Excretion into the Culture Medium of a *Bacillus* β-Glucanase after Overproduction in *Escherichia coli*

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The β-glucanase gene (bgl) from *Bacillus amyloliquefaciens* was expressed in *E. coli* CSH 55 under the control of the P_R promoter of phage λ that is repressed by the thermosensitive repressor C_{1857}. Production of β-glucanase was drastically stimulated by a temperature shift to 42 °C. This overexpression of the bgl gene (about 20% of the total cellular protein) led to an almost complete excretion of the otherwise periplasmic protein into the extracellular medium, β-glucanase accounted for more than 50% of the extracellular proteins.

Col E 1 related plasmid (pEG 1) are amplified in *E. coli* relA strains in response to an amino acid limitation leading to a 10-fold increase in the activity of plasmid encoded genes. In this work we intended to maximize the expression of the bgl gene by a concerted action of a plasmid amplification and temperature induction. Surprisingly we could not increase the β-glucanase production above the level reached by plasmid amplification or temperature induction alone. The reasons for this unexpected result will be discussed. Under all conditions tested the expression of the bgl gene was much lower in the *E. coli* relA strain NF 162 than in *E. coli* CSH 55; the low β-glucanase production was accompanied by a reduced excretion rate of the enzyme.

Introduction

Genetic engineering methods have facilitated the expression of foreign genes in *Escherichia coli*. Two main approaches have been applied in order to enhance the expression of foreign genes: (i) to increase the gene dosage by amplification of chromosomal genes or by plasmid amplification [1–3]; (ii) to bring the foreign gene under the control of a very strong [4] and regulable promoter (for review see [5]).

In this study we tried to combine the amplification of plasmids with the application of strong and regulable promoters in order to maximize the expression of foreign genes in *E. coli*. We found that in *E. coli* relA strains Col E 1-related plasmids were amplified about 5- to 8-fold in response to amino acid starvation [6, 7]. It is noteworthy that stringently controlled strains did not amplify the plasmid under the same conditions [7, 8]. This amplification system is suitable for analyzing the overexpression of plasmid-encoded genes. We studied the expression of a cloned β-glucanase gene (bgl) from *Bacillus amyloliquefaciens* [9]. After amplification of bgl-encoding plasmids an about tenfold increase of β-glucanase production was measured [10].

A further enhancement of bgl expression is expected when plasmid amplification would be combined with the application of strong and regulable expression systems. Therefore the original bgl promoter was replaced by the strong phage lambda promoter P_R which is controlled by the thermosensitive repressor C_{1857} [11]. After temperature shift up we found a very strong stimulation of bgl expression. It is interesting to note that this overproduction of the normally periplasmic β-glucanase [9] led to the excretion of the enzyme into the culture medium. However, we have not succeeded yet in further increasing the bgl expression by a concerted action of plasmid amplification and temperature induction. The reasons for this unexpected result will be discussed.

Materials and Methods

Plasmids pEG 1 [9] and pCEZ 12 [11] were digested with *Eco*RI, the corresponding fragments ligated (Fig. 1) and transformed into several *E. coli* strains (see Table I). The strain CSH 55 was grown at 30 °C under continuous shaking in a synthetic medium [12] supplemented with thiamine (10 mg/l) and glucose (2 g/l). The $relA^+$/relA-strains were

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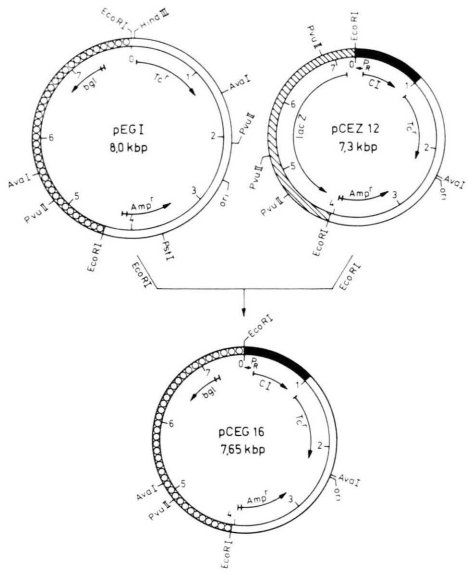

Fig. 1. Construction of pCEG 16. Plasmid pEG 1 contains a 3,6 kb *Eco*R I-fragment of *Bacillus amyloliquefaciens* DNA carrying the bgl gene inserted into the *Eco*R I site of pBR 322 [9]. The *Eco*R I-fragment of pEG 1 containing the bgl gene was ligated with the 4.05 kb *Eco*R I-fragment of pCEZ 12 yielding a construct in which the bgl gene can be expressed from the phage λ promoter P_R.

Table I. *E. coli* strains used in this study.

Strain	Genotype	Source
CSH 55	Δ (lac pro), supE, nalA, thi, (F'lacZ, pro A^+ B)	this lab
NF 161	met, arg, spoT	B. Bachmann
NF 162	met, arg, spoT relA	B. Bachmann

grown in the same medium but supplemented with thiamine (10 mg/l), glucose (6 g/l), $Na_2HPO_4 \times 12$ H_2O (322 mg/l), arginine and methionine (each 25 mg/l). After strains NF 161 and NF 162 had entered the stationary phase as a result of arginine exhaustion, amino acids were added again after 10 h of starvation in order to allow an optimal expression of the amplified DNA. For the inactivation of the C_{1857} repressor one half of the culture was shifted to 42 °C and the other half remained at 30 °C as control.

The plasmid content was determined according to Frenkel and Bremer [13] and the activity of β-glucanase was measured according to Borriss ([14], see also [10]). Enzyme activity is expressed as units per cell mass [$U \times ml^{-1} \times A_{500}{}^{-1}$]. One unit was defined as the amount of β-glucanase releasing sugar residues at 37 °C with a reducing action of 1 μmol glucose.

Cellular β-glucanase was obtained by sonication of washed cell suspensions. The preparation of proteins for electrophoretic analysis was described by Georgiou *et al.* [15]. Proteins were separated in a 10 to 20% SDS polyacrylamide gradient gel containing 0.2% lichenan [16]. The zymogram technique for the detection of β-glucanase in polyacrylamide gradient gels was performed as described previously [17].

Results and Discussion

Plasmid pCEG 16 encodes the bgl gene of *Bacillus amyloliquefaciens*. In addition to its original promoter the construct carries the promoter P_R of phage λ which is controlled by the thermosensitive repressor C_{1857} [11]. At 30 °C C_{1857} represses the P_R promoter but after a temperature shift to 42 °C the repressor is inactivated. Exponentially growing cells of *E. coli* CSH 55 harbouring the plasmid pCEG 16 contained only a low β-glucanase activity at 30 °C due to the original bgl promoter from *Bacillus* [9]. The enzyme activity was mainly localized in the periplasmic space [9]. After a temperature shift to 42 °C the β-glucanase activity was drastically enhanced (Fig. 2). This overproduction of β-glucanase was accompanied by leakage of the otherwise periplasmic protein into the culture medium. Eleven h after the temperature shift up to 80% of the β-glucanase activity was found in the culture medium. The absence of intracellular marker enzymes (*e.g.* glucose-6-phosphate dehydrogenase) in the growth medium indicates that this enzyme excretion was specific and not due to cell lysis.

Our electrophoretic analysis showed that the cloned gene product accounted for about 20% of the total cellular protein (Fig. 3). The β-glucanase was identified on the gel by a zymogram technique (not shown). Densitometric measurements revealed about 60 to 70% of the total extracellular protein to be β-glucanase (Fig. 3). The excreting cells released several other periplasmic proteins into the growth medium too (Fig. 3).

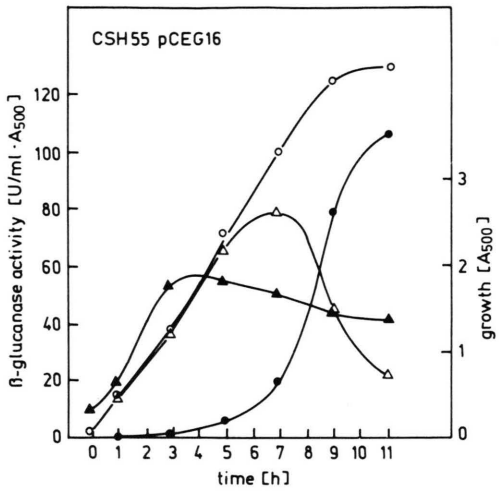

Fig. 2. Time course of β-glucanase production and excretion by *E. coli* CSH 55 pCEG 16 after a temperature shift from 30 °C to 42 °C (temperature shift at 0 h). —○— total β-glucanase activity; —△— cellular β-glucanase activity; —●— extracellular β-glucanase activity; —▲— growth, measured as absorbance at 500 nm (A_{500}).

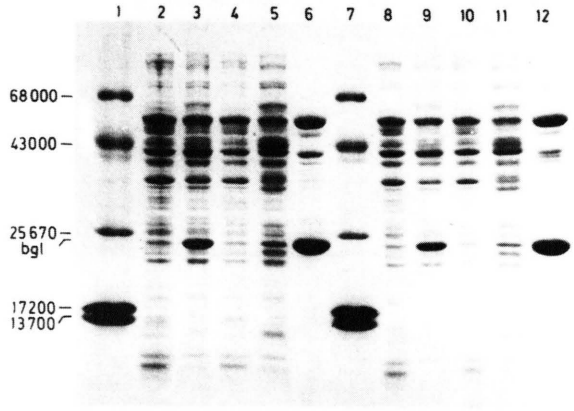

Fig. 3. Detection of β-glucanase in CSH 55 pCEG 16 after SDS-polyacrylamide gel electrophoresis (SDS-PAGE). Separation of cellular (lanes 2 to 5, 40 µg and 8 to 11, 30 µg) and extracellular (lane 6 and 12, 6 µg) protein fractions on SDS-PAGE (10–20%). Staining of proteins was performed with Coomassie brilliant blue G 250. The β-glucanase is marked with bgl.

Lanes: 1, 7	molecular weight markers;
2, 8	7 h, 30 °C (control);
3, 9	7 h after the temperature shift;
4, 10	11 h, 30 °C (control);
5, 11	11 h after the temperature shift;
6, 12	extracellular protein fraction, 11 h after the temperature shift.

In order to enhance the temperature-dependent overexpression by plasmid amplification we transformed the plasmid pCEG 16 into the isogenic relA$^+$/relA-strains of *E. coli* NF 161 and NF 162. After arginine starvation the plasmid content increased about 5-fold in the relA strain (Fig. 4C) but in the stringently controlled counterpart NF 161 the plasmid content did not significantly change (Fig. 4A). In spite of plasmid amplification the β-glucanase activity remained unchanged at 30 °C in amino acid-starved *E. coli* relA cells.

When amino acids were added to this starved cells, an approximate 5-fold increase in enzyme activity per cell mass was observed. This increase in enzyme activity correlated with the preceding plasmid amplification (Fig. 4C). However, β-glucanase activity did not increase after the addition of amino acids to starved cells of *E. coli* NF 161 (relA$^+$) which did not amplify the plasmid (Fig. 4A). Similar results were obtained using the plasmid pEG 1 which encodes the β-glucanase from *Bacillus amyloliquefaciens* under the control of its original promoter [10].

When the readdition of amino acids to starving cells was accompanied by a temperature shift to 42 °C for P_R-induction a strong increase in β-glucanase activity was observed in the relA$^+$ strain (Fig. 4B). In the relA strain which amplified the plasmid there was no further increase above the enzyme level measured at 30 °C (Fig. 4D). The reason for this unexpected behaviour must await further investigation. We could neither find an enrichment of an inactive protein precursor on the polyacrylamide gel nor inclusion bodies as a result of protein precipitation (not shown, see [15]).

The enzyme activities measured in the strains NF 161 and NF 162 were generally lower than the activities determined in CSH 55. The enzyme excretion rates were also lower in the former strains (Fig. 4A–D). These result might be interpreted as a first hint for a correlation between overexpression and enzyme excretion.

In summary we found that overproduction of normally periplasmic proteins may trigger enzyme excretion. This overexpression may disturb the integrity of the outer membrane resulting in the release of periplasmic enzymes without cell lysis. It is tempting to speculate that the high protein concentration in the periplasmic space triggers the leakiness of the outer membrane. Recently the ex-

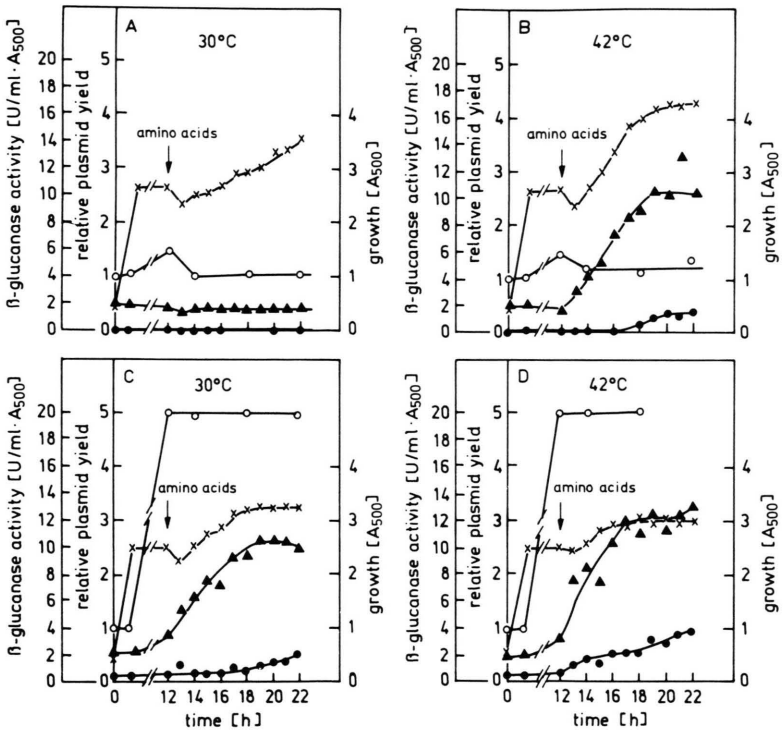

Fig. 4. Time course of β-glucanase production and excretion and plasmid content in amino acid starved *E. coli* strains NF 161 pCEG 16 (relA$^+$, A and B) and NF 162 pCEG 16 (relA, C and D) grown at 30 °C. Ten h after the onset of the stationary phase amino acids were readded and one half of the culture remained at 30 °C (A, C) whereas the other half was shifted to 42 °C (B, D). —▲– β-glucanase activity (cellular); —●— β-glucanase activity (extracellular); —×— growth, measured as absorbance at 500 nm (A_{500}); —○— relative plasmid yield (plasmid content during expotential growth corresponds to 1.).

cretion of periplasmic enzymes and proteins as a result of overproduction have been reported [18–22]. Gormely *et al.* [23], however, found that the secretion of *Bacillus subtilis* C 120 β-glucanase in *E. coli* was not affected by alterations in its rate of synthesis.

Pages *et al.* [18] pointed out that the excretion of PhoS, a periplasmic phosphate-binding protein from *E. coli,* is not caused by a non-specific leakage from the periplasm. Suominen *et al.* [19] suggested that periplasmic protein release is a stress response probably related to activation of autolytic activities. Such autolytic activities could be activated by overproduction of periplasmic proteins. Georgiou *et al.* [21] showed that the permeability of the outer membrane of the excreting cells has been affected, probably by decreasing levels of two outer membrane proteins, OmpA and OmpC [22]. The elucidation of the excretion mechanism, however, must await further investigation.

Furthermore it is noteworthy that cells of *E. coli* relA which amplified the plasmid pCEG 16 in response to amino acid starvation did not show any further increase of β-glucanase activity when the readdition of amino acids was accompanied by a temperature dependent bgl-induction. These results can be explained by a limitation of export sites.

There are indeed hints that export sites might be limited in *E. coli.* A hybrid maltose-binding protein (β-galactosidase) cannot be exported because of jamming of the export machinery [24]. Pages *et al.* [18] found that the overproduction of PhoS led to an accumulation of pre-PhoS both in the cytoplasm and in the inner membrane [25, 26]. However we could not detect any β-glucanase precur-

sors with SDS-polyacrylamide gel electrophoresis. The unexpectedly low production of mature β-glucanase after temperature induction in *E. coli* cells upon amplification of the plasmid pCEG16 remains an interesting problem that needs further investigation.

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