoter fused 78 bp upstream of the phbC structural gene. The native phbC Shine-Dalgarno sequence and ribosome-binding site is retained in this fusion. Thus, expression
of the phb genes is transcriptionally regulated by the tac promoter and translationally regulated by the native phbC Shine-Dalgarno sequence.

This construct proved to be unstable in liquid media. We believe this is because cells containing the plasmid excreted ampicillin, which inactivated the ampicillin. Once this occurred the selective pressure to retain the plasmid was eliminated. Since high levels of expression of the phb genes were deleterious to the cell, plasmidless cells were rapidly selected. For these reasons, a different antibiotic resistance determinant was cloned into a restriction site downstream of the phb operon to increase plasmid stability. The plasmid pJM9227 was digested with EcoR I (Gibco BRL) and the 5' phosphate groups were removed using Calf Intestinal Phosphatase (New England Biolabs) to prevent self-ligation. The Kanamycin Resistance GENBLOCK® (Eco RI) (Pharmacia) restriction fragment was ligated into the EcoR I site located downstream of the phb genes. The resulting plasmid, pJM9228 (Figure 4), was introduced into E. coli strain DH5α by electroporation as previously described. The transformants were spread on LB agar plates containing 50 μg/ml kanamycin (Sigma). The plates were incubated at 37°C in a Fisher IsoTemp Oven Model 350D until colonies were visible. Plasmid DNA was isolated from purified clones. The plasmid-encoded ampicillin resistance gene was inactivated by Dra I (Gibco BRL) digestion to delete a 0.71 kb fragment from the bla structural gene, followed by religation. The resulting plasmid was designated pJM9229 (Figure 4).

This plasmid construct contains the tac promoter fused 78 bp upstream of the phbC structural gene, and results in a leader of approximately 72 bases (see Figure 1, panel b; Seq. ID No. ______). The native phbC Shine-Dalgarno sequence is retained in this fusion. Thus, expression of the phb genes is transcriptionally regulated by the tac promoter and translationally regulated by the native phb ribosome binding sites.

Example 8. Construction of multicopy tac::phb fusion plasmids pJM9230, pJM9231 and pJM9231 containing a phbC leader of approximately 355 base pairs

Plasmid pJM9226 (Figures 2 and 5) was digested with BamH I and the 5' recessed ends were filled in with T4 DNA Polymerase to form blunt ends. A restriction fragment containing the phb operon was fused to the tac promoter
as follows: The plasmid pTZ18U-4c was digested with EcoR I and Hind III. This released a restriction fragment containing the phb structural genes and 290 bp of the upstream leader sequence. The ends of the fragment were filled in using T4 DNA Polymerase (Gibco BRL). The fragment was then excised from an agarose gel and purified using GENECLEAN®. The phb fragment was ligated into the plasmid containing the tac promoter fragment at the filled-in BamHI I site. The resulting plasmid, designated pJM9230 (Figure 5), was introduced into E. coli strain DH5α by electroporation as previously described. The transformed cells were plated on LB agar plates containing 200 μg/ml ampicillin. The presence of the tac::phb operon fusion was confirmed by screening for the PHB⁺ phenotype (i.e., white colonies) on LB plates containing 200 μg/ml ampicillin and 1% glucose.

To increase plasmid stability, a kanamycin resistance gene was cloned into the plasmid as follows: The plasmid was digested with Spe I (New England Biolabs) and the recessed 5'-ends were filled-in with T4 DNA Polymerase to create blunt ends. The 5' recessed ends of a Kanamycin Resistance GENBLOCK® (Eco RI) (Pharmacia) restriction fragment were filled in with T4 DNA Polymerase. The Kanamycin Resistance GENBLOCK® was ligated into the filled-in Spe I site downstream of the phb operon. The recombinant plasmid, designated pJM9231 (Figure 5), was introduced into E. coli strain DH5α by electroporation and transformants were selected on LB agar plates containing 50 μg/ml kanamycin. Single clones were isolated and plasmid DNA isolated from these clones was purified as described above. The ampicillin resistance gene (bla) was inactivated by digestion with Dra I, followed by religation, which removed a 0.71 kb fragment from the structural gene. The resulting plasmid was designated pJM9232 (Figure 5). This construct contains the tac promoter fused 361 bp upstream of the phbC structural gene, resulting in a leader of approximately 355 bases (see Figure 1, panel c; Seq. ID No. _______). The native phbC Shine-Dalgarno sequence and ribosome binding site are retained.
Example 9. Construction of runaway replicon tac::phb fusion plasmids pJM9233, pJM9234, pJM9235 and pJM9236 containing a phbC leader of approximately 72 base pairs

5 The runaway replicon vector pRA89 (Benzon Pharma A/S, Helselholmen 1, P.O. Box 1185, DK-2650 Hvidovre, Denmark) (Figures 3 and 6) was digested with BamH I and the ends were dephosphorylated using Calf Intestinal Phosphatase as described above. The tac::phb fusion was cloned into this vector as follows: The plasmid pJM9227 (Figures 4 and 6) was digested with EcoR I and Hind III and the single stranded ends were removed using T4 DNA Polymerase. The restriction fragments were separated by preparative gel electrophoresis on a 1.0% SeaPlaque (FMC BioProducts) agarose gel in TAE buffer using a BRL Horizon Model 58 Horizontal Gel Electrophoresis System and a BRL Model 200 Power Supply. The DNA was stained with 0.5 μg/ml ethidium bromide for 20 minutes and destained in water for 20 minutes. The bands were visualized using a UV transilluminator. The restriction fragment containing the tac::phb fusion was excised from the gel and purified using GENECLEAN® (BIO101) according to the manufacturer's protocol. Phosphorylated BamH I linkers (New England Biolabs) were ligated to the ends of the fragment using T4 DNA Ligase and the DNA fragment was purified using GENECLEAN® (BIO101). This DNA fragment was then ligated into the BamH I-digested vector pRA89. The recombinant plasmid was introduced into E. coli strain XL1-Blue F':Tn10 (proA+B+ lacI97 Δ(lacZ)M15/ recA1 endA gyrA96 (NalR) thi hsdR17 (rK-mK+ supE44 relA1 lac) by electroporation as previously described except the transformed cells were incubated at 30°C to allow expression of the plasmid-encoded antibiotic resistance factors. The transformants were spread on LB agar plates containing 25 μg/ml chloramphenicol (Sigma). The plates were incubated in a Fisher IsoTemp Oven Model 350D at 30°C to prevent runaway replication. Transformants were purified by picking well-isolated colonies and streaking for single colony isolation on LB agar plates containing 25 μg/ml chloramphenicol. The plates were incubated at 30°C to prevent runaway replication. The purified isolates were screened for the presence of the phb operon by streaking the isolates onto LB agar plates containing 25 μg/ml chloramphenicol + 1.0% glucose. The plates were incubated in a Fisher IsoTemp Oven Model 350D at 37°C to induce runaway replication. White colonies indicated the production of PHB, and thus the presence of the phb genes on the plasmid. Recombinants that exhibited a
PHB\(^+\) phenotype at 37°C were inoculated into LB media containing 25 \(\mu\)g/ml chloramphenicol. The culture was incubated at 30°C for 6 hours, then 100 \(\mu\)l of the culture was used to inoculate 3 ml of LB media containing 50 \(\mu\)g/ml chloramphenicol. The cultures were incubated at 37°C overnight. Plasmid DNA was isolated from the cultures and digested with Kpn I (New England Biolabs) to determine the orientation of the tac::phb insert. The plasmid containing the tac::phb operon fusion with the tac promoter proximal to the chloramphenicol resistance gene (cat) was designated pJM9233 (Figure 6). The plasmid containing the tac::phb operon fusion with the tac promoter proximal to the cI857 gene was designated pJM9234 (Figure 6). These tac::phb fusions were also cloned into the vector plasmid pRA90 (Benzon Pharma A/S, Helseholmen 1, P.O. Box 1185, DK-2650 Hvidovre, Denmark) (Figure 7) by the same procedure. The plasmid containing the tac promoter proximal to the parB locus was designated pJM9235 (Figure 7). The plasmid containing the tac promoter proximal to the chloramphenicol resistance gene (cat) was designated pJM9236 (Figure 7).

As described above for plasmid pJM227, these plasmid constructs contain the tac promoter fused 78 bp upstream of the phbC structural gene, resulting in an approximately 72 bp leader (see Figure 1, panel b; Seq. ID No. ______). These plasmids retain the native phbC Shine-Dalgarno sequence and ribosome binding site.

**Example 10. Construction of runaway replicon tac::phb fusion plasmids pJM9237 and pJM9238 containing a phbC leader of approximately 355 base pairs**

The runaway replicon vector pRA90 (Benzon Pharma A/S, Helseholmen 1, P.O. Box 1185, DK-2650 Hvidovre, Denmark) (Figures 7 and 8) was digested with BamH I and the 5' recessed ends were filled in with T4 DNA Polymerase. An EcoR I-Hind III restriction fragment from plasmid pJM9230 (Figures 5 and 8) containing the tac promoter and the phb structural genes was gel purified as previously described and the single-stranded ends were filled using T4 DNA Polymerase. This fragment was ligated into the filled-in BamH I site of pRA90. The recombinant plasmid was introduced into *E. coli* strain XL1-Blue (Stratagene) by electroporation as previously described except the transformed cells were incubated at 30°C to allow expression of the plasmid-
encoded antibiotic resistance factors. The transformants were spread on LB agar plates containing 25 \( \mu \)g/ml chloramphenicol. The plates were incubated in a Fisher IsoTemp Oven Model 350D at 30°C to prevent runaway replication. Transformants were purified by single colony isolation and screened for the presence of the \( phb \) operon by streaking the isolates onto LB agar plates containing 25 \( \mu \)g/ml chloramphenicol and 1.0% glucose. The plates were incubated at 37°C to induce runaway replication. Recombinants that exhibited a PHB\(^+\) phenotype at 37°C were inoculated into LB media containing 25 \( \mu \)g/ml chloramphenicol. The culture was incubated at 30°C for 6 hours, then 100 \( \mu \)l of the culture was used to inoculate 2.5 ml of LB media containing 50 \( \mu \)g/ml chloramphenicol. The cultures were incubated at 37°C overnight. Plasmid DNA was isolated from the cultures and digested with \( Kpn \) I to determine the orientation of the \( tac::phb \) insert. The plasmid containing the \( tac::phb \) operon fusion with the \( tac \) promoter proximal to the chloramphenicol resistance gene (\( cat \)) was designated pJM9237 (Figure 8). The plasmid containing the \( tac::phb \) operon fusion with the \( tac \) promoter proximal to the \( cl857 \) gene was designated pJM9238 (Figure 8).

As described for plasmid pJM9230, these plasmid constructs contain the \( tac \) promoter fused 361 bp upstream of the \( phbC \) structural gene, resulting in a leader of approximately 355 bases (see Figure 1, panel c; Seq. ID No. ______). The native \( phbC \) Shine-Dalgarno sequence and ribosome binding site is retained.

**Example 11. Analysis of the \( phb \) operon gene products by SDS-PAGE**

The purpose of this experiment was to quantitate induction of the \( phb \) operon gene products in the \( tac::phb \) promoter constructs and to compare induction of the gene products to that observed in the native \( phb \) promoter clones. For this experiment, all of the plasmids were introduced into \( E. coli \) strain HMS174 by electroporation as previously described. To more effectively control expression of the \( tac \) promoter in this and other experiments, the plasmid pMS421 (obtained from G. Weinstock) (Figure 9), was introduced into the strains containing \( tac::phb \) fusions on multicopy plasmids by electroporation. This procedure was performed as previously described, except the transformants were selected on LB agar plates containing 10 \( \mu \)g/ml streptomycin. The plasmid pMS421 is a low copy number vector that confers streptomycin resistance and
contains the *lacI* gene, which overproduces the Lac repressor protein (Muller-Hill and Gilbert, *Proc. Natl. Acad. Sci.* 59:1259, 1968). Five *E. coli* strains were used in this experiment: HSM174, HSM174 pJM9131, HSM174 pJM9232 pMS421, HSM174 pJM9117, and HSM174 pJM9238. *E. coli* strain HSM174 was inoculated into 3 ml of LB medium. *E. coli* strain HSM174 pJM9131 was inoculated into 3 ml of LB medium containing 50 µg/ml kanamycin. *E. coli* strain HSM174 pJM9232 pMS421 was inoculated into 3 ml LB medium containing 50 µg/ml kanamycin and 10 µg/ml streptomycin. These cultures were shaken at 200 rpm at 37°C in a Lab-Line Incubator-Shaker (Lab-Line Instruments, Inc.) for approximately 15 hours. *E. coli* strain HSM174 pJM9117 and *E. coli* strain HSM174 pJM9238 were each inoculated into 3 ml of LB medium containing 25 µg/ml chloramphenicol. These cultures were shaken at 200 rpm at 30°C in a Lab-Line Orbital Environ-Shaker (Lab-Line Instruments, Inc.) for approximately 15 hours. The cultures were diluted 1:100 into 50 ml of the same medium in a 250 ml baffled Erlenmeyer flask (Wheaton), except glucose was added to a final concentration of 1.0%, and the cultures were incubated at the same temperature and agitation as previously described. The growth of the culture was followed by withdrawing aliquots at regular time intervals and measuring the optical density at 600 nanometers using a Shimadzu UV 160U Spectrophotometer. The *phb* operon was induced in *E. coli* strain HSM174 pJM9232 pMS421 by the addition of IPTG to a final concentration of 10 mM at an OD₆₀₀ of 2.75. The *phb* operon was induced in *E. coli* strain HSM174 pJM9117 and *E. coli* strain HSM174 pJM9238 by transferring the cultures to a 41°C waterbath for 30 minutes when an OD₆₀₀ of 0.7 was reached. A sterile stir bar was added and the cultures were mixed at 200 rpm using a Fisher Scientific Electronic Stirrer 2008. Following the heat pulse, the cultures were incubated in a Lab-Line Incubator-Shaker (Lab-Line Instruments, Inc.) at 37°C and shaken at 200 rpm. One milliliter aliquots were withdrawn at time intervals, centrifuged in an Eppendorf Centrifuge 5415C and frozen at -70°C. These samples were subsequently analyzed by 1-D SDS-PAGE analysis. Proteins were separated by gel electrophoresis using precast Mini-PROTEAN II Ready Gels (Bio-Rad). A 12% polyacrylamide gel was used to resolve the thiolase and reductase proteins. The proteins were electrophoresed for approximately 45 minutes using the Mini-PROTEAN II® Electrophoresis Cell (Bio-Rad) and BRL Model 200 Power Supply. Prestained SDS-PAGE Standards (Bio-Rad) were used as molecular weight markers to monitor protein migration through the gels during electrophoresis. The proteins were visualized by silver staining using the Bio-
Rad Silver Stain Plus kit according to the manufacturer's protocol. The gels were dried between cellophane sheets using drying frames (Integrated Separation Systems). The \textit{phb} gene products were quantitated as a relative percentage of total protein by densitometry using a Ultrascan XL Enhanced Laser Densitometer (LKB).

Results: The thiolase and reductase bands were clearly visible after induction in the samples from the \textit{tac::phb} clones, while the synthase was undetectable in all samples. The \textit{phb} gene products were not present in the negative control \textit{E. coli} strain HMS174. In \textit{E. coli} strain HMS174 pJM9131 (\textit{phb} promoter, multicopy) the thiolase and reductase levels remain relatively constant. The thiolase levels were 1.61%-1.86% of total protein, and the reductase levels were 0.38%-0.95% of total protein (Figure 10, panel a). In \textit{E. coli} strain HMS174 pJM9232 pMS421 (\textit{tac} promoter, multicopy) the thiolase and reductase levels rapidly increased after the addition of IPTG. The thiolase level rose from 0.78% of total protein before induction to 4.36% 30 minutes after IPTG addition. The reductase levels increased from 0.73% to 1.63% of total protein 3 hours after IPTG addition (Figure 10, panel b). In \textit{E. coli} strain HMS174 pJM9117 (\textit{phb} promoter, runaway replicon) the thiolase and reductase proteins were undetectable before induction. The thiolase level increased to 2.54% and the reductase level rose to 10.11% of total protein 2.3 hours after the heat pulse (Figure 10, panel c). In \textit{E. coli} strain HMS174 pJM9238 (\textit{tac} promoter, runaway replicon) The thiolase and reductase levels increased from undetectable levels to 15.2% and 9.34% of total protein, respectively, 2 hours after the heat pulse (Figure 10, panel d).

These results indicate that synthase production is regulated post-transcriptionally, since replacement of the \textit{phbC} promoter with the \textit{tac} promoter did not measurably increase the synthesis of the \textit{phbC} gene product. Control of thiolase synthesis is clearly regulated by the \textit{tac} promoter. In both \textit{tac::phb} fusion constructs, this protein was rapidly synthesized to relatively high levels following induction. In contrast, reductase synthesis appears to be related to plasmid type or copy number. In the multicopy plasmids, the highest reductase levels are 0.95% for \textit{E. coli} strain HMS174 pJM9131 and 1.63% for \textit{E. coli} strain HMS174 pJM9232 pMS421. In contrast, the runaway replicon plasmids have significantly higher levels of reductase. The highest reductase level for \textit{E. coli} strain HMS174 pJM9117 was 11.55% of total protein. For \textit{E. coli} strain HMS174 pJM9238, the highest reductase level was 10.01% of total protein.
In summary, synthase production appears to be primarily regulated at the post-transcriptional level. Synthesis of thiolase is regulated at the level of transcription. Synthesis of reductase appears to be a function of plasmid type or copy number, and may be regulated by a gene dosage effect.

Example 12. Replacement of the native phbC ribosome-binding site with the lac ribosome-binding site to create plasmids pJM9375 and pJM9376

1-D SDS-PAGE analysis of the phb gene products indicated that the phbC gene product was not induced to detectable levels in either the native promoter clones or the tac::phb fusions. This indicated that this gene is post-transcriptionally regulated. Inspection of the phb operon DNA sequence indicates that the phbA and phbB genes are each preceded by a putative Shine-Dalgarno sequence that perfectly or nearly matches, respectively, the consensus sequence 5'AGGAG 3' (Gold, *Ann. Rev. Biochem.* 57:199-233, 1988); however, a Shine-Dalgarno sequence similar to that of the consensus could not be found in the phbC ribosome-binding region. Inefficient translation of the phbC gene would result in very low levels of the synthase even under fully induced conditions. In order to more efficiently translate the phbC gene, the native phbC ribosome-binding site was replaced with the lac ribosome-binding site. The lac Shine-Dalgarno sequences closely matches the consensus sequence and thus, ribosomes are predicted to have a higher affinity for this site than for the phbC Shine-Dalgarno sequence. It was hoped that this replacement would result in increased phbC synthesis, with a resulting increase in PHB yields.

The phbC Shine-Dalgarno sequence was replaced as follows. A 50 ml culture of *E. coli* strain XL-1 Blue (Stratagene) pJM9230 was grown to saturation in LB + 50 µg/ml kanamycin in a 250 ml Erlenmeyer flask (Wheaton). Plasmid pJM9230 was purified from the culture using the QIAGEN Plasmid Kit (QIAGEN Inc.) according to the manufacturer's protocol. This procedure is based on a modification of the alkaline lysis method (Birboim and Doly, *Nucl. Acids. Res.* 7:1513-1522, 1979.). The plasmid was digested the BstB I, which cleaves at a site approximately 30 bp upstream of the phbC structural gene. Small deletions were made from this site into the phbC ribosome-binding region with Exonuclease III using the double-stranded Nested Deletion Kit (Pharmacia) according to the manufacturer's protocol, with the following modifications: The
incubation temperature was 30°C and the digestion buffer contained 150 mM NaCl.

To measure the extent of the deletion, an aliquot of DNA from each time point was digested with Not I (New England Biolabs), which cleaves at a site within the phbC structural gene. The DNA was separated by gel electrophoresis in 3.0% Meta-Phor agarose (FMC) in TAE buffer using a BRL Model H5 Horizontal Gel Electrophoresis System and a BRL Model 100 Power Supply. The gel was stained in a solution of 0.5 µg/ml ethidium bromide for 20 minutes and destained in water for one hour. DNA samples in which the deletions extended into the phbC ribosome-binding site but not into the phbC structural gene were chosen for cloning.

Phosphorylated BamH I linkers were ligated to the blunt ends of the deletion endpoints using T4 DNA Ligase and the DNA was introduced into E. coli strain XL-1 Blue cells by electroporation as previously described. Transformants were spread onto LB agar plates containing 200 µg/ml ampicillin and 1.0% glucose. Light-brown translucent (PHB-) colonies were picked and purified by single colony isolation. These mutants were defective in PHB production, presumably because they were unable to synthesize the phbC gene product. Since the promoter region was still intact and the deletions did not extend into the phbC structural gene, this phenotype could only be due to a partial or complete deletion of the phbC ribosome-binding site. Several isolates were picked and purified by single colony isolation. These mutants were inoculated into 3 ml of LB liquid media containing 200 µg/ml ampicillin. The plasmid DNA was purified using the QIAGEN Plasmid Kit (QIAGEN Inc.) according to the manufacturer's protocol and digested with BamH I. Linearization of the DNA indicated that the BamH I linkers were successfully ligated to the endpoints of the deletion. An aliquot of the BamH I-digested samples was digested with Hind III. This removed all DNA sequence upstream of the deletion endpoint including the promoter. The tac promoter GENVBLOCK® restriction fragment was ligated into the Hind III-BamH I pJM9230 fragment using T4 DNA ligase and the recombinant plasmid was introduced into E. coli strain HMS174 pMS421 by electroporation as previously described. The transformed cells were selected on LB agar plates containing 200 µg/ml ampicillin and 10 µg/ml streptomycin. Colonies were replica plated onto LB agar plates containing 200 µg/ml ampicillin + 1.0% glucose + 1 mM IPTG using an Accutan Replica Plater (Schleicher & Schuell). Deletion derivatives
that yielded large white colonies were isolated from the original selection plates and purified. The plasmids were designated pJM9375 and pJM9376 (Figure 11).

Example 13: Determination of the tac GeneBlock-phbC leader fusion joint in plasmids pJM9375 and pJM9376 by sequence analysis.

The precise endpoints of the Exo III deletions were determined by sequence analysis. The plasmid DNA used as template was isolated from 50 ml cultures of *E. coli* strain Hms174 pJM9375 pMS421 and Hms174 pJM9376 pMS421 grown to saturation in LB media containing 200 µg/ml ampicillin + 10 µg/ml streptomycin. The plasmid DNA was purified using a QIAGEN Plasmid Kit (QIAGEN Inc.) as previously described. The DNA was sequenced using a Li-Cor DNA Sequencer Model 4000. The primer used was an infrared dye-labeled M13 17-mer -20 Sequencing Primer (3'TGACCCGCAAGCAAAAAATG5') (Seq. I.D. No. ____________). Sequenase Version 2.0 T7 DNA Polymerase (United States Biochemical) was used to extend the primer. The dideoxy reaction was performed as described in the Li-Cor Model 4000 Quick Start Tutorial (Li-Cor), Section 3, with the following modifications: no DTT was used, and the template and primer were annealed for 1 hour at 55°C.

The sequence of the clones is shown in Figure 1, panels d and e (Seq. ID Nos. ______ to ______). Previous studies (reviewed by Gold, *Ann Rev. Biochem.* 57:199-233, 1988) indicate that optimal spacing between the translational start codon and the Shine-Dalgarno sequence is greater than five nucleotides and less than thirteen. In the pJM9375 clone the spacing between the first (downstream) lac Shine-Dalgarno sequence and the start of the phbC structural gene, 4 bases, is less than optimal. The spacing between the phbC start codon and the second (upstream) lac Shine-Dalgarno sequence, 11 bases, is within the optimal range. In the process of constructing the pJM9375 clone a mutation was made that resulted in an alteration of the phbC start codon. The ATG start codon was replaced with a GTG start codon. Although some mRNAs exhibit the same translational yield with ATG and GTG, the ATG codon usually results in higher translation (Gold, *supra*). In the pJM9376 clone the spacing between the start of the phbC structural gene and the lac Shine-Dalgarno sequence, 18 bases, is greater than the optimal typical range. This construct retains the putative phbC Shine-Dalgarno sequence and the ATG start codon.
Example 14. Comparison of PHB production in native and tac promoter phb multicopy clones

The purpose of this experiment was to compare PHB production of the tac::phb fusion constructs with that of the native phb clones in the multicopy and runaway replicon systems. Four E. coli strains were used in this experiment: HMS174 pJM9131, HMS174 pJM9232 pMS421, HMS174 pJM9117, and HMS174 pJM9238. E. coli strain HMS174 pJM9131 was inoculated into 50 ml of LB medium containing 50 µg/ml kanamycin in a 250 ml Erlenmeyer flask. E. coli strain HMS174 pJM9232 pMS421 was inoculated into 50 ml LB medium containing 50 µg/ml kanamycin and 10 µg/ml streptomycin in a 250 ml Erlenmeyer flask. These cultures were shaken at 225 rpm at 37°C in a G24 Environmental Incubator Shaker (New Brunswick Scientific) for approximately 15 hours. E. coli strain HMS174 pJM9232 and E. coli strain HMS174 pJM9238 were each inoculated into 50 ml of LB medium containing 25 µg/ml chloramphenicol in a 250 ml Erlenmeyer flask. These cultures were shaken at 225 rpm at 30°C in an Innova 4000 Incubator Shaker (New Brunswick Scientific) for approximately 15 hours. The cultures were diluted to a final optical density at 600 nm of 0.10 into 250 ml of the same media in a 1 liter baffled Erlenmeyer flask, except glucose was added to a final concentration of 2.0%, and the cultures were incubated at the same temperature and agitation as previously described. The growth of the culture was followed by withdrawing aliquots at regular time intervals and measuring the optical density at 600 nanometers using a Shimadzu UV 160U Spectrophotometer.

The phb operon was induced in E. coli strain HMS174 pJM9232 pMS421 by the addition of IPTG to a final concentration of 10 mM at an OD600 of 2.75. The phb operon was induced in E. coli strain HMS174 pJM9117 and E. coli strain HMS174 pJM9238 by transferring the cultures to a 41°C waterbath for 30 minutes when an OD600 of 0.7 was reached. (Previous experiments indicated that the optimal cell density for induction of E. coli strain HMS174 pJM9117 in minimal media is at an optical density of 1.0 or slightly lower. When cells were induced at cell densities slightly higher than 1.0, the PHB yield rapidly decreased.) A sterile stir bar was added and the cultures were mixed at 200 rpm using a Fisher Scientific Electronic Stirrer 2008. Following the heat pulse, the cultures were incubated in a 37°C in an Innova Incubator Shaker (New Brunswick Scientific) and shaken at 200 rpm.
Aliquots were withdrawn for dry weight determinations and to quantitate PHB production as follows. For the PHB assay, duplicate 3 ml samples were placed in Pyrex No. 9826 screw-capped tubes and pelleted by centrifugation in a Varifuge RF centrifuge (Heraeus Instruments) for 10 minutes at 2500 rpm. The supernatant was aspirated and discarded, and the tubes containing the cell pellets were placed at -70°C for at least one hour. Uncapped screw-capped tubes containing the frozen pellets were then placed in a Labconco lyophilizer for approximately 2 hours until samples were freeze-dried.

Samples were then subjected to methanolysis as follows. To each tube was added 1.7 ml ACS grade methanol (Mallinkrodt), 2 ml ACS grade chloroform (Mallinkrodt), 0.3 ml concentrated sulfuric acid (added while vortexing tube) and 0.1 ml benzoic acid solution (2 mg/ml). Samples were capped tightly, placed in a heat-block adjusted to 100°C and incubated for 140 minutes. Samples were then removed from the heat block and allowed to cool to room temperature. One ml of deionized water was then added to each tube, the tubes were vortexed for 30 seconds, and then centrifuged in a Varifuge RF centrifuge (Heraeus Instruments) for 10 minutes at 2500 rpm. The upper aqueous phase of each sample was removed by aspiration and the remaining organic phase was pipetted into vials and assayed for PHB production by gas chromatography.

The gas chromatography system consisted of a Shimadzu GC-14A, connected to a CR-4A data processing unit, an AOC-14 autoinjector, and an AOC-1400 autosampler. The carrier gas was UPC grade helium and detection was through a flame ionization detector. The flow rate of the carrier was approximately 5 ml/min. The column used for detection was a Supelcowax 10 column (Supelco Separation Technologies). The column is a 15 meter column, 0.53 mm inner diameter, with a 1 μm thick coating. Samples (1 to 3 μl) were injected into the injection port (temperature 200°C) and carried into the column. The samples were run under temperature profile of 55°C for 5 minutes, followed by a temperature ramp of 5°C per minutes until the column temperature reached 220°C. The temperature was held at 220°C for 5 minutes, followed by a termination of the run and cool-down for the next run. Typically, the solvent peak eluted through the detector (240°C) between 1 and 2 minutes, and the PHB peak eluted between 3 and 4 minutes. Analyses were done using benzoic acid (100 μl of 2 mg/ml solution in methanolysis tubes) as an internal standard. Typically, benzoic acid eluted from the GC column approximately 5 minutes into the run.
PHB samples were weighed out on Sartorius balance and subjected to methanolysis. The area under each curve (integration by Shimadzu data processor) was graphed against the known weights. The resulting line was used to generate an equation that could be used in calculating the PHB content in the experimental samples using the integration area under the PHB peak.

For dry weight determinations, 5.0 ml samples were removed from the cultures and centrifuged for 10 minutes at 3000 rpm in a Varifuge RF centrifuge (Heraeus Instruments). The supernatant was removed by aspiration and the cell pellet was resuspended in 1.0 ml of 0.85% saline solution. This was added to a preweighed aluminum weigh boat and placed in a 80°C drying oven for approximately 30 hours. The dry weight was calculated by subtracting the weight of the empty aluminum weigh boat and the weight of 1.0 ml of 0.85% saline from the total weight. This value was then divided by 5 to obtain the dry weight in mg/ml.

Glucose concentrations were quantitated using the Sigma Diagnostics Glucose Assay Kit (Sigma), Procedure No. 635, p. 5. Test tubes were labeled blank, standard, and test. To each blank tube, 0.1 ml water was added. To the standard tubes, 0.1 ml of diluted Glucose Standard solution (Catalog No. 635-100) were added at concentrations of 1-20 mM. One milliliter of each culture sample to be tested was centrifuged in a Eppendorf Centrifuge 5415C microcentrifuge for two minutes to pellet the cells. To each test tube, 0.1 ml of culture supernatant was added, then 5.0 ml of o-Toluidine Reagent (Catalog No. 635-6) was added. The tubes were mixed by vortexing and placed into a 100°C heat block for 10 minutes. The tubes were removed and cooled to room temperature. The contents of tubes were transferred to cuvettes and the absorbance at 635 nm was read using a Shimadzu UV 160U Spectrophotometer with the blank as reference.

The plasmid copy number was determined for each culture as follows. Two hundred microliters of cell suspension was centrifuged in a Eppendorf Centrifuge 5415C microcentrifuge for one minute. The supernatant was aspirated off and discarded. The cell pellet was resuspended in 50 ml of 10 mM Tris (pH 8.0), 10 mM EDTA, 100 mM NaCl, 20% sucrose, 1.5 mg/ml lysozyme (Sigma), 2 units/ml RNase. The solution was incubated for 30 minutes at 37°C. Fifty microliters of 2% SDS was added and the solution was mixed by vortexing at the maximum setting for two minutes. The solution was frozen at -70°C and thawed for two cycles. Five microliters of a 400 mg/ml proteinase K (BRL) stock solution was added and the tube was incubated for 30 minutes at
37°C. Twenty-five microliters of loading buffer (50% glycerol, 1 mM EDTA, pH 8.0, 0.1% bromophenol blue) was added and 5-15 µl of the sample was loaded on a 0.9% agarose gel in TBE (89 mM Tris-borate, 2 mM EDTA, pH 8.0) buffer. The gel was run for three hours at 75 volts. The gel was stained for 40 minutes in 1 µg/ml ethidium bromide solution. The gel was then destained for 20 minutes in water, rinsed, and destained an additional 20 minutes. The gel was placed on a UV transilluminator (Fotodyne) and photographed using a Polaroid MP-4 Land Camera with Polaroid Type 665 film. The lowest F-stop was used and the shutter was opened for 45 seconds. Immediately after exposure, the negative was placed in fixer solution and agitated gently for approximately 30 seconds (in the dark). The negative was then washed under 65°C running water for 5 minutes and dried. The plasmid and chromosomal bands were quantitated by densitometry using an Ultrascan XL Enhanced Laser Densitometer (LKB) at the following settings: X-width=5, Beam=line, Offset=0, Y-step=1, Smoothing=1, X-step=250, A-axis=normalized, Baseline=2, Peak width=5,5,1. The copy number was calculated by the following formula:

\[
\frac{\text{area in plasmid band}}{\text{area in chromosome band}} \times \frac{\text{kb in plasmid}}{\text{kb in chromosome}}
\]

Results: The presence of the tac promoter in the runaway replicon vector significantly increased the induction rate and yield. In the tac promoter construct *E. coli* strain HMS174 pJM9238, the PHB concentration reached 3 mg/ml (72% of dry weight) approximately 8 hours after induction (Figure 12, panel a). At the same time after induction, the PHB concentration was less than 1.3 mg/ml (46% of dry weight) in the native promoter clone *E. coli* strain HMS174 pJM9117 (Figure 12, panel b) and 1.8 mg/ml (64% of dry weight) and 1.7 mg/ml (57% of dry weight) in *E. coli* strains HMS174 pJM9131 and HMS174 pJM9232 pMS421, respectively (Figure 12, panels c and d). *E. coli* strain HMS174 pJM9238 also retained a higher PHB yield as a percentage of dry weight than strain HMS174 pJM9117 throughout the post-induction period (Figure 13, panel a). This difference was not due to a higher gene dosage for the tac promoter clone. In fact, the pJM9117 copy number was slightly higher than the pJM9238 copy number (Figure 14, panel a). Neither clone utilized all of the glucose by 24 hours, indicating that the conditions of induction were not optimal for either strain in LB medium; however, the comparison is valid since the strains were induced under the same conditions.
In the multicopy plasmid constructs the induction kinetics and yields were not significantly different. In both strains PHB production continued to increase after induction. The yield at 23 hours was 5.2 mg/ml PHB (77% dry weight) for the native promoter clone *E. coli* strain HMS174 pJM9131 (Figure 12, panel c) and 5.6 mg/ml PHB (78% dry weight) for the tac promoter clone *E. coli* strain HMS174 pJM9232 pMS421 (Figure 12, panel d). Both strains had similar PHB yields as a percentage of dry weight after induction, although this value was significantly lower for strain HMS174 pJM9232 pMS421 before the addition of IPTG (Figure 13, panel b), indicating efficient regulation of *phb* expression by the *lac* operator-repressor system. Although the copy number of plasmid pJM9131 was higher than that of plasmid pJM9232 (Figure 14, panel b), this difference had no significant effect on PHB production or yield as a percentage of dry weight.

**Example 15. Comparison of PHB production in the 78 bp leader and the 361 bp leader *phbC tac::phb* fusion constructs**

As described in the section detailing plasmid constructions, two types of *tac::phb* fusions were constructed. In one type of fusion the *tac* promoter was inserted 78 bp upstream of the *phbC* structural gene. In the other type of fusion the *tac* promoter was inserted 361 bp upstream of the *phbC* structural gene. Each type of fusion was cloned into a multicopy vector and runaway replicon vectors. To determine if the leader sequence contained cis-acting elements that regulated the expression of the *phb* genes, PHB production was quantitated in *tac::phb* fusion multicopy clones containing each type of fusion. The *E. coli* strains used in this study were HMS174 pJM9229 pMS421 and HMS174 pJM9232 pMS421. The strains were inoculated into 50 ml of LB media containing 50 μg/ml kanamycin and 10 μg/ml streptomycin in a 250 ml baffled Erlenmeyer flask (Wheaton). The culture was incubated at 200 rpm at 37 °C in a Lab-Line Orbital Environ-Shaker (Lab-Line Instruments, Inc.) for approximately 15 hours. One ml of the stationary phase culture was added to 250 ml of LB media containing 1.0% glucose + 50 μg/ml kanamycin + 10 μg/ml streptomycin in a 1 liter baffled Erlenmeyer flask (Bellco) and incubated at 200 rpm at 37°C. The growth of the culture was followed by withdrawing aliquots at regular time intervals and measuring the optical density at 600 nanometers using a Shimadzu UV 160U Spectrophotometer. At an OD<sub>600</sub> of 2.0, IPTG (United
State Biochemical Corp.) was added to each culture to a final concentration of 10 mM. Samples were withdrawn at regular time intervals for GC analysis.

Results: PHB was not produced in either strain until the addition of the chemical inducer IPTG. PHB production is rapidly induced after the addition of IPTG in both strains (Figure 15). The 361 bp leader tac::phb fusion construct produces more PHB than the 78 bp leader fusion construct. After 5 hours the strain containing plasmid pJM9232 produced twice as much PHB as the strain containing pJM9229, as indicated by GC counts. These results indicate that the leader sequence contains a cis-acting positive regulatory element or elements that increases phb expression. The results also show that the presence of the lacI9 gene on plasmid pMS421 is able to very effectively repress transcription of the phb operon in both of these constructs.

15 Example 16: Optimization of PHB Production at Different Temperatures in E. coli strain HMS174 pJM9238

Preliminary experiments in which PHB production was compared in native and tac promoter phb clones (see Example 14, above) indicated that the conditions for induction were not optimal for E. coli strain HMS174 pJM9238. As one step to determine the optimal conditions for PHB synthesis, the strain was grown at a constant temperature and PHB production was quantitated. E. coli strain HMS174 pJM9238 was inoculated into 50 ml of LB + 25 µg/ml chloramphenicol and the culture was incubated overnight at 30°C. The next morning, 250 ml of LB media containing 2% glucose and 25 µg/ml chloramphenicol in a 1 liter baffled Erlenmeyer flask (Wheaton) was equilibrated to the proper temperature by placing the flask in an incubator. The optical density at 600 nm of the overnight culture was determined and enough volume of the culture was added to the medium to obtain an initial optical density at 600 nm of 0.10. The culture was incubated at a constant temperature of 30°C, 32°C, 34°C, 36°C, 38°C, or 40°C in an Innova 4000 Incubator Shaker (New Brunswick Scientific) at an rpm setting of 175. A total of 5 samples were taken for each culture in the optical density range of 0.4 to 2.0 and used to assay PHB and determine the dry weight as previously described.

Results: PHB levels were 0.1%-2% of the cell dry weight when the cultures were incubated at temperatures of 30°C, 32°C, and 34°C (data not shown). At 36°C the PHB concentration was 10% of the cell dry weight after
about 2 hours of incubation. The PHB concentration increased to over 40% of the cell dry weight after 1.5 hours of incubation at 38°C. At 40°C the PHB concentration was approximately 20% of cell dry weight after 2 hours of incubation (Figure 16). These results indicate that the culture should be incubated at 36°C during the growth phase and shifted to 38°C to initiate PHB production.

Example 17: Determination of the optimal cell density to initiate PHB production in *E. coli* strain HMS174 pJM9238

As a second step to increase PHB production, the optimal cell density at which to induce the PHB biosynthetic pathway was determined. To do this, a culture of *E. coli* strain HMS174 pJM9238 was induced at various cell densities as follows: The strain was inoculated into 50 ml of LB + 25 µg/ml chloramphenicol and the culture was incubated overnight at 30°C. The next morning, 250 ml of LB media containing 2% glucose and 25 µg/ml chloramphenicol in a 1 liter baffled Erlenmeyer flask (Wheaton) was equilibrated to 36°C by placing the flask in an incubator. The optical density at 600 nm of the overnight culture was determined and enough volume of the culture was added to the medium to obtain an initial optical density at 600 nm of 0.10. The culture was incubated at a constant temperature of 36°C in an Innova 4000 Incubator Shaker (New Brunswick Scientific) at an RPM setting of 175. The growth of the culture was followed by measuring the optical density as previously described. Twenty milliliter aliquots of the culture were withdrawn at various times during growth and added to sterile 250 ml baffled Erlenmeyer flasks (Bellco) prewarmed to 38°C in a Lab-Line Incubator-Shaker (Lab-Line Instruments, Inc.). The cultures were incubated at 175 RPM for a total of 24 hours, after which the cells were harvested. PHB production and dry weight of each culture was determined as previously described.

Results: The results indicate that PHB production is highest when the culture is induced at low cell density. When the culture was induced at an optical density of 0.10, the 24 hour culture contained 5.5 mg/ml PHB (71% of dry weight). PHB production was lowest when the culture was induced at mid-log phase. When induced at an optical density of 0.78, the 24 hour culture contained only 2.2 mg/ml PHB (48% of dry weight). PHB production increased as the cells entered late log phase. When the culture was induced at an optical
density of 2.05, the 24 hour culture contained 3.7 mg/ml PHB (66% of dry weight) (Figure 17). These results are different from those obtained with E. coli strain HMS174 pJM9117, where a culture induced by heat shift at an optical density of approximately 1.0 yielded the highest concentration of PHB. The cells were grown in minimal media and the doubling time of the culture was considerable longer. Thus, the growth rate of the culture may be a significant factor in PHB production. In rapidly growing cultures, cell division is able to stay ahead of PHB production. Under these conditions, the cells do not produce enough PHB to inhibit growth. In slow growing cultures, cell division is not rapid enough to stay ahead of PHB production. Consequently, the cells accumulate PHB. Under slow growth conditions it would be better to allow the cell density to increase before initiating PHB production. During rapid growth, the best strategy is to induce PHB production early so that by the time the cells enter late log phase they contain adequate concentrations of the phb gene products and are able to rapidly shift to PHB production.

Example 18: Comparison of PHB production in E. coli strain HMS174 pJM9238 grown in media with and without chloramphenicol

The purpose of this experiment was to compare PHB production in E. coli strain HMS174 pJM9238 in medium containing chloramphenicol and in medium without chloramphenicol. This is a measure of the stability of the plasmid without the selective pressure of the antibiotic under conditions in which PHB is produced. E. coli strain HMS174 pJM9238 was inoculated into 50 ml of LB medium containing 25 μg/ml chloramphenicol in a 250 ml baffled Erlenmeyer flask and incubated at 30°C, 175 rpm overnight. Two 1 liter baffled Erlenmeyer flasks containing 250 ml of LB broth + 2% glucose were prepared. Chloramphenicol was added to one flask to a final concentration of 25 μg/ml. The overnight culture was inoculated into each flask at a starting optical density at 600 nm of 0.10. The cultures were incubated at 38°C, 175 rpm in a Lab-Line Orbital Environ-Shaker (Lab-Line Instruments, Inc.). Multiple 3 ml samples were taken in duplicate over a 7 hour period to determine PHB production by GC analysis.

Results: PHB induction in each culture was nearly identical. At seven hours after induction, the culture containing chloramphenicol produced 1.65 mg/ml PHB, while the culture without chloramphenicol produced 1.58
mg/ml PHB (Figure 18). These results indicate that PHB can be produced efficiently in *E. coli* HMS174 pJM9238 in medium that does not contain chloramphenicol.

Example 19. Quantitation of PHB production in *tac* promoter *lac* Shine-Dalgarno-phbC fusion clones

*E. coli* strains HMS174 pJM9375 pMS421 and HMS174 pJM9376 pMS421 were tested for PHB production in liquid media. The cultures were inoculated into 3 ml of LB media containing 200 µg/ml ampicillin and 10 µg/ml streptomycin in 16x100 mm culture tubes and grown to saturation in a Lab-Line Incubator-Shaker at 37°C with shaking at 200 rpm. The cultures were diluted to an optical density at 600 nm of 0.10 into 50 ml of LB media containing 200 µg/ml ampicillin + 10 µg/ml streptomycin + 2% glucose. At an optical density of 2.0, IPTG was added to the culture to a final concentration of 10 mM. Aliquots of the cultures were withdrawn during growth for measuring optical density, dry weight analysis, and to quantitate PHB production as described above.

Results: PHB started to accumulate 30 minutes after the addition of IPTG to *E. coli* strain HMS174 pJM9375 pMS421 and strain HMS174 pJM9376 pMS421. Six hours after the addition of IPTG, the PHB level in the culture approached 3 mg/ml, compared to approximately 1 mg/ml for strain HMS174 pJM9232 pMS421 (previously determined in Example 15) (Figure 19). These results indicate that the replacement of the *phb* Shine-Dalgarno sequence with the *lac* Shine-Dalgarno sequence resulted in a 3-fold increase in the rate of synthesis of PHB from the multicopy plasmid vector.

Example 20. *Klebsiella* plasmid stability

Strain Constructions: The bacterial strain used in this study was *Klebsiella aerogenes* strain KC2671 *hutC515 recA3011 Δ[bla]-2. Strain KC2671 was streaked onto an LB plate and strain KC2671 pMS421 was streaked onto an LB + 10 µg/ml streptomycin plate from frozen permanents. Single colonies were picked from the plates and patched onto the same medium. These were used as stock plates. Strain KC2671 was inoculated into 3 ml of LB and strain KC2671 pMS421 was inoculated into 3 ml LB + 10 µg/ml streptomycin
and grown to saturation. One ml of each culture was inoculated into a 250 ml Erlenmeyer flask containing 50 ml of the same medium in which they were previously grown. The strains were grown at 30°C to an optical density at 600 nm of 0.7-0.8 and were made electrocompetent as follows (during the following steps the cells were kept ice cold): The cultures were decanted into 50 ml Falcon tubes and placed on ice for 15 minutes, then centrifuged at 3000 rpm for 15 minutes. The supernatant was decanted and the pellets were resuspended in 50 ml of ice cold 10% glycerol. The cultures were centrifuged as before. The supernatant was aspirated off, then the pellets were resuspended in 20 ml of 10% glycerol. The cells were centrifuged as before, then resuspended in 10 ml of 10% glycerol in a 15 ml Falcon tube. The cultures were centrifuged as before, then resuspended in 200 μl 10% glycerol. 40 μl of the cell suspension were pipetted into chilled microfuge tubes and stored at -70°C.

To the KC2671 electrocompetent cells, 1 μl of plasmid pJM9238 was added, and the mixture was pipetted into an ice cold cuvette. To the KC2671 pMS421 electrocompetent cells, 1 μl of plasmid pJM9232 was added and the mixture was pipetted into an ice cold cuvette. The plasmid DNA was introduced into the cells by electroporation as previously described. After electroporation the cells were resuspended in 3 ml of LB medium and incubated at 30°C for 90 minutes, then 1, 10, and 100 μl of each culture was spread onto the appropriate antibiotic plates: strain KC2671 pJM9238 was plated onto LB + 25 μg/ml chloramphenicol and strain KC2671 pMS421 pJM9232 was plated onto LB + 10 μg/ml streptomycin + 50 μg/ml kanamycin. The plates were incubated overnight at 30°C.

Six well-isolated colonies of each strain were picked and patched onto the following plates: strain KC2671 pMS421 pJM9232 was patched onto a) LB + 10 μg/ml streptomycin + 50 μg/ml kanamycin, and b) LB + 10 μg/ml streptomycin + 50 μg/ml kanamycin + 1% glucose + 5 mM IPTG. Both plates were incubated at 30°C overnight. Strain KC2671 pJM9238 was patched onto a) LB + 25 μg/ml chloramphenicol and incubated at 30°C, and b) LB + 25 μg/ml chloramphenicol + 1% glucose and incubated at 37°C.

The glucose-containing plates were inspected and the apparent best PHB producer of each strain was chosen. The strains were then picked from the plates that did not contain glucose (that is, the best-producing cultures were then grown under conditions in which they did not make PHB and were then picked) and inoculated into 50 ml of LB containing the appropriate antibiotics. The cultures were incubated for 6-8 hours at 30°C (until the cultures were in early
stationary phase), then frozen permanents of each strain were made as described above and stored at -70°C.

Plasmid Stability: Four 50 ml cultures in 250 ml Erlenmeyer flasks were started from 500 μl of frozen permanents as follows: Strain KC2671 pJMS421 pJM9232 was inoculated into a) LB medium and b) LB + 10 μg/ml streptomycin + 50 μg/ml kanamycin. Strain KC2671 pJM9238 was inoculated into a) LB medium and b) LB + 25 μg/ml chloramphenicol. The cultures were incubated at a temperature of 30°C in an Innova shaker incubator at 200 rpm. The next morning each culture was diluted into sterile 0.85% saline solution and the 10^-6, 10^-7, and 10^-8 dilutions were spread onto LB plates.

For plasmid preps, 1.0 ml of KC2671 pMS421 pJM9232 culture was pipetted into 1.5 ml microfuge tubes and centrifuged. The pellet was resuspended in 150 μl of SET buffer and stored at -20°C. For strain KC2671 pJM9238, 45 ml of culture was centrifuged in a 50 ml Falcon tube, resuspended in 4 ml of P1 buffer (QIAGEN kit), and stored at -20°C. For the first two days, 0.5 ml of the overnight culture was inoculated into 50 ml of fresh medium and the culture was incubated as before. After the second day, 125 μl of the overnight culture was used as the inoculum. The following day, 50 colonies were picked from each of the four cultures that had been plated, then the selected colonies were patched onto a 50 grid pattern as follows: For strain KC2671 pMS421 pJM9232, colonies were patched onto a) LB + kan, b) LB + strep, c) LB + kan + strep + glucose + IPTG, and d) LB media. The plates were incubated at 30°C. For strain KC2671 pJM9238, colonies were patched onto a) LB + chlor (30°C), b) LB + chlor (37°C), and c) LB (30°C). The next AM, 125 μl of the overnight culture was inoculated into fresh medium, the culture was grown overnight, and plated as described above. After 100 generations, colonies were replica plated onto screening plates for counting.

Results: For strain KC2671 pMS421 pJM9232, after 100 generations 100% (100/100) of the isolates tested from the LB culture and 100% (102/102) of the isolates tested from the LB + 10 μg/ml streptomycin + 50 μg/ml kanamycin culture retained streptomycin and kanamycin resistance. All isolates also produced PHB on plates containing glucose. For strain KC2671 pJM9238, after 100 generations, 99.4% (310/312) of the isolates tested from the culture grown in LB + 25 μg/ml chloramphenicol retained chloramphenicol resistance and produced PHB on plates containing glucose. After 100 generations, 89.8% (212/236) of the isolates tested from the culture grown in LB retained chloramphenicol resistance and produced PHB on plates containing glucose.
The loss of plasmid pJM9238 was first observed after 44 generations. The kinetics of plasmid pJM9238 segregation during growth in nonselective media are depicted in Figure 20, panel a.

Based on the above, when KC2671 pMS421 pJM9232 and KC2671 pJM9238 were grown selectively (i.e., in the presence of antibiotics) the phb genes were not deleted or mutated. This indicates that the presence of the phb operon does not inhibit cell growth. Surprisingly, when the strains were grown nonselectively (i.e., without antibiotics), both the drug resistance markers and the phb genes were retained. Thus, pJM9232 and pJM9238 are stable in this strain, and are suitable for use in a large scale fermentation without the presence of antibiotics in the culture.

Example 21. Klebsiella steady-state tac-phb induction studies

Klebsiella strain KC2671 pJM9238 was grown to saturation overnight in 50 ml of LB + 25 µg/ml chloramphenicol in a 250 ml Erlenmeyer baffled flask at 30°C with shaking at 175 rpm. An aliquot of the culture was inoculated into 250 ml of LB media containing 2% glucose and 25 µg/ml chloramphenicol in a 1 liter Erlenmeyer baffled flask to yield an initial optical density at 600 nm of 0.10. The culture was incubated at a given temperature in the range of 30°C to 40°C with shaking at 175 rpm. The growth of the culture was followed by measuring the optical density at 600 nm. During exponential growth, samples of the culture were harvested for analysis of PHB production.

Results: PHB could be detected in all of the cultures 2 to 4 hours after inoculation. The results for two temperature induction experiments are shown in Figure 20, panel b. In the culture grown at 31°C, PHB production rose from 1.054 µg/ml (0.405% of dry weight) at 3.0 hours after inoculation to 234.4 µg/ml (19.2% of dry weight) at 6.7 hours, then to 716.7 µg/ml (33.8% of dry weight) at 24 hours. In the culture grown at 33°C, PHB production rose from 10.4 µg/ml (1.305% of dry weight) at 2.75 hours after inoculation to 758 µg/ml (32.5% of dry weight) at 5.8 hours, then to 5.416 mg/ml (55.7% of dry weight) at 24 hours. Increasing the temperature above 33°C did not significantly increase the rate of PHB production relative to production at 33°C, and actually resulted in significantly smaller cells (as observed under light microscopy) and decreases yields. This may be a consequence of incubating the bacteria at a temperature well above the optimal range. These results indicate that in the Klebsiella strain KC2671, the tac::phb operon is repressed at 31°C, but expressed at 33°C.
Example 22. KC2671 pJM9238 fermentation

*Klebsiella aerogenes* strain KC2671 pJM9238 was tested for PHB production during fed-batch fermentation. The fermentor used in this study was a B. Braun Type ES10 Biostat E 15 liter fermentor. The parameters were controlled using the Micro-MFCS computer control system (B. Braun Melsungen AG) with a Hyundai Super-386C computer. The strain was inoculated from a frozen permanent into 50 ml of LB medium containing 25 μg/ml chloramphenicol and grown at 31°C to saturation. Approximately 10 ml of this culture was then inoculated into each of two 1 liter Fernbach flasks containing 250 ml of LB + 25 μg/ml chloramphenicol to obtain an initial optical density at 600 nm of 0.10. The cultures were incubated at 31°C, 175 rpm. At an optical density at 600 nm of approximately 3.5, the cultures were inoculated into 5 liters of media containing the following components: 6 g/L Na₂HPO₄ anhydrous, 6 g/L KH₂PO₄ anhydrous, 5 g/L (NH₄)₂SO₄, 0.35 g/L MgSO₄·7H₂O, 3 ml/L trace elements, 5 g/L yeast extract. Chloramphenicol was added to the medium at a final concentration of 25 μg/ml. The feed media was composed of the following components: 33 g/L (NH₄)₂SO₄, 400 g/L glucose, 7 g/L MgSO₄·7H₂O, 5 ml/L trace elements, 5 g/L yeast extract. The culture was incubated at 31°C to an optical density at 600 nm of approximately 3.0, at which time the temperature was shifted to 33°C. Aliquots were harvested at approximately 1 hour intervals for determination of dry weight, PHB content, and glucose concentrations. PHB content and dry weight were determined as previously described in Example 11. Glucose was quantitated using the Sigma Diagnostics Glucose Assay Kit (Sigma), Procedure No. 635, p. 5, as previously described in Example 15.

Results: The results are depicted in Figure 21, panel a. PHB production was effectively repressed in KC2671 pJM9238 when the culture was grown at 31°C. Prior to thermal induction, PHB levels were at or below 0.0441 mg/ml. After the incubation temperature was increased to 33°C, PHB synthesis was rapidly induced. At the 12 hour time point (approximately 6 hours after the temperature shift to 33°C) the PHB concentration was 7.017 mg/ml, an increase of over 150-fold. At the 24 hour time point (approximately 18 hours after the temperature shift) the PHB concentration was 27.4 mg/ml, an increase of over 600-fold. The rate of PHB synthesis observed in strain KC2671 pJM9238 was significantly higher than that previously observed in strain KC2671 pJM9131, as
shown in Figure 21, panel b. For example, at the 20 hour time point, the KC2671 pJM9238 culture contained 23 mg/ml PHB, while KC2671 pJM9131 contained only 10 mg/ml PHB.

The present embodiments of the present invention are to be considered in all respects as illustrative and not restrictive, the scope of the invention being indicated by the appended claims rather than by the foregoing description, and all changes that come within the range and equivalency of the claims are therefore intended to be embraced therein.
Claims

1. A runaway replicon nucleic acid vector construct, comprising a promoter that is negatively regulated by a repressor molecule, b) an operator region capable of binding said repressor molecule, c) a desired nucleic acid sequence, wherein said promoter is operably linked to said operator region and to said desired nucleic acid sequence, and d) a stabilization locus.

2. The vector construct of claim 1 wherein said promoter comprises a -35 region of a trp promoter operably linked to a -10 region of a lac promoter, and b) an operator region of the lac promoter.

3. The vector construct of claim 2 wherein said promoter is a tac promoter.

4. The vector construct of claim 1 wherein said construct further comprises a consensus Shine-Dalgarno sequence operably linked to said desired nucleic acid sequence.

5. The vector construct of claim 4 wherein said consensus Shine-Dalgarno sequence is a lac Shine-Dalgarno sequence.

6. The vector construct of claim 1 wherein said stabilization locus is parB.

7. The vector construct of claim 1 wherein said runaway replicon vector construct includes a λ pR promoter operably linked to a repA gene.

8. A method of inducing a vector construct comprising,

   a) introducing into a host cell a runaway replicon vector construct, comprising i) a promoter that is negatively regulated by a repressor molecule, ii) an operator region capable of binding said repressor molecule, iii) a desired nucleic acid sequence, wherein said promoter is operably linked to said operator region and to said desired nucleic acid sequence, and iv) a stabilization locus; and
b) increasing the temperature of said host cell, thereby expressing said desired nucleic acid sequence.

9. The method of claim 8 wherein step b) further comprises culturing said host cell on an appropriate medium, then increasing said temperature of said host cell, and then further culturing said host cell for a time sufficient to produce a desired product from said desired nucleic acid sequence.

10. The method of claim 9 further comprising the step of isolating said desired product from said host cell.

11. The method of claim 8 wherein said runaway replicon vector construct comprises a λ pR promoter operably linked to a repA gene, and λcI857 gene.

12. The method of claim 8 wherein said host cell is an Enterobacteriaceae host cell.

13. The method of claim 12 wherein said Enterobacteriaceae host cell is E. coli.

14. The method of claim 12 wherein said Enterobacteriaceae is Klebsiella.

15. The method of claim 14 wherein said Enterobacteriaceae is Klebsiella aerogenes.

16. The method of claim 8 wherein said prokaryotic host cell includes an operable lacIq gene.

17. The method of claim 8 wherein said prokaryotic host cell includes an operable lacI gene and does not include an operable lacIq and gene.

18. The method of claim 8 further comprising, during step (b), determining whether said culture of said host cells is growing rapidly or slowly; and then increasing said temperature early in a log phase of a growth cycle of said culture
when said culture is slow-growing, or increasing said temperature late in a log phase of a growth cycle of said culture when said culture is fast-growing.

19. The method of claim 18 wherein said Enterobacteriaceae host cell is *Klebsiella* and said temperature is increased to at least 33°C.

20. The method of claim 19 wherein said Enterobacteriaceae is *E. coli* and said temperature is increased to at least 36°C.

21. The method of claim 9 wherein step b) further comprises growing said host cell for multiple generations without adding a selective pressure agent to said medium.

22. The method of claim 21 wherein said selective pressure agent is an antibiotic.

23. An Enterobacteriaceae host cell containing a vector construct according to any one of claims 1 to 7.

24. The Enterobacteriaceae of claim 23 wherein said Enterobacteriaceae is *E. coli*.

25. The Enterobacteriaceae of claim 23 wherein said Enterobacteriaceae is *Klebsiella*.

26. A method of inducing a vector construct comprising,
   (a) introducing into a host cell a runaway replicon vector construct, comprising (i) a promoter that is negatively regulated by a repressor molecule, (ii) an operator region capable of binding said repressor molecule, and (iii) a desired nucleic acid sequence, wherein said promoter is operably linked to said operator region and to said desired nucleic acid sequence;
   (b) culturing said host cell for multiple generations in an appropriate medium without adding a selective pressure agent to said medium;
   (c) increasing the temperature of said host cell, thereby expressing said desired nucleic acid sequence.
27. The method of claim 26 wherein said selective pressure agent is an antibiotic.

28. The method of claim 26 wherein said method further comprises, after increasing said temperature, further culturing said host cell for a time sufficient to produce a desired product from said desired nucleic acid sequence.

29. The method of claim 26 wherein said host cell is an Enterobacteriaceae host cell.

30. The method of claim 29 wherein said Enterobacteriaceae host cell is E. coli.

31. The method of claim 29 where said Enterobacteriaceae host cell is Klebsiella and said increase in temperature is to about 33°C.

32. The method of claim 31 wherein said Enterobacteriaceae host cell is Klebsiella aerogenes.
phb promoter and phbC Shine-Dalgamo (pJM9131, pJM9117)

\[
\begin{align*}
\text{TTGACAGCGGTGCAGTGCAAGGCAACATGGACCTCAAA...288 bp...AGAGAGACATCAATCATG} \\
-35 & -10 & +1 & \text{putative SD Met}
\end{align*}
\]

TAC promoter and phbC Shine-Dalgamo (72 bp leader) (pJM9229, pJM9236)

\[
\begin{align*}
\text{TTGACAAATATTACATCGGCTCGTATATAATGTGGAATT...58 bp...AGAGAGACATCAATCATG} \\
-35 & -10 & +1 & \text{putative SD Met}
\end{align*}
\]

tac promoter and phbC Shine-Dalgamo (355 bp leader) (pJM9232, pJM9238)

\[
\begin{align*}
\text{TTGACAAATATTACATCGGCTCGTATATAATGTGGAATT...334 bp...AGAGAGACATCAATCATG} \\
-35 & -10 & +1 & \text{putative SD Met}
\end{align*}
\]

tac promoter and laco Shine-Dalgamo (pJM9375)

\[
\begin{align*}
\text{TTGACAAATATTACATCGGCTCGTATATAATGTGGAATT...20 bp...CACAGGAAACAGGATCCCCTG} \\
-35 & -10 & +1 & \text{SD SD SD Met}
\end{align*}
\]

tac promoter and laco and phbC Shine-Dalgamo (pJM9376)

\[
\begin{align*}
\text{TTGACAAATATTACATCGGCTCGTATATAATGTGGAATT...20 bp...CACAGGACGGATCCCAGACAAATCATG} \\
-35 & -10 & +1 & \text{SD SD putative SD Met}
\end{align*}
\]

**Fig. 1**
Fig. 3A
Fig. 5
SUBSTITUTE SHEET (RULE 26)
Fig. 8
Fig. 9
Fig. 11
Comparison of PHB Induction in multicopy tac::phb clones

![Graph showing PHB induction over time for pJM9229 and pJM9232 clones.]

**Fig. 15**

PHB Production in E.coli strain HMS174 pJM9238 at different Incubation Temperatures

![Graph showing PHB production at different incubation temperatures (36°C, 38°C, 40°C).]

**Fig. 16**
Fig. 17

Fig. 18
Comparison of PHB Synthesis in Transcriptional and Translational Fusions

Fig. 19
Stability of p/M9238 in strain KC2671
(no chloramphenicol added)

Fig. 20A

PHB Production in KC2671 p/M9238
at Different Temperatures

Substitute sheet (Rule 26)  Fig. 20B
**Fig. 21A**

**KC2671 pJM9238 Fermentation**

- **Dry Cell Weight**
- **PHB**

**Fig. 21B**

**Comparison of PHB Yield in strain KC2671 containing different phb plasmids**

- **pJM9131**
- **pJM9238**
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N15/69 C12N15/70 C12N15/74 C12P7/62

According to International Patent Classification (IPC) or to both national classification and IPC:

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched:

Electronic database consulted during the international search (name of data base and, where practical, search terms used):

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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<tbody>
<tr>
<td>X</td>
<td>WO, A, 93 24633 (CENTER FOR INNOVATIVE TECHNOLOGY) 9 December 1993 cited in the application</td>
<td>1,6-13, 16-18, 20-24, 26-30</td>
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<tr>
<td>Y</td>
<td>see the whole document.</td>
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<td>Y</td>
<td>BIOTECHNOL. PROG., vol. 9, 1993 pages 31-39, A.P. TOGNA ET AL.; 'Effects of plasmid copy number and runaway plasmid replication on overproduction and excretion of beta-lactamase from Escherichia coli' see the whole document.</td>
<td>2,3</td>
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Further documents are listed in the continuation of box C.

 Patent family members are listed in annex.

Date of the actual completion of the international search

2 May 1995

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk
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Authorized officer

Yeats, S
<table>
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<tr>
<td>Y</td>
<td>NUCL. ACIDS RES., SYMPOSIUM SERIES, vol. 12, 1983 pages 87-90, M. NOMURA ET AL.;  'Construction of expression plasmids producing high levels of human immune interferon in E. coli' see the whole document.</td>
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<td>Patent document cited in search report</td>
<td>Publication date</td>
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<td>WO-A-9324633</td>
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