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Reduction of Wobble-position GC Bases in *Corynebacteria* Genes and Enhancement of PCR and Heterologous Expression

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Abstract

Corynebacteria **codon usage exhibits an overall GC content of 67%, and a wobble-position GC content of 88%. *Escherichia coli*, on the other hand has an overall GC content of 51%, and a wobble-position GC content of 55%. The high GC content of *Corynebacteria* genes results in an unfavorable codon preference for heterologous expression, and can present difficulties for polymerase-based manipulations due to secondary-structure effects. Since these characteristics are due primarily to base composition at the wobble-position, synthetic genes can, in principle, be designed to eliminate these problems and retain the wild-type amino acid sequence. Such genes would obviate the need for special additives or bases during *in vitro* polymerase-based manipulation and mutant host strains containing uncommon tRNA's for heterologous expression.**

We have evaluated synthetic genes with reduced wobble-position G/C content using two variants of the enzyme 2,5-diketo-D-gluconic acid reductase (2,5-DKGR A and B) from *Corynebacterium*. The wild-type genes are refractory to polymerase-based manipulations and exhibit poor heterologous expression in enteric bacteria. The results indicate that a subset of codons for five amino acids (alanine, arginine, glutamate, glycine and valine) contribute the greatest contribution to reduction in G/C content at the wobble-position. Furthermore, changes in codons for two amino acids (leucine and proline) enhance bias for expression in enteric bacteria without affecting the overall G/C content. The synthetic genes are readily amplified using polymerase-based methodologies, and exhibit high levels of heterologous expression in *E. coli*.

Introduction

2,5-diketo-D-gluconic acid reductases (2,5-DKGR; E.C. 1.1.1.-) from *Corynebacterium* catalyze the NADPH-dependent reduction of 2,5-diketo-D-gluconic acid (2,5-DKG) to 2-keto-L-gulonic acid (2-KLG) (Sonoyama *et al.,* 1982). 2-KLG is a key intermediate in the commercial synthesis of L-ascorbic acid (vitamin C) (Anderson *et al.,* 1985; Miller *et al.,* 1987; Grindley *et al.,* 1988). Two variants of this enzyme, 2,5-DKGR A and 2,5-DKGR B, have been identified with 41% identity at the DNA level and 38% identity at the amino acid level (Sonoyama and Kobayashi, 1987). Both *Corynebacterium* genes have high GC content; form A having 68% (Anderson *et al.,* 1985) and form B having 71% (Grindley *et al.,* 1988). Sequencing and PCR amplification of the 2,5-DKGR genes have proven problematic (Anderson *et al.,* 1985; Powers, 1996), presumably due to regions of high melting temperature or residual secondary structure in G/C-rich regions of the DNA duplex. Heterologous expression of *Corynebacterium* 2,5-DKGR A has been demonstrated in *Erwinia herbicola* (Anderson *et al.,* 1985), while expression attempts in *E. coli* have proven unsuccessful (Powers, 1996). Heterologous expression of 2,5-DKGR B in *E. coli* has been reported, but the level of expression was not evaluated (Grindley *et al.,* 1988).

Analysis of codon statistics for *Corynebacterium* is limited by a relatively small sample population but indicates that there is an overall bias for G/C residues of 67%, with 67% G/C content in the first position, 45% in the second, and 88% in the wobble-position (Genbank). *E. coli*, on the other hand, has an overall bias for G/C residues of 51%, with 59% G/C content in the first position, 41% in the second, and 55% in the wobble-position (Genbank). Therefore, reduction of the G/C content of *Corynebacterium* genes may be achieved by appropriate substitutions at the wobble-position base, while retaining the corresponding amino acid sequence. Such altered genes may exhibit improved properties with regard to polymerase-based manipulations. Furthermore, appropriate alterations at the wobble positions may additionally increase the preferred codon usage for heterologous expression in enteric bacteria. Such synthetic genes for 2,5-DKGR A and B were designed and assembled in a two-step PCR method (Dillon and Rosen, 1990) and their PCR and heterologous expression properties evaluated.

Results

A total of 155 codons (out of 278 total) in 2,5-DKGR A, and 163 codons (out of 277 total) in 2,5-DKGR B were changed in the design of the synthetic genes. In 2,5-DKGR A, 116 codon changes result in a decrease in the G/C content, 31 result in no change, and 8 result in an increase in G/C content (Table 1). In 2,5-DKGR B, 125 codon changes result in a decrease in G/C content, 30 result in no change and 8 result in an increase in G/C content (Table 2). A total

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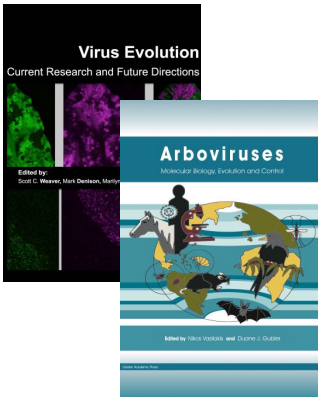

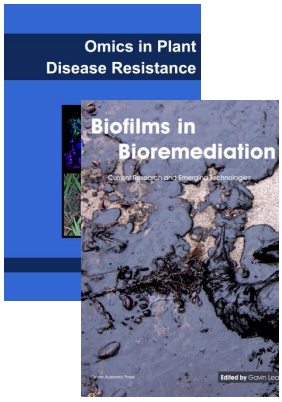

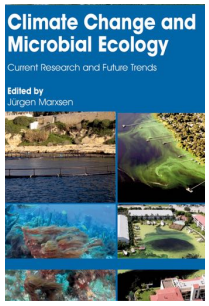

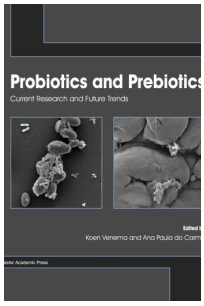

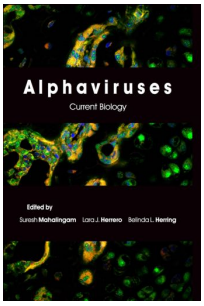

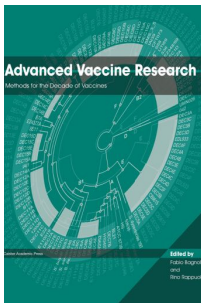

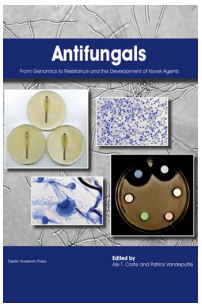

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Table 1. The Most Significant Codon Substitutions in the Construction of the Synthetic 2,5-DKGR A Gene. The relative effects upon codon wobble position G/C content and bias in relationship to enteric bacteria codon preference are listed.

Residue	From	To	ΔWobble G/C	ΔBias
ALA	GCG(17), GCC(17)	GCT	-34	5.78
ARG	CGC(10), CGG(1)	CGT	-11	5.64
GLU	GAG(13)	GAA	-13	7.28
GLY	GGC(12), GGG(2)	GGT	-14	3.66
LEU	CTC(17)	CTG	0	12.92
LYS	AAG(9)	AAA	-9	4.32
PRO	CCC(7)	CCG	0	5.39
SER	AGC(5), TCG(4)	TCT	-9	2.8
THR	ACG(3)	ACC	0	1.44
VAL	GTC(11), GTG(12)	GTT	-23	9.04

of 154 codon changes (out of 155) in 2,5-DGKR A, and a total of 160 codon changes (out of 163) in 2,5-DKGR B, result in an increase in the preferred codon bias for the *E. coli* host. The resulting nucleotide sequences for 2,5-DKGR A and B reduce the overall GC content from 68% to 55% and from 71% to 56%, respectively, and increase the average codon bias for enteric bacteria from 44% to 66% and from 41% to 68% respectively.

The results of the initial PCR for the construction of the nascent template indicate the presence of several PCR products, most of which are smaller than the desired full-length 2,5-DKGR genes (Figure 1). Nonetheless, the second PCR step, using outer primers, resulted in the production of a DNA product with a size appropriate for the full-length genes (Figure 1). Thus, the initial PCR step resulted in the successful assembly of full-length genes, in addition to various partial gene fragments. Sequence analysis of the pFASTBAC1 subcloned PCR product indicated two point mutations within the 2,5-DKGR A gene and one point mutation within the 2,5-DKGR B gene. Repeated PCR experiments resulted in similar numbers of point mutations, albeit at different locations. The correct synthetic gene sequences were thus produced by subsequent site-directed mutagenesis upon genes within the pFASTBAC1 vector. Re-sequencing in the pET-21(+) expression vector confirmed the correct desired sequences.

Induction of expression by IPTG in the pET-21(+) expression vector in the BL21(λDE3) *E. coli* host resulted in the production of a ~34 kDa protein for 2,5-DKGR A and a ~31 kDa protein for 2,5-DKGR B (Figure 2). The control cells with no added IPTG showed no such proteins. This level of expression indicates that 2,5-DKGR A and B represent the major proteins in the induced cells. The expression reached maximum levels within 4 hours after induction by IPTG. Purified 2,5-DKGR A and B exhibit enzyme activity towards both dihydroxy acetone phosphate and 2,5-DKG substrate (data not shown).

Table 2. The Most Significant Codon Substitutions in the Construction of the Synthetic 2,5-DKGR B Gene. The relative effects upon codon wobble position G/C content and bias in relationship to enteric bacteria codon preference are listed.

Residue	From	To	ΔWobble G/C	ΔBias
ALA	GCG(14), GCC(7)	GCT	-21	3.01
ARG	CGC(16), CGG(6)	CGT	-22	12.28
GLU	GAG(19)	GAA	-19	10.64
GLY	GGC(14), GGG(6)	GGT	-20	6.36
LEU	CTC(15)	CTG	0	11.4
LYS	AAG(3)	AAA	-3	1.44
PRO	CCC(8)	CCG	0	6.16
SER	AGC(11), TCG(5)	TCT	-16	5.02
THR	ACG(5)	ACC	0	2.4
VAL	GTC(12), GTG(10)	GTT	-22	8.78

Discussion

With the exception of AGC to TCT mutations for the codon corresponding to serine (5 total in 2,5-DKGR A and 11 in 2,5-DKGR B) all mutations in design of the synthetic genes comprised point mutations at the codon wobble position. The greatest contribution to changes in GC content for 2,5-DKGR A included alanine, valine, glycine, glutamate, arginine, serine and lysine codons (Table 1). A similar analysis for 2,5-DGKR B identifies valine, arginine, alanine, glycine, glutamate, and serine codons (Table 2). Codon changes that did not affect GC content, but did improve codon bias for heterologous expression in *E. coli*, included leucine, proline and threonine codons for both 2,5-DKGR A and B (Tables 1 and 2).

The 2-step PCR method used here to produce synthetic 2,5-DKGR A and B genes has been applied in the construction of a variety of genes, gene libraries, and plasmids (Rauscher *et al.,* 1990; Ye *et al.,* 1992; Stemmer *et al.,* 1995). DNA sequences in the range of ~200 bp to 5 Kb can be assembled from chemically synthesized oligonucleotides in a single reaction (Stemmer *et al.,* 1995). However, the construction of synthetic 2,5-DKGR A and B genes using the described two-step PCR method did not result in genes that were free from sequence errors. In several different experiments we observed between one and five point mutations in the final PCR product. These mutations may be the result of long PCR reactions (Stemmer *et al.,* 1995). Barnes *et al.* has suggested that the addition of a proofreading polymerase may be important to ensure efficient long PCR reactions by combining high processivity with proofreading (Barnes, 1994). However, it has been demonstrated that similar mutations were found with or without proofreading polymerase (Chen *et al.,* 1994). The most expedient approach to obtain a correct sequence did not appear to be repeating the PCR steps, but to perform site-specific mutagenesis on the incorrect full-length synthetic genes. Similar results have been noted by other groups using this method (Beattie and Fowler, 1991).

Another approach for the construction of synthetic genes involves an annealing/ligation protocol of oligonucleotides comprising the entire sequence of a desired gene (Sproat and Gait, 1985; Wosnick *et al.,* 1989; Climie and Santi, 1990). In this method, oligonucleotides are annealed in a piecemeal fashion followed by joining with T4 DNA ligase. The method used here has some advantages over the annealing/ligation method. First of all, the two-step PCR method can be completed within 1 working day, however, annealing and ligation of overlapping sets of complementary oligonucleotides can often require considerably longer time periods (*i.e.* weeks) to complete (Beattie and Fowler, 1991). Another advantage of the present method is that it is more economical than

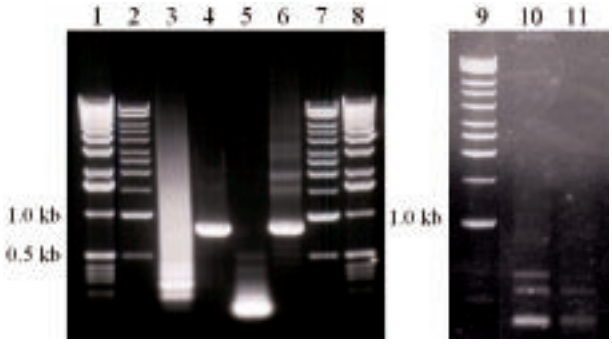

Figure 1. Analysis of synthetic 2,5-DKGR A and B genes by 1% agarose gel electrophoresis. Lanes 1, 2, 7 and 8, DNA size markers; lanes 3 and 5, products of the first PCR step in construction of the synthetic 2,5-DKGR A and B genes, respectively; lanes 4 and 6, the end products of the second PCR for synthetic 2,5-DKGR A and B gene, respectively. Lane 9, DNA size marker; lanes 10 and 11, PCR of wild-type 2,5-DKGR A and B genes, respectively, using outer primers (as described for the second PCR reaction for the synthetic genes).

annealing/ligation methods (Di Donato *et al.,* 1993). A total of 20 oligonucleotides (~60mers) were used to construct both 2,5-DKGR A (834 bases) and B (831 bases) synthetic genes. The number of bases involved is approximately 25% lower than the number of bases required by the established methodology of total synthesis using ligation of complementary oligonucleotides.

One of our primary goals in the development of synthetic genes for 2,5-DKGR A and B was to improve the ability to perform polymerase-based methodologies, including PCR, mutagenesis and sequencing. Prior reports describing sequencing or mutagenesis efforts with 2,5-DKGR A or B have detailed problems with polymerase-based sequencing and PCR (Anderson *et al.,* 1985; Powers, 1996). In our own hands, the sequencing of wild-type 2,5-DKGR A has been very difficult to achieve - requiring proprietary commercial sequencing reagents and methods. Since the method utilized for the construction of the synthetic 2,5-DKGR genes relies upon PCR under standard buffer conditions, the successful construction of a full-length gene indicates that the problems associated with PCR and the wild-type genes have been eliminated. Furthermore, the sequencing of the resulting synthetic 2,5-DKGR A and B genes proceeds without the difficulty experienced with the wild-type genes. The results support the hypothesis that the high GC content of 2,5-DKGR A and B contributes to problematic polymerase-based methodologies, and that appropriate reduction in GC content can solve this problem.

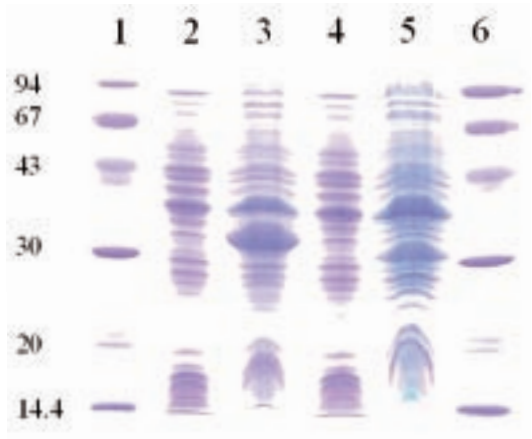

Figure 2. Expression of synthetic 2,5-DKGR A and B genes in pET21 expression vector and *E. coli* BL21(λDE3) host. Lanes 1 and 6, molecular weight markers; lanes 2 and 4, synthetic 2,5-DKGR A and B genes in pET21 expression vector, respectively, non-induced; lanes 3 and 5, synthetic genes for 2,5-DKGR A and B in pET21 expression vector induced by 1 mM IPTG, respectively.

A second goal in the development of synthetic genes for 2,5-DKGR A and B was to allow high-levels of expression in an *E. coli* host. SDS PAGE of the IPTG-induced BL21(λDE3) *E. coli* host indicates that high levels of expression of both 2,5-DKGR A and B are achieved (Figure 2). *Acetobacter* has been used for the heterologous expression of 2,5-DKGR A primarily because expression in *E. coli* has proven unsuccessful (D. Powers, personal communication). Heterologous expression of 2,5-DKGR B in *E. coli* has been reported, but the levels of expression were not detailed (Grindley *et al.,* 1988). In our hands, we were never able to successfully PCR the wild-type 2,5-DKGR A or B genes for subcloning purposes, thus, we were unable to construct and evaluate expression of the wild-type gene sequence. The results shown here demonstrate that high-level heterologous expression of synthetic 2,5-DKGR A and B genes has been achieved in *E. coli*, presumably due to the improvement in codon bias for enteric bacteria. Additional experiments with heterologous expression of the synthetic 2,5-DKGR A gene indicate that approximately 30 mg of purified active protein can be isolated from 1.0 liter of bacterial culture in M9 minimal media. Although problematic *in vitro* polymerase-based procedures can sometimes be obviated by the inclusion of various additives in the reaction mixture (Baskaran *et al.,* 1996), and improved heterologous expression can be achieved in hosts containing supplemental tRNA's for rare codons (Carstens and Waesche, 1999), the development of the synthetic genes in the present report eliminates both of these restrictions. Due to the characteristically high GC content at the wobble position, the present methodology represents a generally applicable approach to allow efficient polymerase-based manipulation as well as efficient heterologous expression of *Corynebacteria* genes.

Experimental Proceedure

Pwo DNA polymerase and T4 DNA Ligase were from Boehringer Mannheim Co. (Indianapolis, IN). Subcloning vector pFASTBACI, restriction enzymes (*Nde*I, *Hin*dIII, and *Stu*I), Calf Intestinal Alkaline Phosphatase (CIAP), and T4 Polynucleotide Kinase were from New England Biolabs or GIBCO BRL (Gaitherburg, MD). Expression vector pET-21a(+) was from Novagen (Madison, WI). *E. coli* strains DH5α and BL21(λDE3) were from GIBCO BRL. Long oligonucleotides (~60 nucleotides) were synthesized and further purified using polyacrylamide gel electrophoresis (PAGE) by Integrated DNA Technologies, Inc. Short oligonucleotides (~20 oligonucleotides) were synthesized by the Bioanalysis Sequencing and Synthesis Laboratory at the Florida State University. QuikChange™ Site-Directed Mutagenesis Kit was purchased from Stratagene (La Jolla, CA).

Design of the Synthetic 2,5-DKGR A and B Genes

Four general criteria were included in the design of synthetic genes for 2,5-DKGR A and B: 1) Nucleotide sequences for 2,5-DKGR A and B were chosen to maintain the amino acid sequence as deduced from the wild-type nucleotide sequences (Anderson *et al.,* 1985; Powers, 1996). 2) In the case of amino acids with degenerate codons, codons were chosen to minimize G/C content at the wobble position. 3) Codons were chosen to maximize observed codon bias in enteric bacteria (Grosjean and Fiers, 1982). In cases where the preferred (A/T-rich) codon(s) had poor enteric bacteria bias (e.g. <0.1) preferred codons were chosen over A/T rich codons. 4) The cut-off limit of acceptable free energies for hairpin, dimerization and false priming for 60mer test oligonucleotides were -7.0, -13, and -23 kcal/mol, respectively. Regions of possible hairpin formation, false priming and primer dimerization within the synthetic nucleotide sequences were identified and ranked by free energy calculations using the program Primer Premier (Premier Biosoft International). Based on the above criteria, a total of 20 oligonucleotides, each approximately 60 nucleotides in length, were synthesized for the construction of both 2,5-DKGR A and B genes. For construction purposes, these long oligonucleotides were designed with regions of complementary overlap (~20 bases in length) with neighboring oligonucleotides.

Construction of Synthetic 2,5-DKGR A and B Genes

A two-step PCR method was used for the construction of the synthetic 2,5-DKGR A and B genes (Dillon and Rosen, 1990). Template DNAs corresponding to the full-length synthetic genes were generated using the complete set of 20 overlapping long oligonucleotides in a single PCR. Non-phosphorylated oligonucleotides (each 50 pmol), dNTPs (50 mM), *Pwo* polymerase (5 units) and PCR reaction buffer were mixed together in a 100 μl sample. The assembled genes from this initial PCR were used as templates in a second PCR using phosphorylated outer primers. Templates (1 μl of first PCR reaction), dNTPs (20 mM), primers (each 20 pmol), *Pwo* polymerse (2.5 units) and PCR reaction buffer were mixed together in a 100 μl sample. Both PCR reactions were carried out in a Pelkin-Elmer thermal cycler for 30 cycles. Each cycle comprised denaturation, annealing and extension conditions of 94°C for 1 minute, 60°C for 1 minute, and 72°C for 1 minute, respectively. An initial denaturation step of 94°C for 5 min was applied for each PCR reaction. Polynucleotide products from both the first and second PCR were analyzed using ethidium bromide stained 1% Agarose gel electrophoresis.

Subcloning of the Synthetic 2,5-DKGR A and B Genes into Heterologous Expresssion Vector

After the second PCR amplification, the 2,5-DKGR A and B genes were extracted from agarose gel and subcloned into *Stu*I digested, and calf intestinal phosphatase treated, pFASTBAC1 vector (GIBCO BRL) via blunt end ligation. The choice of pFASTBAC1 for this step of subcloning was simply to expedite subsequent subcloning via restriction by *Nde*I and *Hin*dIII endonucleases. The synthetic genes for both 2,5-DKGR A and B were sequenced after subcloning into pFASTBAC1 by vector-specific primers. The synthetic genes were restricted from the pFASTBAC1 vector using *Nde*I and *Hin*dIII restriction endonucleases and purified using 1% Agarose gel electrophoresis. The gel-extracted DNA fragments were ligated with *Nde*I/*Hin*dIII restricted pET-21a(+) expression vector (Novagen). After this final subcloning step, both genes were sequenced again in the pET-21a(+) vector to confirm their sequence.

Heterologous Expression of Synthetic 2,5-DKGR A and B Genes in *E. coli*

2,5-DKGR A and B in the pET-21a(+) expression vector were transformed into *E. coli* strain BL21(λDE3). The transformed *E. coli* was grown at 37°C in M9 minimal media (Sambrook *et al.,* 1989) to an optical density of A_{600}=1.2, at which point the temperature was shifted to 28°C and expression of the synthetic 2,5-DKGR A and B genes was induced by the addition of 1 mM isopropyl-ß-D-thiogalactopyranoside (IPTG). The cells were allowed to grow for an additional 4.0 h and were then harvested by centrifugation (8,000 X g for 10 min). The cell pastes were stored frozen at –20°C before use. Induction of 2,5-DKGR A and B proteins was evaluated using sodium dodecylsulfate (SDS) PAGE.

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