Altered temperature induction sensitivity of the lambda \( p_R/cI857 \) system for controlled gene \( E \) expression in \emph{Escherichia coli}

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Abstract

Cell lysis of Gram-negative bacteria can be efficiently achieved by expression of the cloned lysis gene \( E \) of bacteriophage \emph{PhiX174}. Gene \( E \) expression is tightly controlled by the rightward \( \lambda p_R \) promoter and the temperature-sensitive repressor \( cI857 \) on lysis plasmid \( pAW12 \). The resulting empty bacterial cell envelopes, called bacterial ghosts, are currently under investigation as candidate vaccines. Expression of gene \( E \) is stringently repressed at temperatures up to 30°C, whereas gene \( E \) expression, and thus cell lysis, is induced at temperatures higher than 30°C due to thermal inactivation of the \( cI857 \) repressor. As a consequence, the production of ghosts requires that bacteria have to be grown at 28°C before the lysis process is induced. In order to reflect the growth temperature of pathogenic bacteria in vivo, it seemed favorable to extend the heat stability of the \( \lambda p_R \) promoter/\( cI857 \) repressor system, allowing pathogens to grow at 37°C before induction of lysis. In this study we describe a mutation in the \( \lambda p_R \) promoter, which allows stringent repression of gene \( E \) expression at temperatures up to 36°C, but still permits induction of cell lysis at 42°C.

Keywords: Gene expression; \( \lambda cI857/p_R \); \emph{PhiX174} gene \( E \); Safety cassette; Candidate vaccine

1. Introduction

Empty bacterial cell envelopes (bacterial ghosts) raised from pathogenic Gram-negative bacteria are under investigation as vaccine candidates and carriers of foreign antigens [1–5]. The production of ghosts by protein \( E \)-mediated lysis is based on the tightly controlled expression of the cloned lysis gene \( E \) of bacteriophage \emph{PhiX174}. The gene \( E \) encodes a hydrophobic polypeptide of 91 amino acids that forms a transmembrane tunnel in the bacterial cell wall through which the cytoplasmic contents are expelled [6–11]. The resulting bacterial ghosts share functional and antigenic determinants of the envelope with their living counterparts, as the protein \( E \) does not cause physical or chemical denaturation processes to bacterial surface structures [8,12]. In various lysis plasmids gene \( E \) expression, which is lethal to the host cell, is controlled by the rightward phase \( \lambda p_R \) promoter and the corresponding temperature-sensitive repressor, \( cI857 \), which is inactivated at temperatures higher than 30°C [13]. Using shuttle
lysis plasmids we have been able to produce ghosts of a variety of Gram-negative bacteria [3].

Bacterial lysis due to expression of gene $E$ is induced by a temperature shift of the growing culture from 28°C to 42°C. In $E. coli$ growth of the culture stops approximately 10 to 15 min after the induction of gene $E$ expression. Since pathogenic bacteria infecting humans or animals mostly grow at an ambient temperature of 37°C or more, it is favorable to cultivate these bacteria at their natural ambient growth temperature. Another advantage of cultivating bacteria at temperatures higher than 28°C is the faster growth rate and higher cell mass production. Thus, the aim of this study was to extend the heat stability of the $cI857\lambda prl$ gene $E$ expression cassette allowing bacterial growth around 37°C and lysis at 42°C. In this study, we describe a mutation in the $O_{pl2}$ operator region of the rightward $\lambda prl$ promoter which increases the temperature stability of the $V_{plR}/cI857$ gene expression system. Thus gene $E$ expression is stringently repressed at temperatures up to 36°C, but cell lysis is allowed at 38°C or higher. The mutation introduced by random mutagenesis and identified by phenotypic selection was confirmed by site-directed mutagenesis to be responsible for the altered temperature sensitivity of the $\lambda prl/cI857$ system.

2. Materials and methods

2.1. Strains, plasmids, and growth conditions

$E. coli$ strains NM522 [14], ES1578 [15], JM103 [16], CJ236 [17], MC4100 [18] and plasmids pAW12 [8], pRS415 [19] as well as phage $M13mp18$ [20] have been described. Cultures were grown in Luria broth [21]. When used, supplements were added at the following concentrations: tetracycline, $10 \mu g/ml$; ampicillin, $100 \mu g/ml$ and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (XGal), $0.04 \mu g/ml$. Growth and lysis of bacterial cultures were monitored by measuring the optical density at 600 nm (OD$_{600}$).

2.2. Materials

Restriction enzymes, DNA-modifying enzymes and T4 gene 32 protein were obtained from New England Biolabs or Boehringer Mannheim, and were used as specified by the supplier. Plasmid DNA and DNA fragments were prepared, analyzed and manipulated by standard procedures [22]. Sequence analysis was performed by the sequencing department (OLIGO-OOCM) of the Vienna Biocenter (cycle sequencing with infrared (IRD41) labeled primer).

2.3. Random mutagenesis

Plasmid pAW12 was transformed into the mutator strain $E. coli$ ES1578 according to Hanahan [23] and transformants were allowed to grow on LB-agar plates containing 10 $\mu g/ml$ tetracycline for 2 days at room temperature. After replica plating onto LB-agar plates supplemented with 10 $\mu g/ml$ tetracycline and 1% SDS, the plates were incubated at 37°C and 42°C and colonies growing at 37°C were further analyzed.

2.4. Site-directed mutagenesis and construction of phages $M13WJ1$, $M13WJmut$ and plasmid $pAWJ$

In order to reconstruct the mutation introduced by random mutagenesis of pAW12, a site-directed mutagenesis was performed. A 2190 bp DNA fragment containing the $\lambda prl$ promoter, $\lambda cI857$ repressor and the ColE1 origin of replication (ori) was isolated from pAW12 using the unique $PstI$ and $AflIII$ sites. It was then cloned between the unique $PstI$ and $SalI$ sites of M13mp18 after treatment of the $AflIII$ and $SalI$ restriction sites with Klenow polymerase. The resulting phage M13WJ1 was used for site-directed mutagenesis according to Kunkel et al. [17] except for the addition of 1 $\mu g$ T4 gene 32 protein per $\mu g$ ssDNA to the extension mix. The oligonucleotide 5'-GTAAAATAGTCAACACGC GGGTGTTAGATATTTATC-3' was used for the base substitution (mismatch base is underlined). Mutated plasmids were identified by the use of the restriction enzyme BsrUI, as this restriction site was newly introduced by the site-directed mutagenesis. The base substitution in the resulting plasmid M13WJmut was confirmed by sequence analysis. From M13WJmut a 2190 bp was obtained by digestion with $PstI$ and
AflIII and used to replace the analogous DNA fragment of pAW12. The resulting plasmid pAWJ therefore is analogous to pAW12 except for the single base-pair exchange in the \( \lambda p_R \) promoter.

2.5. Construction of plasmids pAW12-lac and pAWJ-lac

A 4.7 kbp \( \text{EcoRI} \) DNA fragment of pAW12 lacking gene \( E \) was treated with Klenow polymerase. A 3.2 kbp DNA fragment containing the \( \text{lacZ} \) gene was obtained from pRS415 by digestion with \( \text{DraI} \) and \( \text{SmaI} \) and inserted into the blunt end sites of the DNA fragment of pAW12. This resulted in pAW12-lac, where the \( \text{lacZ} \) gene is under control of the wild-type \( \lambda p_R \) promoter. The same procedure described above was performed with plasmid pAWJ, resulting in pAWJ-lac, where the \( \text{lacZ} \) gene is under control of the mutated \( \lambda p_R \) promoter. Positive clones were isolated on the basis of the blue colony color in the presence of XGal at 37\(^\circ\)C. The \( \beta \)-galactosidase activity was determined as described [21].

3. Results and discussion

3.1. Random mutagenesis and detection of a mutation in the \( \lambda p_R \) promoter

The E-specific expression cassette was modified by random mutagenesis to allow growth of the bacteria at 37\(^\circ\)C and lysis induction at 42\(^\circ\)C. The lysis plasmid pAW12, in which the gene \( E \) is under control of the \( \lambda p_R \) promoter and the temperature-sensitive cI857 repressor (Fig. 1A), was used for random mutagenesis. \( E. coli \) bacteria transformed with pAW12 exhibited lysis within 15–30 min after temperature shift-up, whereas bacteria growing at 36\(^\circ\)C (○) exhibit lysis after 90 min.

Fig. 1. A: Lysis plasmid pAW12. The E-specific lysis cassette consists of the lysis gene \( E \) of bacteriophage PhiX174 under transcriptional control of the rightward \( \lambda p_R \) promoter and the corresponding temperature-sensitive repressor cI857. ColE1, origin of replication ColE1; tet, tetracycline resistance gene; E, lysis gene \( E \); cI857, repressor gene cI857; \( \lambda p_R / cI857 \), promoters of phage lambda. B: Growth and lysis curves of \( E. coli \) NM522 harboring the wild-type lysis plasmid pAW12. At temperature shift-up the culture was shifted from 28\(^\circ\)C (●) to 36\(^\circ\)C (○), 38\(^\circ\)C (△) and to 42\(^\circ\)C (□) as indicated by an arrow. At 38\(^\circ\)C (△) and 42\(^\circ\)C (□) onset of lysis starts 15–30 min after temperature shift-up, whereas bacteria growing at 36\(^\circ\)C (○) exhibit lysis after 90 min.
strain ES1578 and transformants were incubated at room temperature for 2 days. A mutant clone was selected for its capability to grow at 37°C and to lyse at 42°C by replica plating and incubation under selective conditions. Plasmid pAW12-10 was isolated, transformed into E. coli strain NM522 and the growth and lysis profile of this clone was determined (Fig. 2). At 36°C bacteria were able to grow with a normal doubling rate, whereas at 42°C lysis of bacteria could be detected 15 min after temperature shift-up as observed for bacteria harboring the wild-type plasmid (pAW12). At 38°C lysis can be observed 2 h after temperature shift-up. A 1570 bp DNA fragment containing the E-specific lysis cassette of plasmid pAW12-10, the cI857 repressor, the $\lambda_{pR}$ promoter and the lysis gene $E$, was used as a template for sequencing reactions. Both DNA strands were sequenced twice and a T-C base exchange in the $O_{R2}$ operator region of the $\lambda _{pR}$ promoter was detected (Fig. 3).

3.2. The T$\rightarrow$C mutation in the $\lambda_{pR}$ is responsible for the altered phenotype

To prove that the T$\rightarrow$C mutation in the $O_{R2}$ region of the $\lambda_{pR}$ promoter of the plasmid pAW12-10 was responsible for the extension of heat stability of the $\lambda_{pR}$/cI857 system, the same mutation was reproduced in the wild-type plasmid pAW12. For this purpose the promoter region of plasmid pAW12 was subcloned into M13mp18, resulting in M13WJ1. Using single-stranded DNA of M13WJ1 as a template the T$\rightarrow$C base exchange was generated by site-directed mutagenesis [17]. The promoter region of pAW12 was then exchanged with the mutated promoter region of M13WJ1mut, the M13 vector carrying the $\lambda_{pR}$ promoter with the T$\rightarrow$C base exchange created by site-directed mutagenesis. The resulting plasmid, pAWJ, was identical with pAW12 except for the T$\rightarrow$C mutation in the $O_{R2}$ region of the $\lambda_{pR}$. To compare the growth and lysis kinetics of E. coli pAW12-10 with E. coli pAWJ, the plasmid pAWJ was transformed into E. coli NM522 and the growth and lysis phenotypes were determined. The growth and lysis of E. coli NM522 (pAW12) were similar to that of E. coli NM522 harboring pAW12-10, the mutant plasmid produced by random mutagenesis in the mutator strain E. coli ES1578. No lysis of bacteria was detected at 36°C. However, cell lysis was induced 15 and 90 min after temperature shift to 42°C and 38°C, respectively. These results show clearly that the single base mutation is responsible for the increased temperature stability of the $\lambda_{pR}$/cI857 system.

3.3. Comparison of the promoter activity of the mutated and wild-type $\lambda_{pR}$ promoter

In order to determine the promoter activity of the mutated and wild-type $\lambda_{pR}$ promoters, we substituted the lysis gene $E$ in plasmids pAW12 and pAWJ with the $\beta$-galactosidase reporter gene lacZ resulting in two plasmids pAW12-lac and pAWJ-lac, respectively. To quantify the $\beta$-galactosidase expression driven from the wild-type and the mutated $\lambda_{pR}$ promoter, the plasmids pAW12-lac and pAWJ-lac were transformed into E. coli MC4100 lacking the lac operon in order to avoid background expression of lacZ. The expression of the lacZ gene driven from the mutated and the wild-type $\lambda_{pR}$ promoter at 28°C, 36°C and 42°C was determined [21]. At 28°C both promoters were efficiently repressed allowing only background expression of about 0.5% of a fully induced wild-type $\lambda_{pR}$ promoter at 42°C.

Fig. 3. Operator/promoter sequence of the mutated rightward $\lambda_{pR}$ promoter. The T$\rightarrow$C base exchange in the $O_{R2}$ region of the mutated $\lambda_{pR}$ promoter responsible for the altered temperature sensitivity of the expression system is indicated by an arrow.
At 42°C, the mutated \( V_pR \) promoter showed nearly the same high level of activity (70%) as the wild-type \( V_pR \) promoter. However, the activity of the mutated \( V_pR \) promoter decreased to about 25% of the wild-type \( V_pR \) promoter at each time point after temperature shift to 36°C (Fig. 4).

Apparent, the T→C mutation in the \( OR2 \) region of the \( V_pR \) promoter decreases the \( V_pR \) promoter activity. At 42°C and 36°C the expression rate of the \( lacZ \) gene driven by the mutated \( p_R \) promoter is lower when compared to the wild-type \( p_R \) promoter. Both promoters show decreased activity at 36°C when compared to 42°C, suggesting that at 36°C the cI857 repressor affects gene expression driven from the \( p_R \) promoter. We assume that at 42°C the cI857 repressor is inactivated immediately without influencing gene expression from the \( p_R \) promoter. The mutated \( p_R \) promoter when compared to the wild-type \( p_R \) promoter showed a more dramatic decrease in gene expression at 36°C than at 42°C (from 70% to 25%) suggesting that the lower temperature is not the only factor influencing gene expression driven from the mutated \( p_R \) promoter.

It has been reported that mutations inactivating the \( V_pR \) promoter facilitate open complex formation of RNA polymerases (RNAPs) at \( V_pRM \), suggesting that \( p_R \)-bound RNAP interferes with open complex formation at \( p_RM \) [24-27]. Similarly the T→C mutation decreasing the \( V_pR \) promoter activity may facilitate open complex formation of RNAPs at \( p_RM \), resulting in a stronger expression from the \( p_RM \) promoter and an increased synthesis of cI857. Whereas at 42°C even a high amount of cI857 repressor is inactivated in a sudden way, at 36°C the higher amount of cI857 repressor molecules expressed from a stronger \( p_RM \) might be responsible for the decreased promoter activity of the mutated \( p_R \) compared to wild-type \( p_R \).

Such mutated E-specific lysis cassettes may represent a powerful safety system when introduced into bacterial live vaccines. The risk of bacterial live vaccines to exhibit virulence in the vaccinated host is a concern in the medical and veterinary field. Returning to virulence, bacterial live vaccines should induce fever in the host, thus raising the body temperature to 38°C or more. Bacteria harboring such E-specific safety cassettes might then be inactivated by the E-mediated lysis event triggered by the increased body temperature. As gene \( E \) can be replaced with any other gene of interest, it is now possible to regulate gene expression with the altered cI857/\( V_pR \) expression system at other temperatures than used in the wild-type system. Thus, the altered temperature regulation of gene expression using the mutated \( p_R \) promoter has general applications for controlled gene expression in bacteria.
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References