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Extracellular expression of human epidermal growth factor encoded by an *Escherichia coli* K-12 plasmid stabilized by the *ytl2-incR* system of *Salmonella typhimurium*

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A plasmid stabilization system, active in high copy-number plasmids, was cloned from the large resident plasmid, pSLT, of *Salmonella typhimurium*. The *ytl2* gene, together with a 249-bp region (termed *incR*) downstream of the gene, imparted $>10^4$-fold stability to a pBR322-based plasmid. The *ytl2-incR* region was then used to stabilize a recombinant plasmid carrying the human epidermal growth factor gene (with the *Escherichia coli* K-12 *ompA* signal sequence), behind the *lacUV5* promoter. In shake flask tests to optimize expression of human epidermal growth factor, loss of recombinant plasmid was <1% when growth (both before and after induction with isopropyl-β-D-galactopyranoside) took place even in the absence of antibiotic selection, and the specific activity of secreted human epidermal growth factor was *ca* 20 μg per 10^8 cells at harvest, compared to a figure of *ca* 3 μg per 10^8 cells when a comparable plasmid, but devoid of the *ytl2-incR* region, was employed, as outgrowth of plasmid-free cells after induction severely compromised the specific activity of the secreted product.

Keywords: hEGF; plasmid pSLT; *ytl2-incR* stabilization

Introduction

Instability of recombinant plasmids during bacterial fermentations decreases the yield of the recombinant product, and the problem may be particularly acute if the plasmid-free cells grow faster than those retaining the plasmid. In this case, the specific activity (wt of recombinant material/No. of live cells) of product may be adversely affected, thus creating product purification problems [4]. Among plasmid stabilization systems tested for use in fermentations, that encoded by the *sop* region of the F plasmid was the most effective, in comparative tests with *parA* and *parB* genes from plasmid R1, and *ccd* from the F plasmid [4]. The *sop* region stabilized a pBR322-based plasmid by a factor of *ca* 10^3. More recent work on the maintenance of recombinant plasmids used the *hok/sok* system of the R1 plasmid (which causes plasmid-free cells to be killed) [16]. In this work, cells of *E. coli* strain BK6, carrying a *hok/sok*-stabilized plasmid expressing β-galactosidase from an inducible (by IPTG) promoter, were efficiently killed upon plasmid loss, as a culture with 10% plasmid-free cells was obtained only 39 h post-induction. For the control strain, containing a plasmid lacking *hok/sok*-mediated stabilization, the figure was 2 h.

Earlier [5], we obtained, in plasmid pADE50, a 4.3-kb *Pst*I-*Mlu*I fragment from the virulence plasmid, pSLT, of *Salmonella typhimurium* which conferred stability upon high copy-number plasmids in which it was cloned. Analysis of this fragment [5,6] defined four regions of interest (Figure 1a, top), termed *parA*, *parB*, *parS*, and *incR*. Tinge and Curtiss [13] cloned a similar fragment, and identified regions which imparted stability to the low copy-number plasmid pACYC184. Sequencing [6] suggested that the stabilization (partition) function identified by Tinge and Curtiss [13] was attributable to the expression of the *parA* and *parB* genes (Figure 1a, top), with subsequent binding of a ParA/ParB complex to *parS*, as has been shown for (highly homologous) well-understood partition systems of phages P1 and P7 [1,10]. Such systems function poorly in high copy-number plasmids [8,13], so it was not surprising that a high copy-number pJRD158B-based plasmid (pADE57) carrying only the pSLT *parA-parB-parS* region (and with little of the remaining DNA of the 4.3-kb *Pst*I-*Mlu*I fragment) was somewhat less stable than the control vector [5].

These data suggested that the 4.3-kb *Pst*I-*Mlu*I fragment contained a second stabilization system, active in high copy-number plasmids. Indeed, stabilization of pJRD158B-based plasmids by the 4.3-kb *Pst*I-*Mlu*I fragment was not compromised by elimination of *parB* (as in plasmids pADE81 and pADE82; [5]) or the truncation of *parA* (pADE82). It seemed possible that the function might be useful to stabilize recombinant plasmids in biotechnological fermentations. This paper addresses this possibility, using *Escherichia coli* strains encoding secretable human epidermal growth factor (hEGF) as a model system.

Materials and methods

Materials

Enzymes active on DNA were purchased from Gibco BRL (Gaithersburg, MD, USA), Pharmacia (Uppsala, Sweden), and Promega (Madison, WI, USA). The hEGF used as an ELISA standard was the product of PeproTech (Rocky Hill,

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NJ, USA). The hEGF secreted by bacterial cultures was assayed directly from culture supernatants after removal of bacterial cells by centrifugation. The ELISA assay was performed according to a standard procedure [2] using rabbit anti-hEGF antibody from CalBiochem (Cambridge, MA, USA), and goat anti-rabbit IgG conjugated with alkaline phosphatase (Pierce, Rockford, IL, USA). Isopropyl-β-d-galactopyranoside (IPTG) was purchased from Sigma (St Louis, MO, USA). All other chemicals were molecular biology grade. DNA techniques followed standard protocols [12]. PCR primers were synthesized by Gibco BRL, and purified on a reverse phase C18 spun column before use. PCR was effected with a Pharmacia Gene ATAQ controller.

Media

In small-scale work, strains were usually grown in a nutrient broth (1.6% w/v, Difco, Detroit, MI, USA) with NaCl (0.5%, w/v). Nutrient agar plates contained 4% (w/v) Difco blood base agar and were supplemented with ampicillin (50 μg ml^{-1}) when required. Minimal medium was M9 [7] with 0.5% (w/v) glucose, Difco casamino acids (0.5%, w/v), thiamine (0.5 μg ml^{-1}), and tryptophan (2 μg ml^{-1}), and minimal plates were made with the same medium, but solidified by the addition of Difco Bacto-Agar (4%, w/v). Bacterial growth was normally effected at 37°C.

In work with the hEGF-expressing strains, growth (30–50 ml cultures) was effected in MBL medium [17], supplemented with glucose (5 g L^{-1}) and with $MgSO_4 \cdot 7H_2O$ at 1 g L^{-1}. The host strain was *E. coli* JM101 [12]. As the hEGF gene was behind the *lacUV5* promoter in all constructs, induction was effected with IPTG (0.1 mM), when the cell density reached *ca* 10^{10} ml^{-1}. In hEGF-expression tests, bacterial growth was at 30°C. Both before and after induction, the frequency of plasmid-containing cells was measured by enumeration of dilutions of the cultures on plates with or without ampicillin (50 μg ml^{-1}). Plasmid loss at frequencies of $>$10% could be detected in this manner. To estimate plasmid loss frequencies in the 1–10% range, 100 colonies from an antibiotic-free plate were picked to antibiotic-containing solid medium.

Source of hEGF-encoding DNA

Synthesis of the hEGF gene has been previously described [14]. The gene was fused in-frame behind the *E. coli* K-12 *ompA* signal peptide in an expression cassette in which transcription of the cloned sequence is under the control of the *lacUV5* promoter ([15]; Wong *et al*, manuscript in

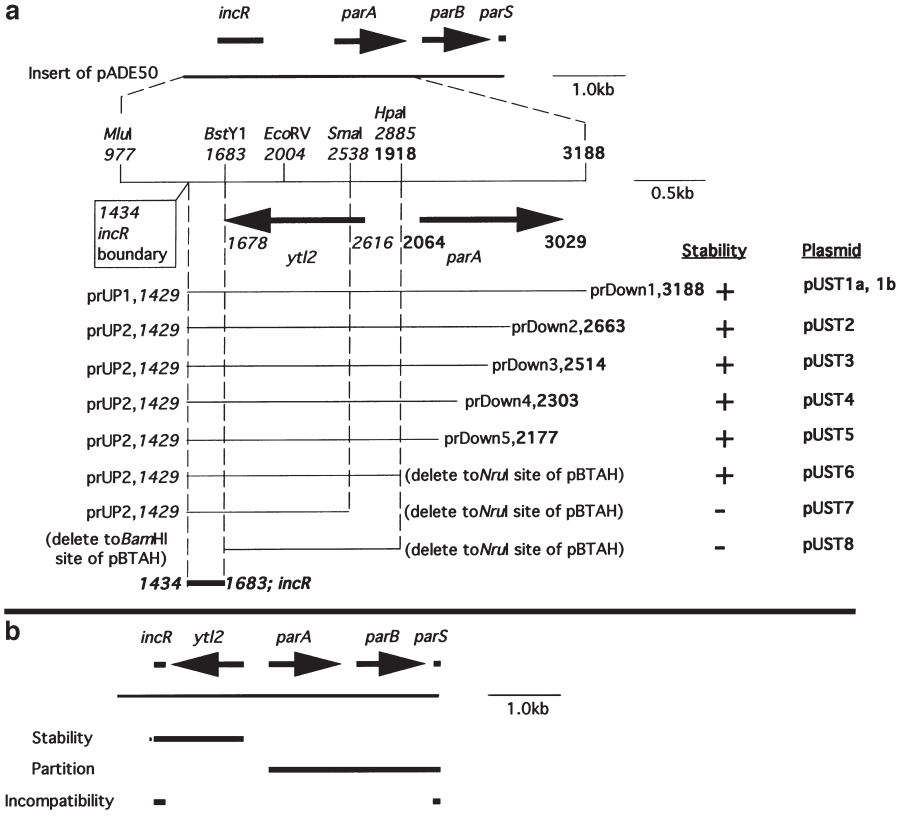

Figure 1 Localization of a new plasmid stabilization function of pSLT. (a) The insert DNA of plasmid pADE50 contains *parA, parB,* and *parS* genes, and an *incR* region, as shown [5,6]. Portions of pADE50 insert DNA, in the *parA-incR* region, which also contains the *ytl2* gene of unknown function, were cloned into pBTAH, to give plasmids pUST1a, pUST1b, and pUST2–pUST8 (see text). The numbers in italics are nt positions from GenBank submission Z29513, which includes the *ytl2* and *incR* regions. The numbers in bold are nt positions from GenBank submission m97752, which includes the *parA, parB,* and *parS* genes. The two sequences overlap, and are shown here joined at the *Hpa*I site (*2885,* **1918**). When restriction enzyme sites are shown, the nt number refers to the first nt in the recognition sequence, regardless of the position of DNA cleavage. (b) Summary of plasmid stabilization functions of pSLT. Plasmid stabilization may be achieved either by the *parA-parB-parS* partition system [13] or by the *ytl2-incR* complex, while incompatibility between pSLT and an incoming plasmid requires that both *parS* and *incR* be present on the new plasmid [5].

preparation). A 402-bp fragment including the promoter and the cloned hEGF gene was amplified with the PCR primers 5′-ggttgaattcGCATACTTACTCCCCATCCC-3′ and 5′-ggttgaattcTTAAAAAAAAGCCCGCTCAT-3′, in which upper-case letters represent sequences binding to the cloned DNA, and the lower-case letters are primer tails carrying *Eco*RI sites (underlined). This fragment was cloned, after digestion with *Eco*RI, into an *Eco*RI plasmid site (see Results).

PCR primers binding to pSLT DNA

Below, nucleotides (nts) homologous to pSLT sequence are capitalized, while other nts in the primers are shown in lower-case, with restriction sites underlined. Two primers were made with reference to sequence Z29513 (sequence positions italicized), and were: (i) prUp1: 5′ctcaaatgtcgac(*1429*)GGGAATGGTCCGGCCGGAATGC TGT(*1453*), used in cloning of amplified fragments with *Sal*I; and (ii) prUp2: 5′ggctggatcc(*1429*)GGGAATGG TCCGGCCGGAAT(*1448*) used in cloning of amplified fragments with *Bam*HI. Five primers were made with reference to sequence m97752 (sequence positions in bold), and were: (i) prDown1: 3′(**3164**)TACTACCTTCGTGGCT CCGCGAATT (**3188**)cagctgtaaactc, used in cloning of amplified fragments with *Sal*I; (ii)–(v): four other primers, each with contained termination codons, used in cloning (with *Sph*I) of *parA* gene fragments. These were: prDown2: 3′(**2647**)GTTAATAGGGCCCGCAA(**2663**)*atc*c cgtacgggcgaa; prDown3: 3′(**2500**)AGGACCGGTAGCTA*A* (**2514**)*tc*gtacgcgtccgt; prDown4: 3′(**2292**)CCGTTATGGTAC (**2303**)*atc*gtacgctactgc; and prDown5: 3′(**2162**)CGAAT GGCTCAAGATA(**2177**)*atc*cgtacgtcgtttc. In each of prDown2–prDown5, the termination codon (atc or Atc) is italicized.

Stability tests

Plasmid pBTAH [3] is pBR322 with a 1.1-kb *Hin*dIII fragment encoding the *thyA* gene of *E. coli* K-12. When this plasmid is transformed into the *thyA*$^-$ strain J357 (*S. typhimurium* LB5010, but cured of pSLT, and *thyA*; [5]), the organism becomes sensitive to trimethoprim. Spontaneous plasmid loss renders the bacteria trimethoprim-resistant; such bacteria may be easily enumerated on trimethoprim-containing solid medium (the medium must cater for the thymine and amino acid auxotrophies of the host strain).

Strain J357 recombinants harboring pBTAH derivatives, or control vector, were grown for up to 100 generations in M9 minimal medium supplemented with thymine (50 μg ml^{-1}). Under these conditions, there is no selection for the plasmids, and spontaneous curing may be effected. At various times during growth, samples were taken and viable counts were performed on thymine-containing M9 medium minimal plates with or without trimethoprim (10 μg ml^{-1}).

Results

Isolation of a stabilization function from the pSLT plasmid

As removal of *parB*, and truncation of *parA*, did not eliminate a plasmid stabilization function (active in high copy-number vectors) of the 4.3-kb *Mlu*I-*Pst*I pSLT fragment [5], it was of interest to delineate this stabilization function further. As both *parS* and *incR* were required, *in trans*, to eliminate pSLT from *S. typhimurium* cells [5], it was suspected that *incR* might bind a protein active in the stabilization process. Attempts to obtain a minimal stabilizing fragment (Figure 1a) thus retained *incR*. It was shown earlier [5,6] that nt*1434* was the leftward *incR* boundary (Figure 1a). While the 4.3-kb *Mlu*I-*Pst*I pSLT fragment carries *parA*, *parB* and *parS* genes, the *ytl2* gene, which could encode a protein of mol wt 35319D, of unknown function, is also on this fragment, left of *parA* (Figure 1a). Accordingly, plasmids pUST1a, pUST1b, and pUST2–pUST5 were initially constructed, to contain: (a) varying amounts of the *parA* gene (from the entire gene in the pUST1a and pUST1b plasmids, to very little in pUST5; the downstream primers had in-frame termination codons for *parA*, as explained in Materials and Methods); (b) all of the *ytl2* gene, and (c) all of *incR*. To make pUST1a, pUST1b, and pUST2–pUST5, PCR primers (prUp1, prUp2, prDown1–prDown5; see Materials and Methods) were used to amplify the fragments shown. The fragments were cut with *Sal*I

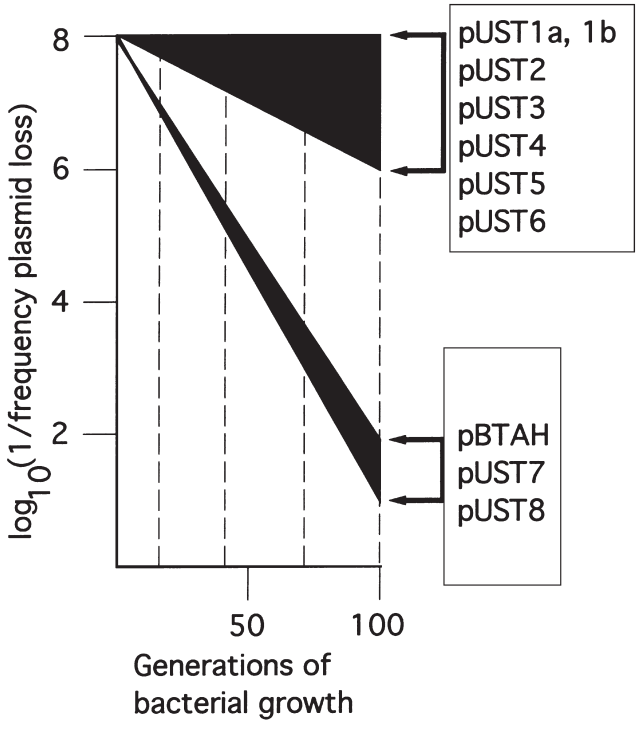

Figure 2 Stability of plasmids carrying *ytl2* and *incR*. Various *thyA*$^+$ plasmids (see text) were transformed to a *thyA*$^-$ strain of *S. typhimurium*, and the transformants grown in liquid culture in a thymine-supplemented minimal medium permissive for plasmid loss. At various times post-inoculation (shown by the dashed vertical lines), culture samples were taken, diluted, and plated on minimal medium-based plates both for total viable counts (the plates contained thymine) and for enumeration of plasmid-free cells (the plates contained both thymine and trimethoprim, to kill plasmid-containing cells). With pUST1a, pUST1b, and pUST2–pUST6, plasmid-free cells were detected at a maximal frequency of 1:10^6 (there were no significant differences in the stabilities of these plasmids). The loss frequencies of pBTAH, and plasmids pUST7 and pUST8, were between 1% and 10% over 100 generations (again, no significant differences in plasmid stabilities were seen). In triplicate tests with each strain, all figures fell into the areas delimited by the shaded triangles.

(inserts for pUST1a and pUST1b only; both orientations of the *Sal*I insert were obtained) or *Bam*HI and *Sph*I (inserts for pUST2–pUST5), and cloned into appropriately-cut pBTAH. The plasmids were assessed for stability. While pBTAH was lost at a frequency of $1:10^1$–$1:10^2$ over 100 generations, plasmids pUST1a, pUST1b, and pUST2–pUST5 were lost at a much lower frequency. Thus, plating of 10^8 bacteria carrying any of these plasmids yielded no more than 10^2 Tmp-resistant colonies. The stabilization effected was thus at least 10^4-fold (Figure 2). Also, the orientation of the stabilizing insert, with reference to the replication region of the vector plasmid, was not important, as both pUST1a and pUST1b were equally stable.

It appeared that the *parA* gene might not be important for the stabilization effect, and, indeed, deletion of all of *parA* (pUST6) did not affect the stabilization function (Figure 2). To construct pUST6, pUST5 was cut with *Hpa*I (the site is in the insert) and *Nru*I (in the vector) and religated. Attention was directed to the *ytl2* gene, and plasmids pUST7 and pUST8 were constructed. To make pUST7, pUST5 was cut (partially) with *Sma*I (there are two sites in the insert), and *Nru*I, and religated. To construct pUST8, pUST6 was cut with *Bst*Y1 (in the insert) and *Bam*HI (one of the cloning sites) and religated. Plasmid pUST7 lacked the N-terminal region of *ytl2*, while pUST8 expressed an essentially intact *ytl2* gene but lacked the 249 bp of DNA from the defined [5] leftward *incR* boundary to the end of *ytl2*. Plasmids pUST7 and pUST8 were assessed for stability, and were no more stable than was the vector (Figure 2).

The ytl2-incR system stabilizes a hEGF-encoding plasmid during recombinant growth

Plasmid pUST6 was used as the base to construct pWKW1 containing a 402-bp *Eco*RI-*Eco*RI cassette encoding secretable hEGF under the control of the *lacUV5* promoter (Materials and Methods; Figure 3). Next, the *thyA* gene of pWKW1 was removed to give pWKW2 (Figure 3). As a *ytl2-incR*-negative control, pWKW3 (Figure 3) was constructed to retain the hEGF-encoding cassette but to lack the *ytl2-incR* stabilization system. Plasmids pWKW2 and pWKW3 were transformed to *E. coli* JM101 and grown in either the presence or absence of ampicillin (70 μg ml^{-1}) to a cell density of *ca* 10^{10} ml^{-1}. Induction of the *lacUV5* promoter with IPTG was then effected.

When growth took place either with or without ampicillin, both plasmids pWKW2 and pWKW3 were essentially stable during the pre-induction phase (Figure 4a); <1% of cells lacked the plasmid at the time of induction. After induction of cells containing pWKW3, three events were noteworthy. First, the viability of the culture fell by *ca* 90–95% in the 5 h post-induction. A deleterious effect of induction has been shown previously when the synthesis of a secretable foreign protein (a *Cellulomonas fimi* exoglucanase) was induced in *E. coli* JM101 [9], and may be attributable to alterations in the cytoplasmic membrane of the induced cell. Second, plasmid-free (ampicillin-sensitive) cells became detectable in the culture *ca* 5 h post-induction (even in ampicillin-supplemented cultures; the antibiotic was presumably destroyed by β-lactamase), and grew to constitute *ca* 80% of total viable cells 20 h post-

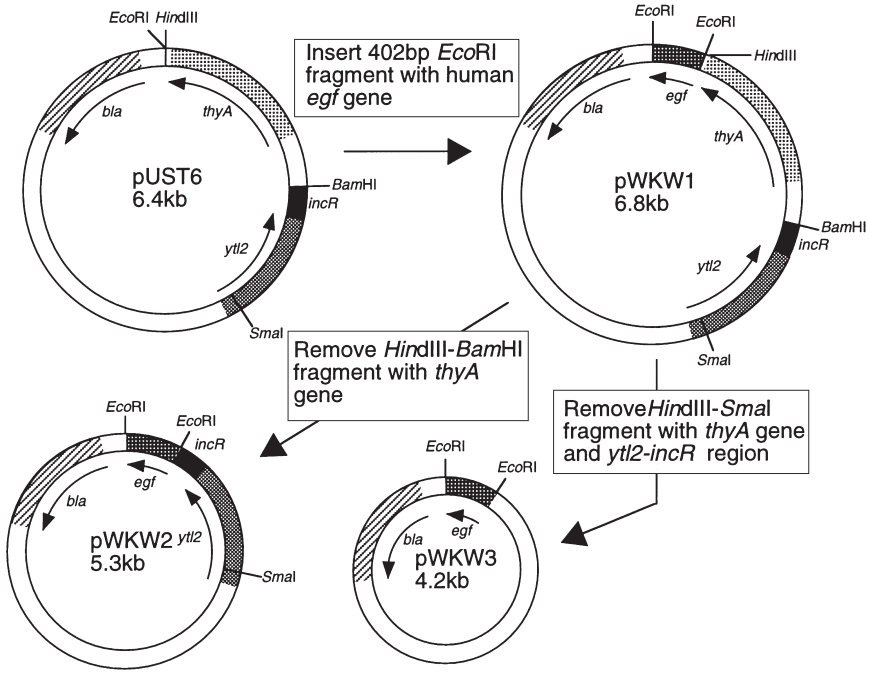

Figure 3 Construction of plasmids carrying the hEGF gene. Plasmid pUST6 (which carries the *ytl2-incR* stabilization system) was opened at the sole *Eco*RI site, and a 402-bp PCR-amplified fragment, bounded by *Eco*RI sites, and containing the hEGF gene (with the *E. coli* K-12 *ompA* signal sequence), was inserted, to give plasmid pWKW1. Removal of a *Hin*dIII-*Bam*HI fragment (containing the *thyA* gene) from pWKW1 gave pWKW2, which retained *ytl2-incR* DNA. As a control (lacking the *ytl2-incR* stabilization system), pWKW3 was made by excision of a *Hin*dIII-*Sma*I fragment (containing both the *thyA* gene and the *ytl2-incR* region) from pWKW1.

induction, when growth was stopped and hEGF assayed in culture supernatants (Figure 4a). While hEGF levels of 107–140 μg ml^{-1} were attained, 20 h post-induction, in four tests, the outgrowth of plasmid-free cells during the induction period greatly reduced the specific activity of the hEGF secreted. The average value was *ca* 3 μg per 10^8 cells 20 h post-induction (Figure 4b). The hEGF specific activity peaked at *ca* 5 μg per 10^8 cells at 12 h post-induction, before outgrowth of plasmid-free cells maximally compromised specific activity. Finally, plasmid-containing cells (presumably synthesizing and secreting hEGF) did not increase in number from 5–20 h post-induction. Either cell division and cell death were in balance, or the cells did not grow and divide because the synthesis and secretion of the recombinant product inhibited some normal cellular processes.

With the strain carrying pWKW2, induction also reduced cell viability *ca* 90%, and plasmid-containing cells did not further grow and divide. The outgrowth of plasmid-free cells post-induction, as noted with *E. coli* JM101/pWKW3, however, was not seen (Figure 4a). Secretion of hEGF from the *E. coli* JM101/pWKW2 culture was similar, in rate and extent, to that seen from *E. coli* JM101/pWKW3, but, as much less cell mass was present at harvesting, the specific activity of the secreted hEGF was *ca* 20 μg per 10^8 cells 20 h post-induction, which represented a 6.7-fold improvement over the average hEGF specific activity afforded by using *E. coli* JM101/pWKW3 (Figure 4b). This improvement was seen even when ampicillin was absent during both pre-induction growth and the induction phase.

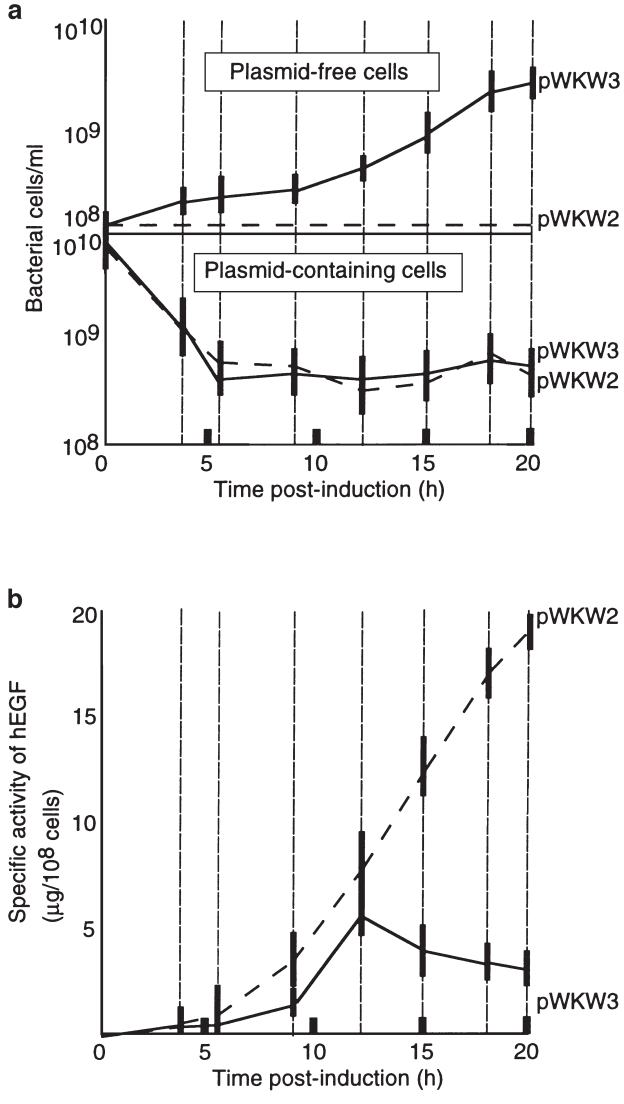

Figure 4 Stability of an hEGF-encoding plasmid, carrying the *ytl2-incR* stabilization system, upon induction of hEGF synthesis. Both plasmids pWKW2 and pWKW3 carry the hEGF gene (with the *E. coli* K12 *ompA* signal peptide) behind the *lacUV5* promoter, and plasmid pWKW2 also contains the *ytl2-incR* stabilization system, lacking in plasmid pWKW3. *E. coli* JM101 strains harboring the plasmids were grown to *ca* 10^{10} bacteria ml^{-1} in shake flasks (30–50 ml of medium) at 30°C, and hEGF syntheis was then induced with IPTG. At the times shown by the vertical dashed lines (to 20 h post-induction) samples were taken and assayed for (a) plasmid-free and plasmid-containing cells by differential plating, and (b) hEGF specific activity in the culture supernatants. All tests were performed with four different cultures, and standard error bars are shown where possible (as the frequency of pWKW2 loss was <1%, and only 100 colonies were screened for plasmid, no error bars can be given). The presence (two cultures) or absence (two cultures) of ampicillin (added at the time of inoculation, *ca* 18 h prior to induction) in the medium did not affect the results seen.

Discussion

The *ytl2-incR* plasmid stabilization system functions in the high copy-number pBR322-based plasmid pBTAH to give at least 10^4-fold stabilization (Figure 2) of a pBR322-encoding plasmid. Among other plasmid stabilization systems tested for use in fermentations, the *sop* region of the F plasmid was the most effective, stabilizing a pBR322-based plasmid by a factor of *ca* 10^3 [4]. Recombinant plasmids may also be maintained by the *hok/sok* system of the R1 plasmid (which causes plasmid-free cells to be killed) [16]. With this system, *E. coli* cells with a *hok/sok*-stabilized plasmid expressing β-galactosidase from an inducible promoter, were efficiently killed upon plasmid loss. A culture with 10% plasmid-free cells was obtained only 39 h post-induction (for the control strain lacking the *hok/sok* maintenance system, the figure was 2 h). In the work with hEGF-expressing plasmids described here, 10% of an induced *E. coli* JM101/pWKW3 culture was plasmid-free after *ca* 4 h (Figure 4a), while <1% of *E. coli* JM101/pWKW2 was plasmid-free after 20 h.

The use of the *ytl2-incR* system also allowed the elimination of ampicillin, as a selective agent, in growth (to 10^{10} ml^{-1}), and subsequent IPTG-induced induction over 20 h, of an *E. coli* JM101 strain secreting recombinant hEGF.

It is hoped that the *S. typhimurium* pSLT *ytl2-incR* system, in combination with other plasmid stabilization approaches based upon single or multiple [11] systems for killing plasmid-free cells, will have applications in biotechnological fermentations. As *ytl2-incR* stabilization does not appear to have a mode of action similar to that of (for example) *hok/sok*, it may be that the use of both *ytl2-incR* and a *hok/sok*-type system in the same vector will have a complementary effect on plasmid stabilization.

Acknowledgements

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References

1 Abeles AL, SA Friedman and SJ Austin. 1985. Partition of unit-copy miniplasmids to daughter cells. III. The DNA sequence and functional organisation of the P1 partition region. J Mol Biol 185: 261–272.
2 Ausübel FM, R Brent, RE Kingston, DD Moore, JG Seidman, JA Smith and K Struhl. 1993. Current Protocols in Molecular Biology, vol 2. pp 11.2.1–11.2.5. John Wiley and Sons, New York, NY.
3 Belfort M, GF Maley and F Maley. 1983. Characterization of the *Escherichia coli thyA* gene and its amplified thymidylate synthetase product. Proc Natl Acad Sci USA 80: 1858–1861.
4 Boe L, K Gerdes and S Molin. 1987. Effects of genes exerting growth inhibition and plasmid stability on plasmid maintenance. J Bacteriol 169: 4646–4650.
5 Cerin H and J Hackett. 1989. Molecular cloning and analysis of the incompatibility and partition functions of the virulence plasmid of *Salmonella typhimurium*. Molecular Pathogenesis 7: 85–99.
6 Cerin H and J Hackett. 1993. The *parVP* region of the *Salmonella typhimurium* virulence plasmid pSLT contains four loci required for incompatibility and partition. Plasmid 30: 30–38.
7 Davis RW, D Botstein and JR Roth. 1980. Advanced Bacterial Genetics: a Manual for Genetic Engineering. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
8 Funnell BE. 1988. Mini-P1 plasmid partitioning: excess ParB protein destabilizes plasmids containing the centromere *parS*. J Bacteriol 170: 954–960.
9 Lam T-L, RSC Wong and W-K R Wong. Enhancement of extracellular production of a *Cellulomonas fimi* exoglucanase in *Escherichia coli* by the reduction of promoter strength. Enzyme Microb Technol 20: 484–488.
10 Ludtke DN, BG Eichorn and SJ Austin. 1989. Plasmid-partition functions of the P7 prophage. J Mol Biol 209: 393–406.
11 Pecota DC, CS Kim, K Wu, K Gerdes and TK Wood. 1997. Combining the *hok/sok*, *parDE* and *pnd* postsegregational killer loci to enhance plasmid stability. Appl Environ Microbiol 63: 1917–1924.
12 Sambrook J, EF Fritsch and T Maniatis. 1989. Molecular Cloning—a Laboratory Manual, 2nd edn. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.
13 Tinge SA and R Curtiss III. 1990. Isolation of the replication and partitioning regions of the *Salmonella typhimurium* virulence plasmid and the stabilisation of heterologous replicons. J Bacteriol 172: 5266–5277.
14 Wong WKR and ML Sutherland. 1993. US patent 5 223 407.
15 Wong WKR, A Ali, WK Chan, V Ho and NTK Lee. 1998. The cloning, expression and characterization of a cellobiase gene encoding a secretory enzyme from *Cellulomonas biazotea*. Gene 207: 79–86.
16 Wu K and TK Wood. 1994. Evaluation of the *hok/sok* killer locus for enhanced plasmid stability. Biotechnol Bioengin 44: 912–921.
17 Yadwad VB, S Wilson and OP Ward. 1994. Production of human epidermal growth factor by an ampicillin resistant recombinant *Escherichia coli* strain. Biotechnol Lett 16: 885–890.