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The toxicity of recombinant proteins in *Escherichia coli*: a comparison of overexpression in BL21(DE3), C41(DE3), and C43(DE3)

Laurence Dumon-Seignovert, Guillaume Cariot, and Laurent Vuillard*

Avidis SA, Biopole Clermont Limagne, 63360 St Beauzire, France

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Abstract

Two mutant strains of *Escherichia coli* BL21(DE3), called C41(DE3) and C43(DE3) and originally described by Miroux and Walker, are frequently used to overcome the toxicity associated with overexpressing recombinant proteins using the bacteriophage T7 RNA polymerase expression system. Even when the toxicity of the plasmids is so high that it prevents transformation in the strain BL21(DE3), the toxic proteins can often be expressed successfully in C41(DE3) and/or C43(DE3). In this work, using a range of plasmids coding for several types of proteins, we investigated in BL21(DE3), C41(DE3), and C43(DE3) their ability to undergo transformation and to express. While transformation was always possible in C41(DE3) and C43(DE3), we could not obtain transformants in BL21(DE3) for 62% of the expression vectors tested. Moreover, after induction, the expression of heterologous proteins in both mutant strains is generally better than in BL21(DE3). In this study, we also enhanced the stability of plasmids in culture during the expression of proteins by adding the *par* locus from the plasmid pSC101 to the vector backbone. The stability of a subset of the plasmids (measured 3 h after induction) was determined in C41(DE3) and C43(DE3) and varies from 62 to 92% for C43(DE3) and from 10 to 90% for C41(DE3). This study demonstrates the usefulness of these strains C41(DE3) and C43(DE3) in solving the problem of plasmid instability during the expression of toxic recombinant proteins.

The bacteriophage T7 promoter expression system is widely used for the overexpression of both prokaryotic and eukaryotic proteins. In the commonly used *Escherichia coli* host strain BL21(DE3), the T7 RNA polymerase is produced from the lysogenic λ prophage DE3, and its expression is under the control of the IPTG-inducible lac UV5 promoter. The target gene cloned downstream from the T7 promoter is transcribed from the vector by bacteriophage T7 RNA polymerase [1].

Although thousands of proteins have been successfully expressed to very high levels in BL21(DE3), very often significant over-production cannot be achieved because of the toxicity of the target protein, which may even cause bacterial cell death. From BL21(DE3) two mutant host strains, called C41(DE3) and C43(DE3), were selected that grew to high saturation density, and continue to produce proteins at an elevated level without toxic effects [2].

The C41(DE3) and C43(DE3) strains have been used to produce proteins that were either expressed poorly in BL21(DE3), or for which the expression plasmids could not be transformed into BL21(DE3) [3–5]. Therefore, in these examples, strains C41(DE3) and C43(DE3) were generally superior to BL21(DE3) as a host for protein overexpression [2].

In the present report, the toxicity of a set of plasmids coding for a variety of recombinant proteins has been compared in three strains: BL21(DE3), C41(DE3), and C43(DE3). Furthermore, in this study we have modified the expression vector used (pRSETA) in order to reduce the problem of plasmid instability often occurring when cultures are expanded for large-scale production. Following the work of Skogmann et al. [6] with tryptophan-operon bearing plasmids, the *par* (or partition) locus described by Miller et al. [7] of the plasmid pSC101 has been added to the pRSETA vector. The plasmid stability

* Corresponding author. Fax: +33-0-473-644-393.
E-mail address: lvuillard@avidis.fr (L. Vuillard).

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in C41(DE3) and in C43(DE3) was studied in the presence or absence of the *par* fragment for a set of proteins. With these practical examples, we demonstrate that C41(DE3) and C43(DE3) can efficiently solve problems of transformation and/or toxicity observed with the BL21(DE3) strain.

Materials and methods

Plasmids and strains

Both the original plasmid pRSETA and the strain BL21(DE3) were obtained from Invitrogen (USA). Both C41(DE3) and C43(DE3) were commercial batches from Avidis S.A. (OverExpress strains; www.avidis.fr).

The *par* fragment was obtained from the pMTL21P vector [8]. To insert the *par* fragment into pRSETA vector, both vectors were digested by the restriction enzyme *Bgl*I. Two *Bgl*I sites are present in each vector. The 1256 bp fragment of pMTL21P containing the *par* locus was ligated into the 1630 bp fragment of pRSETA containing the T7 promoter. In the resulting vector, named pRSETA-*par* (2886 bp), the f1 origin of pRSETA was replaced by the *par* fragment.

Transformation efficiency

For C41(DE3) and C43(DE3), 28 plasmids were tested in each strain, against 26 for BL21(DE3). Out of the 12 plasmids reported as non-expressing in BL21(DE3), 10 corresponds to plasmids that we could not transformed in this strain.

The transformation success rate corresponds to the ratio of the number of plasmids leading to the presence of growth on LB–ampicillin-agar plates to the total number of tested plasmids.

Toxicity test

The toxic effects of the overexpression of heterologous proteins cloned into the pRSETA-*par* vector on the growth of the strains BL21(DE3), C41(DE3), and C43(DE3) were tested on two sets of LB–ampicillin-agar plates (bacto tryptone 10 g/L, yeast extract 5 g/L, NaCl 10 g/L, and agar 15 g/L), one set containing 1 mM IPTG (isopropyl-β-D-thiogalactoside) and the second one lacking this inducer. Both sets contained ampicillin 100 μg/ml. The plasmid toxicity was defined as absence of colonies on plates containing ampicillin and inducer (1 mM IPTG) despite transformation and growth in the presence of ampicillin. The percentage of toxic plasmids corresponds to the ratio of the number of plasmids leading to an absence of growth on LB–ampicillin–IPTG agar plates to the total number of tested plasmids.

Plasmid stability

The plasmid stability was checked in both strains C41(DE3) and C43(DE3) using the following protocol:

Plasmids were transformed using the calcium chloride protocol described in Sambrook et al. [9]. The following day, a single colony was used to inoculate 2 ml LB–ampicillin (100 μg/ml) medium. The culture was incubated overnight at 37 °C with shaking. This pre-culture was then used to inoculate 10 ml LB–ampicillin (inoculum 1% v/v). These cultures were grown at 37 °C with vigorous shaking. During the exponential phase ($0.6 < A_{600\,nm} < 0.8$), cultures were induced by the addition of 0.5 mM IPTG for 3 h. Following measurement of culture turbidity at 600 nm, a series of dilution was prepared and for each dilution, 100 μl were immediately plated either on plain LB-agar plates, or on LB-agar plates containing ampicillin (100 μg/ml). It is important to note that, for both C41(DE3) and C43(DE3), it is essential to use freshly transformed cells.

Result and discussion

Toxicity test

The toxicity of plasmids and the expression of heterologous proteins encoded by these plasmids were compared in three *E. coli* strains: BL21(DE3), C41(DE3), and C43(DE3). The transformation success rate corresponds to the ratio of plasmids leading to the presence of growth on LB–ampicillin-agar plates to the total number of tested plasmids. And the plasmid toxicity was defined as the absence of colonies on plates containing ampicillin and inducer despite transformation and growth in the presence of ampicillin only (i.e., without inducer). Results are summarized in Table 1. First, for 62% of the plasmids tested, we could not transform in BL21(DE3) since no colony was obtained after transformation on LB–ampicillin-agar plates, despite repeated attempts and successful transformations in parallel of the C41(DE3) and C43(DE3) strains. In comparison, both of the strains C41(DE3) and C43(DE3) always produced colonies on LB–ampicillin-agar plates after transformation (transformation success rate of 100%). Furthermore, 96% of tested plasmids were toxic in BL21(DE3) leading to an absence of colonies in the presence of inducer (1 mM IPTG). Toxicity was observed in 50% of cases for C41(DE3), and only in 4% of cases for C43(DE3). However, it should be pointed out that even when toxicity existed in C41(DE3), the target protein were well overexpressed. For C41(DE3) and C43(DE3), respectively, 86 and 81% of the heterologous proteins from this study were expressed after 3 h of induction with IPTG at 37 °C. These percentages were higher than with BL21(DE3) in which only 54% of these proteins

Table 1
Comparison of parental strain BL21(DE3) with the mutant strains C41(DE3) and C43(DE3) for transformation efficiency, expression-induced toxicity, and expression of heterologous proteins

Strains	Transformation			Toxicity			Expression in liquid media		
	Yes	No	Transformation success rate (%)	No	Yes	Percentage of toxic plasmids (%)	Yes	No	Percentage of expressing plasmids (%)
BL21(DE3)	16	10	62	1	25	96	14	12	54
C41(DE3)	28	0	100	14	14	50	24	4	86
C43(DE3)	28	0	100	27	1	4	23	5	81

Transformation. Corresponds to the growth on LB–ampicillin-agar plates after transformation with a plasmid. The figure in the "Yes" column corresponds to the number of plasmid leading to the presence of colonies. The figure in the "No" column corresponds to the number of plasmid leading to the absence of colonies. The transformation success rate corresponds to: [(Number of plasmid leading to the presence of colonies/Number of plasmid tested) × 100].

Toxicity. Corresponds to the growth on LB–ampicillin–IPTG agar plates after transformation with a plasmid. Column "Yes" corresponds to the number of plasmid leading to the presence of colonies. Column "No" corresponds to the number of plasmid leading to the absence of colonies on LB–ampicillin–IPTG agar plates. The percentage of toxic plasmids corresponds to: [(Number of plasmid leading to the absence of colonies/Number of plasmid tested) × 100].

Expression in liquid media: Yes, observation of the heterologous protein in total cell pellet on Coomassie-stained SDS–PAGE. No, absence of the heterologous protein in total cell pellet on Coomassie-stained SDS–PAGE. The percentage of expressing plasmids corresponds to: [(Number of expressed proteins/Number of plasmid tested) × 100].

were expressed. For BL21(DE3), in these 54% we have considered that the 10 plasmids where colonies did not grow on LB–ampicillin-agar plates (i.e., that we could not transform) also correspond to heterologous proteins that cannot be expressed in this strain.

The absence of colonies or the presence of only very small colonies on LB–ampicillin-agar plates when using BL21(DE3) might be explained by an insufficient repression of the T7 RNA polymerase. For the mutant C41(DE3) and C43(DE3) strains, the locations of the mutations are unknown, but a plausible hypothesis [2] for the resistance of these strains to the toxicity of heterologous proteins is that the mutations either affect the activity of the T7 RNA polymerase or reduce the level of production of the polymerase.

It is important to note that, for both C41(DE3) and C43(DE3), it is essential to use freshly transformed cells.

Plasmid stability

To reduce the problem of plasmid instability, we inserted the *par* locus into pRSETA producing the plasmid pRSETA-*par*. Into both of the strains C41(DE3) and C43(DE3), the stability of pRSETA and pRSETA-*par* was compared to that of a plasmid lacking the T7 promoter, namely pUC18. The plasmid stability was defined as the ration of the number of colonies on LB–ampicillin-agar plates under the number of colonies on LB-agar plates. In C41(DE3) and C43(DE3), the pUC18 plasmid has a stability of 86 and 80%, respectively (Table 2). The stability of the pRSETA plasmid was less than 10% in C41(DE3) but when the *par* locus was present (pRSETA-*par*), this stability increased to 81%. This result is in full agreement with previous data [6]. Surprisingly, for the strain C43(DE3), the addition of the *par* locus within pRSETA did not affect the plasmid stability (near 85%).

Table 2
Comparison of the stability of empty vectors with and without the presence of *par* locus in both strains C41(DE3) and C43(DE3)

Strains	Percentage of plasmid stability		
Vector:	pUC18 (%)	pRSETA (%)	pRSETA-par (%)
C41(DE3)	86	<10	81
C43(DE3)	80	85	93

pRSETA-*par*, vector pRSETA with the *par* locus from pSC101. The percentage of plasmid stability corresponds to: [(Number of colonies on LB–ampicillin-agar plates/Number of colonies on LB-agar plates) × 100].

To test more broadly plasmid stability in C41(DE3) and C43(DE3), several heterologous proteins (metabolic enzymes, chaperones, proteases, and complement protein domain) were chosen. The results obtained are summarized in Table 3. The presence of plasmids within both strains was measured after 3 h of induction with 0.5 mM IPTG at 37 °C. Our data showed that all the tested plasmids were relatively stable in the strain C43(DE3) with a stability at 3 h varying between 62 and 91%. For C41(DE3), the plasmid stability was more variable. Nevertheless, the protein was very well expressed even in the absence of inducer. For the eleven (well-expressed) proteins tested in C41(DE3), three had a low plasmid stability (<10%) but for all the other plasmids, the percentage of plasmid stability ranged from 40 to 92%. However, this stability may not necessarily reflect the situation at the protein level as in the case of a very well-expressed protein, (the human chaperonin Cpn10 which can be produced up to 100 mg/L culture in shakeflasks), the plasmid stability after 3 h was less than 10%.

Conclusion

The expression of toxic proteins in *E. coli* strains causes plasmid instability: bacteria which have lost the

Table 3
Plasmid stability for several heterologous proteins in the strains C41(DE3) and C43(DE3)

Protein name	Function	C41(DE3) (%)	C43(DE3) (%)
β-Subunit of *E. coli* ATPase	Transport protein [10]	14	76
GroEL	Chaperone	92	91
AppA protein	Histidine acid phosphatase for feed additives	<10	ND
DsbA protein	Protein disulfide isomerase	46	75
DsbC protein	Disulfide bond isomerase	86	92
Metabolic enzyme	L-serine hydrolyase	46	62
Complement protein domain	Complement regulation	85	72
DsbA fused to complement domain	Disulfide bond locking	70	76
Human Cpn10	Chaperone	44	84
Affinity tag fused to Human Cpn10	Chaperone	40	ND
Hirudin fused to Cpn10	Protease inhibitor	<10	85

The percentage of plasmid stability corresponds to: [(Number of colonies on LB–ampicillin-agar plates/Number of colonies on LB-agar plates) × 100].

expression plasmid rapidly overgrow those retaining it. The presence of the *par* locus and the use of either the C41(DE3) or C43(DE3) strains can minimize this phenomenon of plasmid instability for toxic proteins.

Plasmid stability naturally depends on the encoded proteins, but it is clear that the plasmid stability is, in general, better in the strain C43(DE3). Based on these results, when plasmid stability is a issue, such as in large-scale production, we recommend the preferential use of the strain C43(DE3) rather than C41(DE3).

As seen from the work described here, for approximately two-fifths of heterologous proteins, transformation was even not possible in BL21(DE3). The mutant strains C41(DE3) and C43(DE3) are clearly superior to the parental strain BL21(DE3) for the expression of many heterologous proteins.

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