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An *Escherichia coli* Host Strain Useful for Efficient Overproduction of Cloned Gene Products with NaCl as the Inducer

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Salt-induced overexpression of genes cloned downstream of the phage T7 φ10 promoter was demonstrated in an *Escherichia coli* strain (GJ1158) which carries a single chromosomally integrated copy of the gene for phage T7 RNA polymerase under transcriptional control of the *cis*-regulatory elements of the osmoresponsive *proU* operon. Plasmids that have been constructed to obtain overproduction of individual target gene products in strain BL21(DE3) (by addition of isopropyl-β-D-thiogalactopyranoside as an inducer) can directly be transformed into GJ1158. The NaCl induction regimen was also shown to be associated with a decreased propensity for sequestration of overexpressed target proteins within insoluble inclusion bodies.

Escherichia coli is widely used in basic research studies as a host strain for the overproduction of proteins from cloned genes, and several proteins of pharmaceutical value are also produced on a commercial scale in *E. coli*. In the two most commonly used strategies, a target gene is overexpressed (directly or indirectly) by temperature induction of a phage λ promoter or induction with isopropyl-β-D-thio-galactopyranoside (IPTG) of the *lac* promoter and its variants (reviewed in reference 13).

Some problems associated with temperature-induced overexpression of recombinant gene products include (i) difficulty in achieving rapid temperature upshift, particularly when handling larger culture volumes, (ii) increased likelihood of formation of insoluble inclusion bodies at higher incubation temperatures (20), and (iii) induction of several proteases in *E. coli* upon heat shock (4). With the *lac* promoter-based systems as well, the problem of inclusion body formation may be significant, in addition to the high cost and toxicity of IPTG (13).

In this study, we have developed a simple, efficient, and generally applicable method for the overproduction of recombinant proteins in *E. coli* that makes use of NaCl as an inducer. For this purpose, we have constructed a host strain in which synthesis of the RNA polymerase (RNAP) of bacteriophage T7 has been placed under control of the osmotically inducible *proU* promoter of *E. coli* (6). Any target gene which is cloned downstream of a phage T7 promoter (e.g., φ10) and introduced into this strain will be overexpressed, through a two-tier amplification strategy, upon salt addition. We also demonstrate that the propensity for inclusion body formation may be significantly reduced with the NaCl induction protocol.

Methods. Most of the genetic and recombinant DNA procedures used have been described earlier (5, 19). Induction of target genes by 1 mM IPTG in derivatives of strain BL21(DE3) was done as previously described (21, 22). NaCl induction experiments were done as follows. Ten-milliliter cultures were grown at 37°C to an A_{600} of around 0.8 in low-osmolarity LBON medium (Luria-Bertani medium [19] with NaCl omitted, containing [per liter] 10 g of tryptone and 5 g of yeast extract [pH adjusted to 7.0 with NaOH]) supplemented with appropriate antibiotics. Each culture was then divided into two halves, and NaCl (from a 5 M stock) was added to one to a final concentration of 0.3 M to achieve osmotic induction while the other served as an uninduced control. All cultures were harvested 2 to 3 h after inducer addition and suspended in sample buffer (19); approximately the same amount of total protein from each was then subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis as previously described (19).

To determine the relative proportions of overexpressed proteins in the soluble and insoluble (i.e., inclusion body) fractions, cells were lysed as previously described (10) and each lysate was centrifuged at 10,000 × g for 10 min. Proteins in each of the supernatant (soluble) and pellet (insoluble) fractions obtained from a single volume of the crude lysate were then dissolved in sample buffer and analyzed by gel electrophoresis.

Construction of a *proUp*-T7 RNAP operon fusion. Previous studies in our laboratory had established that a 1.25-kb fragment from the *proU* locus present on plasmid pHYD272 carries all of the *cis* elements necessary to confer a several hundredfold range of osmoresponsivity on a reporter gene (3). This fragment encompasses two promoters, P1 and P2 (that are recognized, respectively, by the σ^S- and σ^{70}-bearing RNAP holoenzymes), along with a promoter-downstream negative regulatory element that partially overlaps the first structural gene, *proV* (3, 18). The *proU* fragment used in this work (*proUp*) is almost identical to that in pHYD272, with the sole modification that an *Nde*I site in the *proV* coding region has been destroyed; this modification had no effect on osmotic regulation of transcription from *proUp* (data not shown).

Although overexpression of a few target genes had been achieved earlier by their direct fusion to the *cis*-regulatory elements of *proU* followed by steady-state growth of strain derivatives carrying these fusions in media with elevated osmolarity (8), we reasoned that the maximal activity of the *proU* promoter(s) is, by itself, not strong enough to permit general applicability of such a single-tier amplification strategy (particularly for synthesis of proteins which may be toxic and therefore would need to be produced by instantaneous induction [13]). We therefore adopted a two-tier amplification strategy in which NaCl induction of T7 RNAP constituted the first tier.

Plasmid pGP1-3 (Fig. 1) carries the T7 RNAP gene downstream of the λ p_L promoter (23). Derivative plasmid pHYD501, lacking the λ p_L promoter but retaining the intact T7 RNAP gene, was identified following linearization of pGP1-3 with *Eco*RI, controlled digestion with exonuclease III and S1 nuclease, and recircularization after *Eco*RI linker addition. The

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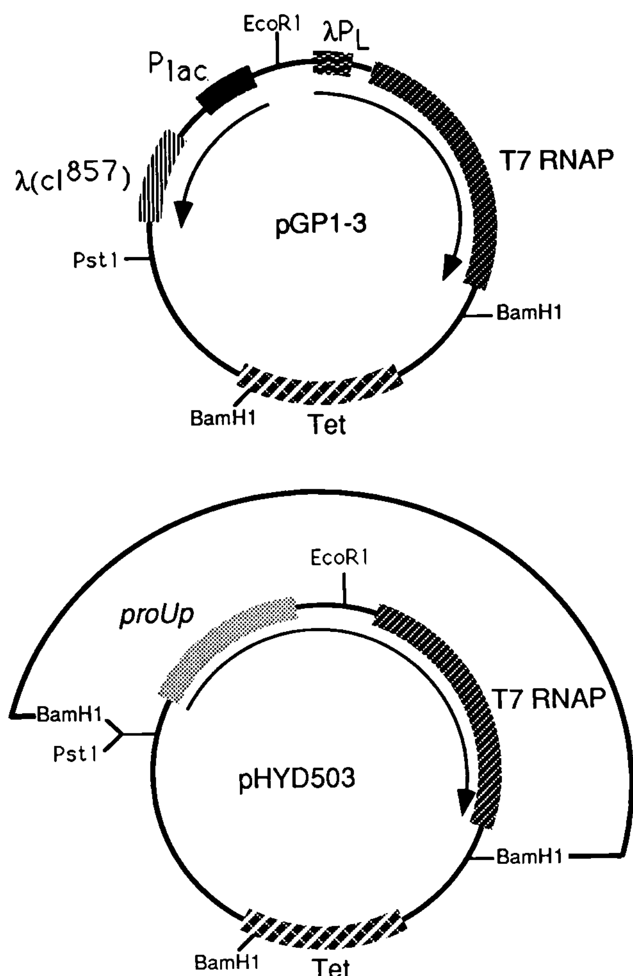

FIG. 1. Structures (not to scale) of plasmid pGP1-3 (23) and its derivative plasmid pHYD503. For details, see the text. Arrows depict the direction and extent of transcription from the P_{lac}, λ P_L, and *proUp* promoters. The large arc depicts the *Bam*HI fragment carrying *proUp*-T7 RNAP that was used in the construction of plasmid pHYD507.

proUp cassette was then cloned on a *Pst*I-*Eco*RI fragment (restriction sites derived from multiple cloning site sequences flanking *proUp*) into the *Pst*I-*Eco*RI sites of pHYD501 to generate pHYD503. As depicted in Fig. 1, plasmid pHYD503 carries a *proUp*-T7 RNAP operon fusion.

Chromosomal integration of *proUp*-T7 RNAP fusion. Stable single-copy integration of the *proUp*-T7 RNAP fusion into the *E. coli* chromosome was obtained by a strategy modified from that previously described (7). The *proUp*-T7 RNAP fusion was cloned on a *Bam*HI fragment from plasmid pHYD503 (Fig. 1) into a unique *Bgl*II site upstream of the promoterless *tet* gene in plasmid pOM41 (24). The resultant plasmid pHYD507 has the *proUp*-T7 RNAP fusion flanked by sequences derived from the divergently transcribed *malTPQ* locus; because of osmoresponsivity of transcription from *proUp*, plasmid pHYD507 confers Tet^r only in high-osmolarity media.

Plasmid pHYD507 was transformed into strain pop2249, which carries a chromosomal *malQ*::*lac*Zhyb11 gene fusion transcribed from *malPp* and which is therefore Lac^+ on maltose-supplemented media (7). Colonies in which a reciprocal promoter exchange by homologous recombination had occurred between the *malTPQ* sequences on the chromosome and that on the plasmid (with the *malPp* promoter now directing *tet* expression on the plasmid and the *proUp*-T7 RNAP fusion resident upstream of *malQ*::*lacZ* on the chromosome) were identified as Tet^r Lac^- on maltose-supplemented, low-osmolarity (LBON)–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) plates. Spontaneous plasmid-cured derivatives of these strains were then identified, and one representative clone was designated GJ1150.

A new Tet^r insertion (designated *zhf-900*::Tn*10*dTet) 98% cotransducible with the *malTPQ* locus was obtained essentially by the method described by Miller (15) and was used to transduce the *proUp*::T7 RNAP::*malQ-lacZ* construct from GJ1150 into *E. coli* B strain BL21 (which is recognized as a good host for polypeptide overproduction [21]). A spontaneous Tet^s derivative (14) selected from one of the latter transductants was designated GJ1158. Strain GJ1158 is therefore very similar to strain BL21(DE3) of Studier and coworkers (21, 22), with the difference that the former carries the *proUp*-T7 RNAP fusion integrated at a mutant *malTPQ* locus and the latter carries a λ prophage with the *lacUV5*-T7 RNAP fusion.

NaCl-induced polypeptide expression in GJ1158. We have successfully demonstrated NaCl-induced overexpression in small-volume cultures of every one of the approximately 20 genes tested, each cloned downstream of the T7 ϕ10 promoter on a plasmid and introduced into GJ1158. Some examples are shown in Fig. 2 and 3. We were also able to demonstrate

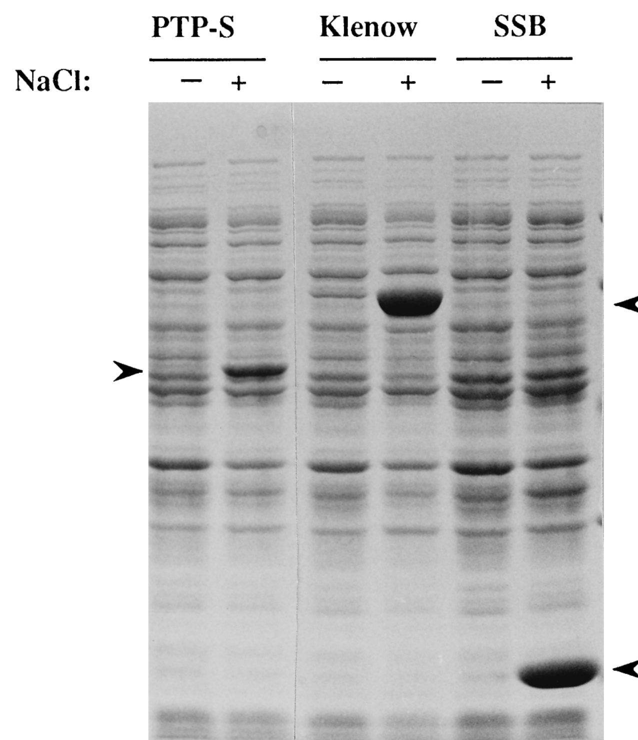

FIG. 2. Induction by NaCl of heterologous proteins in derivatives of strain GJ1158. Protein extracts were prepared from uninduced (−) and NaCl-induced (+) cultures of GJ1158 derivatives carrying plasmids with genes encoding the following target proteins, each cloned downstream of the phage T7 ϕ10 promoter (plasmid designations and references are in parentheses): rat protein tyrosine phosphatase PTP-S (pET-PTP; 17), the Klenow fragment of *E. coli* DNA polymerase I (pET-3a-K; 11), and the *E. coli* SSB protein (pGK2; 12). Proteins in the extracts were visualized by staining with Coomassie blue following gel electrophoresis. Bands corresponding to the target proteins are indicated by arrowheads.

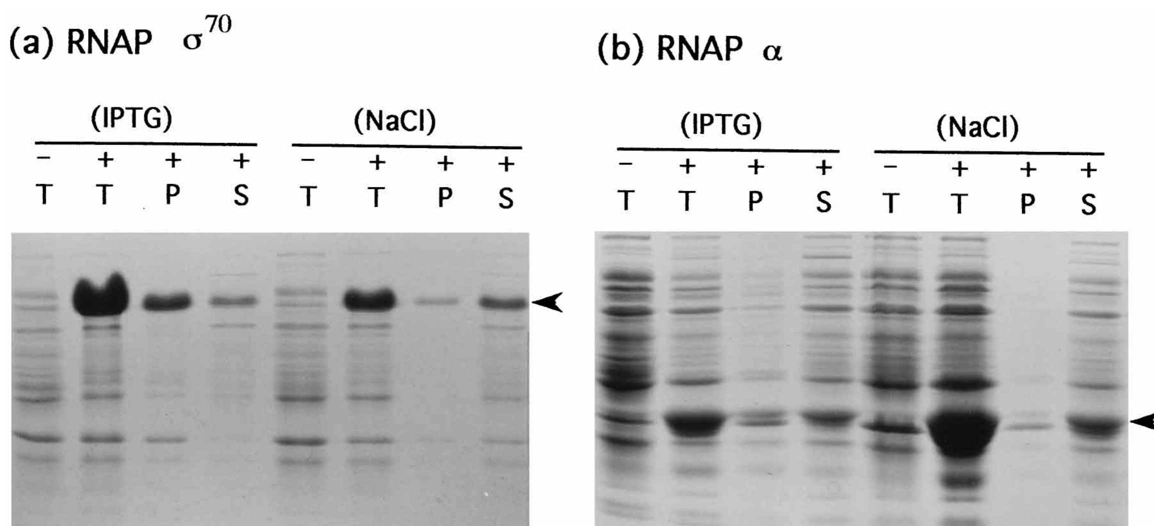

FIG. 3. Propensity for inclusion body formation after NaCl induction in GJ1158 compared to that after IPTG induction in BL21(DE3). Plasmids pGEMD (10) and pGEMAX185 (10) carrying, respectively, genes encoding *E. coli* RNAP subunits σ^{70} (a) and α (b) downstream of the phage T7 ϕ10 promoter were introduced into BL21(DE3) for IPTG induction (IPTG lanes) or into GJ1158 for NaCl induction (NaCl lanes). Proteins were visualized by staining with Coomassie blue following gel electrophoresis. Lane: −T, total (i.e., unfractionated) lysate of uninduced culture; +T, total lysate of induced culture; +P, pellet fraction of induced lysate; +S, supernatant fraction of induced lysate. One-eighth (σ^{70}) or one-twelfth (α) of the lysate volume loaded in each +T lane was separated into pellet and supernatant fractions for loading, respectively, in the corresponding +P and +S lanes. Arrowheads mark the positions of the target proteins. The α protein subunit (b) is the upperband of the pair of closely migrating bands. Strains BL21(DE3) and GJ1158 are closely related *E. coli* B derivatives whose full genotypes are, respectively, *ompT hsdS gal dcm* (λ DE3) and *ompT hsdS gal dcm* Δ*malAp510 malP*::*(proUp*-T7 RNAP) *malQ*::*lacZ*hyb11 Δ(*zhf-900*::Tn*10*dTet).

substantial protein overexpression upon NaCl addition to 500-ml volumes of several cultures (data not shown).

For several target genes, we also compared the magnitude of overexpression achieved by NaCl induction in GJ1158 with that achieved by IPTG in strain BL21(DE3) and found it to be more or less similar (data not shown, but see Fig. 3). Several target gene constructs (including, for example, that encoding rat PTP-S [17]) which could be expressed in the latter strain only in the presence of plasmid pLysS (which encodes phage T7 lysozyme, an antagonist of T7 RNAP, and therefore serves to reduce the uninduced level of target gene expression [22]) were well maintained and expressed in GJ1158 even in the absence of pLysS (Fig. 2; other data not shown). An induction period of 2 to 3 h following NaCl addition was usually optimal, although the temporal pattern of induction did vary from one protein to another (data not shown). As for BL21(DE3), we too noted that GJ1158 derivatives carrying a T7 ϕ10-target gene fusion are best subcultured to the minimum possible extent to avoid enrichment for faster-growing, nonoverproducing mutants (21).

NaCl induction is associated with an increased proportion of overexpressed protein in the soluble fraction. The sequestration of a significant fraction of overexpressed polypeptides in inactive, insoluble inclusion bodies represents a major challenge to the overproduction of several target proteins. An earlier report had demonstrated that cytoplasmic accumulation of glycine betaine could alleviate this problem for at least the one protein that was tested (1). Yeast extract, a component of LBON medium, contains both glycine betaine (16) and its precursor, choline (9), and strains such as GJ1158 are expected to exhibit intracellular accumulation of glycine betaine upon osmotic upshock in this medium (2). We therefore tested the extent of inclusion body formation under these conditions for two proteins (*E. coli* RNAP α and σ^{70} subunits) that had earlier been reported to form inclusion bodies or aggregates in BL21(DE3) upon IPTG induction (10).

The *E. coli* RNAP α and σ^{70} subunits were induced by IPTG and NaCl in derivatives of BL21(DE3) and GJ1158, respectively, and a crude extract of each induced culture was fractionated into insoluble (pellet) and soluble (supernatant) fractions as described above. The relative proportion of target protein in each fraction was then determined following gel electrophoresis (Fig. 3, compare lane pairs +P and +S for each strain). Densitometric analysis (data not shown) revealed that the distribution ratio of σ^{70} protein between the insoluble and soluble fractions was around 2.7:1 following IPTG induction in BL21(DE3), whereas this ratio was reversed to around 1:3.8 in the NaCl-induced GJ1158 derivative. Similarly, the relative proportion of the overexpressed α subunit in the insoluble fraction declined from around 20% in the IPTG-induced culture to $<5\%$ following NaCl induction.

These results suggest that proteins synthesized by NaCl induction in GJ1158 are less prone to form inclusion bodies within the cells. Furthermore, it is likely that the propensity for inclusion body formation by particular proteins can be reduced even further by performing the NaCl induction at lower growth temperatures (1, 20).

Concluding remarks. We have developed a generally applicable procedure for obtaining NaCl-induced overexpression of cloned gene products in *E. coli*. The system is based on expression from the osmoresponsive *proU* promoters and on an amplification step involving phage T7 RNAP. A variety of vectors (such as the pET series of vectors [22]) that have been developed for, and which are widely used with, strain BL21(DE3) can also be used without alteration in GJ1158 to achieve comparable salt-induced expression. Overproduced proteins are less likely to form inclusion bodies in GJ1158, and it is also noteworthy that all of our overexpression results have been achieved in the absence of plasmid pLysS. With the aid of the linked Tn*10*dTet insertion, the chromosomal *proUp*-T7 RNAP fusion can also be transduced into other (for example, protease-deficient) *E. coli* strains that are suited for polypeptide

overproduction (13). We anticipate that virtually any protein (the gene for which has been appropriately cloned downstream of a phage T7 promoter) can be overproduced by this approach, although conditions such as time of NaCl addition and duration of induction may have to be individually optimized in each instance.

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