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pOSEX: vectors for osmotically controlled and finely tuned gene expression in *Escherichia coli*

(Osmoregulation; glycine betaine; *proU* promoter; heterologous gene expression)

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SUMMARY

Expression of the *proU* operon of *Escherichia coli* is directly proportional to the osmolarity of the growth medium. The basal level of *proU* transcription is very low, but a large increase is triggered by a sudden rise in the external osmolarity. This increased expression is maintained for as long as the osmotic stimulus persists. We have capitalized upon these regulatory features of the *proU* operon and have constructed a series of expression vectors (pOSEX) permitting osmotically controlled expression of heterologous genes governed by regulatory signals of *proU*. The pOSEX vectors carry the *proU* promoter, an upstream region required for high-level expression, and part of the first structural gene (*proV*), which acts as a silencer and is necessary to maintain low-level expression in low osmolarity media. An extended multiple cloning site (MCS) positioned at the 3′ end of *proV*′ permits the cloning of heterologous genes into the pOSEX plasmids, and efficient transcription terminators derived from the *rrnB* operon prevent deleterious read-through transcription into the vector portion. The properties of the pOSEX expression vectors were tested by positioning a promoterless *lacZ* (encoding β-galactosidase) gene from *E. coli* and the *gcdA* (encoding carboxytransferase) gene from the Gram$^+$ bacterium *Acidaminococcus fermentans* under the control of the *proU* regulatory region. Efficient, osmoregulated and finely tuned expression of both *lacZ* and *gcdA* was achieved, and the amount of β-galactosidase and carboxytransferase synthesized were simply controlled by adjusting the osmolarity of the growth medium with various concentrations of NaCl.

INTRODUCTION

Bacteria maintain an osmotic pressure of the cytoplasm that is higher than that of the surrounding environment, resulting in an outward directed pressure, the turgor. A sudden increase in the external osmolarity triggers a rapid efflux of water from the cytoplasm, causing loss of turgor and dehydration of the cytoplasm. To combat these deleterious effects, *Escherichia coli* has evolved mechanisms that sense changes in the environ-

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Abbreviations: *A*, absorbance (1 cm); aa, amino acid(s); Ap, ampicillin; βGal, β-galactosidase; bp, base pair(s); *bla*, gene encoding ampicillin resistance; Δ, deletion; dNTP, deoxyribonucleoside triphosphate; GCDA, glutaconyl-CoA decarboxylase; *gcdA*, gene encoding GCDA; kb, kilobase(s) or 1000 bp; LBON, Luria Bertani (medium) without NaCl; MCS, multiple cloning site(s) (polylinker); nt, nucleotide(s); ONPG, *o*-nitrophenyl-β-D-galactopyranoside; PAGE, polyacrylamide-gel electrophoresis; PCR, polymerase chain reaction; PolIk, Klenow (large) fragment of *E. coli* DNA polymerase I; RBS, ribosome-binding site(s); *p*, promoter; ProP, low-affinity transport system for glycine betaine; *proP*, gene encoding ProP; ProU, binding protein-dependent transport system for glycine betaine; *proU* (*proV*, *proW*, *proX*), operon encoding the ProU transport system; *rrnB*, operon encoding ribosomal RNA; SDS, sodium dodecyl sulfate; *tet*, gene encoding tetracycline resistance; [], denotes plasmid-carrier state; ′ (prime), denotes a truncated gene at the indicated side.

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mental osmolarity and respond to these changes in a timely and efficient manner (Csonka and Hanson, 1991; Lucht and Bremer, 1994). One response mechanism is the intracellular accumulation of organic osmolytes, the so-called compatible solutes. These compounds can be amassed to high intracellular concentrations without disturbing essential metabolic functions of the cell. In addition, they stabilize the integrity of cell components and protect proteins from denaturation in solutions of high ionic strength (Arakawa and Timasheff, 1985). One of the most effective compatible solutes is glycine betaine, which has been adopted across the animal, plant and microbial kingdoms as a potent osmoprotectant (Csonka and Hanson, 1991; Lucht and Bremer, 1994). In *E. coli*, uptake of glycine betaine from the environment is mediated by two osmoregulated transport systems: the low-affinity ProP transporter and the high-affinity, binding-protein-dependent ProU transport system (May et al., 1986; Growishankar, 1989; Culham et al., 1993).

Expression of the *proU* operon (*proV, proW, proX*) sensitively responds to increases in medium osmolarity (for an overview, see Lucht and Bremer, 1994). The basal level of *proU* transcription is very low, but transcription is rapidly and strongly stimulated upon a sudden osmotic shock. This higher level of expression is maintained as long as the osmotic stimulus persists. The strength of *proU* expression is directly proportional to the osmolarity of the growth medium, thus allowing the bacterial cell to finely tune the level of ProU synthesis. Osmotically regulated expression of the *proU* operon can be triggered in both minimal and rich media by a variety of ionic (e.g., NaCl) or non-ionic (e.g., sucrose) osmolytes. Glycine betaine, the main substrate for the ProU transport system, does not act as an inducer for *proU* expression (Barron et al., 1986; May et al., 1986). The unique regulatory properties and the strength of the *E. coli proU* promoter make this system an attractive candidate for the overexpression of heterologous genes. We describe in this communication the construction of a set of multi-copy vectors, pOSEX, that permit the efficient and finely tuned gene expression under the control of the regulatory circuit that governs the osmotically regulated transcription initiating from the *proU* promoter.

EXPERIMENTAL AND DISCUSSION

(a) Construction of expression vectors carrying the osmotically controlled *proU* regulatory region

To achieve full and osmoregulated expression directed by the *E. coli proU* promoter, DNA sequences located outside the *proU* −10 and −35 regions are required. These DNA segments comprise a region upstream from the *proU* promoter that is necessary for maximal *proU* transcription (Lucht and Bremer, 1991). A DNA region located at the 5′ end of the first gene (*proV*) in the *proU* operon serves as a transcriptional silencer that functions to keep *proU* expression repressed in media of low osmolarity and also is a target for the high-affinity nucleoid-associated DNA-binding protein H-NS (Dattananda et al., 1991; Ueguchi and Mizuno, 1993; Lucht et al., 1994). As a first step in the construction of the pOSEX vectors, we positioned through a series of cloning steps an extended MCS and the strong transcription terminator sequences *T1* and *T2* from the *E. coli rrnB* operon at the 3′ end of a 612-bp segment from the *proU* regulatory region. The physical and genetic organization of the resulting plasmid, pOSEX2, is shown in Fig. 1. The *proU*-containing DNA segment present in pOSEX2 carries all of the DNA sequences required in *cis* to achieve efficient and sensitively regulated *proU* transcription in response to increases in medium osmolarity (Lucht and Bremer, 1991, Lucht et al., 1994). To reduce the size of pOSEX2 and to remove several restriction sites present in both MCS and the vector segment of pOSEX2, two deletion derivatives of this plasmid were constructed, plasmids pOSEX3 and pOSEX4 (Fig. 1A). The deletion present in pOSEX4 affects the *rop* region (Fig. 1A), which is involved in the copy-number control of pBR322-derived plasmids (Balbás et al., 1986). We therefore expect that pOSEX4 has a higher copy-number than the vectors pOSEX2 and pOSEX3, which carry an intact *rop* region (Fig. 1A).

(b) The pOSEX vectors mediate efficient and sensitively regulated gene expression

To test the properties of the pOSEX expression vectors, we cloned the *E. coli lacZ* gene and the *gcdA* gene from *Acidaminococcus fermentans* into plasmids pOSEX3 and pOSEX4, respectively, and monitored βGal and GCDA synthesis in whole cell extracts of strains grown at various osmolarities. To construct a pOSEX3 derivative expressing the *lacZ* gene under *proU* control, we excised a 3.95-kb *Bam*HI-*Dra*I fragment carrying a promoterless *lacZ* gene from the promoter probe vector pMLB1010 (Silhavy et al., 1984) and inserted it into the *Bam*HI and *Eco*RV sites present in the MCS of pOSEX3 (Fig. 1B), yielding plasmid pBH4. This plasmid was transformed into strain MKH13 [Δ(*putPA*)*101* Δ(*proP*)*2* Δ(*proU*)*608*] (M. Haardt and E.B., unpublished results), a derivative of the *E. coli* K-12 strain MC4100 (Casadaban, 1976). Synthesis of βGal was assessed by SDS-PAGE of whole cell extracts of strain MKH13[pBH4] grown in LB media of different osmolarities. There was little synthesis of βGal when strain MKH13[pBH4] was grown in low osmolarity media

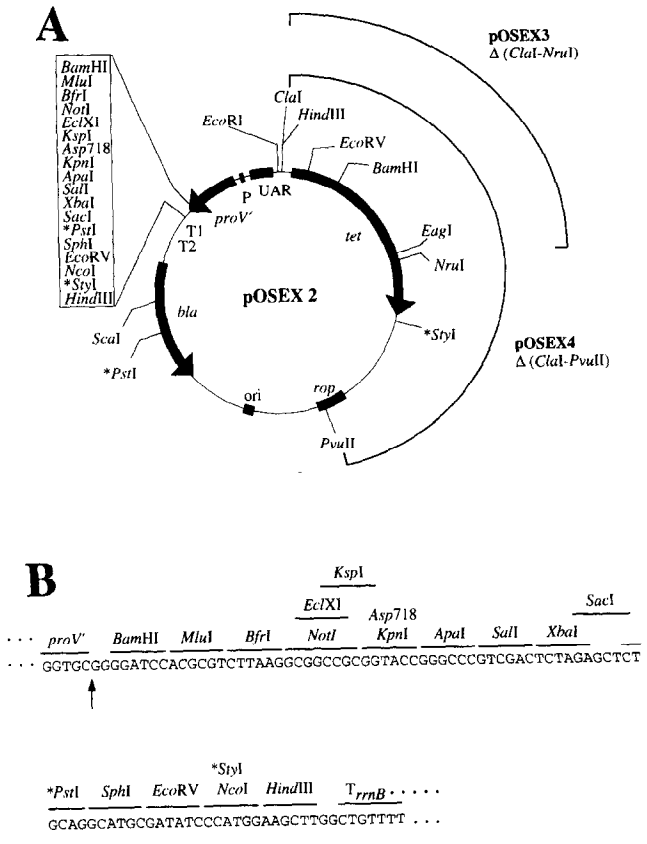

Fig. 1. Genetic and physical organization of the pOSEX expression vectors. (**A**) Vector maps. (**B**) Nucleotide sequence of the MCS following the truncated *proV'* gene. The restriction sites that are not unique in the MCS of the pOSEX expression vectors are marked with asterisks (*). **Methods:** Standard procedures were used for the isolation of plasmid DNA, restriction digests, DNA ligations and transformations (Silhavy et al., 1984; Sambrook et al., 1989). For the construction of plasmid pOSEX2, a *Eco*RI-*Bam*HI fragment was excised from plasmid pJL32-3 carrying *proU* material extending from 385 to 997-bp (J.M. Lucht and E.B.; unpublished results) and inserted into the extended MCS of plasmid pUC-BM20 (Boehringer-Mannheim, Mannheim, Germany), yielding plasmid pOSEX1. The *proU*-derived DNA segment and MCS were excised from pOSEX1 with *Eco*RI + *Hin*dIII and the resulting 704-bp restriction fragment was cloned between the *Eco*RI and *Hin*dIII sites in MCS of plasmid pTrc99A (Amann et al., 1986), resulting in plasmid pBH3. This cloning step positioned the strong transcription terminator sequences *T1* and *T2* from the *E. coli rrnB* operon downstream from the *proU* material. Plasmid pBH3 was then cut with *Eco*RI + *Sca*I and the resulting 1534-bp DNA fragment was inserted between the *Eco*RI and *Sca*I sites of plasmid pBR322, yielding plasmid pOSEX2 (5.38 kb). Two derivatives of pOSEX2 were constructed by deleting part of the plasmid pBR322-derived vector backbone. DNA of plasmid pOSEX2 was cleaved with *Cla*I + *Nru*I, the overhanging ends were filled-in with PolIk and the plasmid was then religated, resulting in plasmid pOSEX3 (4.43 kb). In a similar fashion we removed a *Cla*I-*Pvu*II fragment from pOSEX2 and obtained plasmid pOSEX4 (3.34 kb). The nt sequences of MCS present in the pOSEX plasmids were verified by sequence analysis (Sanger et al., 1977) using double-stranded DNA as the template and a primer (5'-GCTGATTGATGGTGTGG) complementary to a segment of the *proV'* gene (Gowrishankar et al., 1989). The *proV'* portion present in the pOSEX vectors encodes 103 N-terminal aa of the *E. coli* ProV protein; the junction between *proV'* and MCS is indicated by an arrow.

(LBON), but βGal production was strongly induced when the osmolarity of the LB medium was increased by the addition of 300 mM NaCl. Strikingly, the amount of βGal synthesized in strain MKH13[pBH4] could be controlled sensitively by simply varying the osmolarity of the growth medium in small increments with NaCl (Fig. 2A). The low basal level of transcription and the strong osmotic induction mediated by the *proU* regulatory elements were also apparent when we quanitated βGal enzyme activity in strain MKH13[pBH4] grown at low (LBON) or high (LBON with 300 mM NaCl) osmolarity. Under these growth conditions, cultures of strain MKH13[pBH4] produced 2.9 and 101.6 specific βGal units (μmol ONPG cleaved/min per mg protein), respectively, whereas strain MKH13 carrying the vector pOSEX3 produced 0.3 βGal units both in low- and high-osmolarity media.

The *gcdA* gene from the Gram$^+$ bacterium *A. fermentans* encodes the carboxytransferase subunit of the biotin-dependent Na^+ pump glutaconyl-CoA decarboxylase (GCDA) (Bendrat and Buckel, 1983). The *gcdA* gene, lacking its own promoter, was isolated on a 2.05-kb *Hin*dIII restriction fragment from plasmid pKB31 (Bendrat and Buckel, 1983) and cloned in both orientations into the unique *Hin*dIII site of the expression vector pOSEX4 (Fig. 1). The resulting plasmids pBH8a and pBH8b, were transformed into strain MKH13, and GCDA production in cultures grown at low and high osmolarity was followed by SDS-PAGE of whole cell extracts. The *gcdA* gene present on plasmid pBH8b is improperly oriented with respect to the osmoregulated *proU* promoter and hence strain MKH13[pBH8b] did not produce GCDA (Fig. 2B). However, under high osmolarity growth conditions the GCDA enzyme became the predominant protein in whole cell extracts of strain MKH13[pBH8a], in which the *gcdA* gene is under *proU* control (Fig. 2B). The identity of the newly synthesized polypeptide with GCDA was proven by Western immunoblotting using a GCDA-specific antiserum (data not shown). The level of GCDA production in strain MKH13[pBH8a] correlated with the osmolarity of the growth medium. We noticed that less NaCl was required to trigger maximal expression of the cloned genes in plasmid pOSEX4 than in plasmid pOSEX3 (compare the level of βGal and GCDA production; Fig. 2A and B). We attribute the differences in the properties of these two pOSEX expression plasmids to the presumably higher copy number of plasmid pOSEX4. Taken together, the data presented above show that the pOSEX vectors can be readily used for the efficient and sensitively regulated overproduction of heterologous proteins under *proU* control by simply varying the osmolarity of the growth medium.

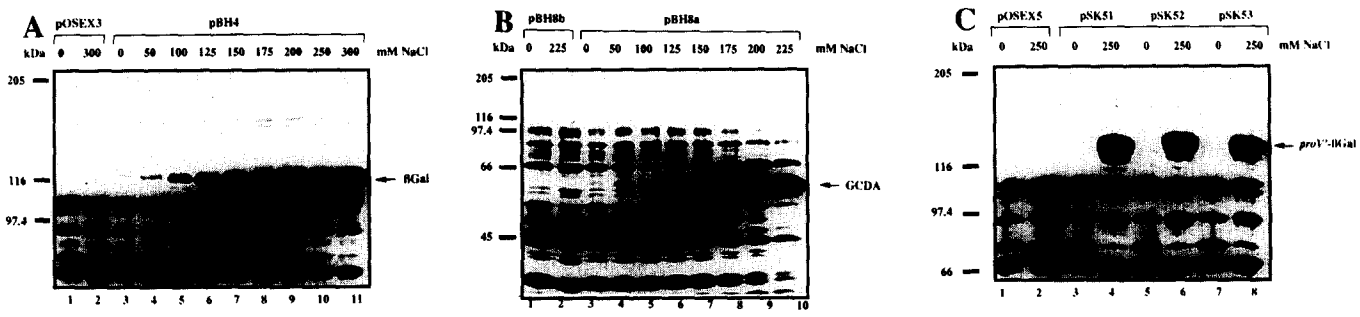

Fig. 2. Osmotically regulated overexpression of heterologous genes under the control of the *proU* regulatory elements. (**A**) Overproduction of the βGal protein from *E. coli*, (**B**) synthesis of the GCDA protein from *A. fermentans* and (**C**) osmotically controlled overexpression of a *'lacZ* gene lacking its transcription and translation start signals. **Methods:** Cultures of strain MKH13 harbouring the various plasmids were grown overnight in LBON in the presence of Ap (100 μg/ml) at 37°C and 0.1 ml were used to inoculate 7-ml cultures in LBON whose osmolarity was varied with NaCl. The cultures were grown for 3 to 4 h at 37°C, the $A_{578\ nm}$ was adjusted to compensate for their different growth of the various low and high osmolarity cultures and cells from a 2-ml portion ($A_{578\ nm}=1$) were pelleted by centrifugation. The pellet was resuspended in 80 μl of sample buffer (Silhavy et al., 1984; Sambrook et al., 1989), the cells were broken by boiling for 3 min and the viscosity of the cell extract was reduced by incubation for 1 h at 37°C in the presence of 25 units of Benzonase (Merck, Darmstadt, Germany). Aliquots (40 μl) of the cell extracts were then applied to 7% (**A**, **C**) and 12% (**B**) polyacrylamide-0.1% SDS gels and the cellular proteins were visualized by staining with Coomassie blue. To construct plasmids expressing *'lacZ* genes lacking their own transcription and translation initiation signals, a 3.14-kb *Eco*RI-*Dra*I fragment was isolated from the *'lacZ* protein fusion plasmids pNM480, pNM481 and pNM482 (Minton, 1984) and inserted into the *proU* expression plasmids pOSEX5A, pOSEX5B and pOSEX5C (Fig. 3), respectively, yielding the *lacZ*$^+$ plasmids pSK51, pSK52 and pSK53.

(c) Construction of pOSEX derivatives with translation initiation signals

We also constructed a set of pOSEX expression vectors that provide an RBS, an ATG start codon and an MCS and, therefore, allow the insertion of DNA fragments in all three reading frames. We amplified the translation start signals and the MCS from plasmids pTrc99A, pTrc99B and pTrc99C (Amann et al., 1986) and cloned the amplified DNA segments into plasmids pOSEX3 and pOSEX4. The nt sequence of the translation initiation region of the resulting pOSEX vectors is shown in Fig. 3. To test the characteristics of these new expression vectors, we cloned a *'lacZ* gene lacking its own transcription and translation initiation signals into plasmids pOSEX5A, pOSEX5B and pOSEX5C (Fig. 3). We followed βGal production in cells of strain MC4100 carrying the resulting plasmids pSK51, pSK52 and pSK53, respectively. The basal level of βGal synthesis was very low when the cells were propagated in media of low osmolarity, but growth at high osmolarity triggered the massive production of a large polypeptide (Fig. 2C) that cross-reacted on Westernblots with a βGal antiserum (data not shown). Thus, these pOSEX vectors can be successfully used to strongly overexpress genes lacking their own translation initiation signals. We noticed, however, that the size of recombinant proteins produced in cells carrying the various pSK *lacZ* expression plasmids was larger than that expected for the authentic βGal (116 kDa) monomer (Fig. 2C). We believe that the produced polypeptides are fusion proteins composed of an N-terminal segment of ProV and a C-terminal portion of βGal. Synthesis of such hybrid proteins is possible because there is no stop codon in the reading frame between the end of the truncated *proV'* gene and the ATG start codon in the MCS (Fig. 3). Using site-directed mutagenesis we introduced an in-frame TAA stop codon into this region in plasmids pOSEX5A, pOSEX5B and pOSEX5C (Fig. 3) but the artificially created stop codon caused a strong polar effect on the expression of genes cloned into the MCS (data not shown).

(d) Conclusions

We have constructed and characterized a set of gene expression vectors, pOSEX, that capitalize on the regulatory properties of the environmentally controlled *proU* operon.

(*1*) Transcription directed by the *proU* promoter occurs at very low basal level in cells grown in low osmolarity media. This important feature for the expression of heterologous genes is preserved when the *proU* promoter is present on multi-copy plasmids.

(*2*) An increase in medium osmolarity triggers high-level *proU* transcription, permitting the massive overproduction of heterologous proteins under the control of the *proU* regulatory elements. Increased *proU* expression is maintained as long as the osmotic stimulus persists.

(*3*) Over a wide range of osmolarities, the strength of *proU* expression is directly proportional to the osmolarity of the growth medium. This unique feature of the *proU* promoter allows one to alter the level of gene expression by simply adjusting the osmolarity of the growth medium.

(*4*) *proU*-mediated gene expression can be triggered by a variety of ionic or non-ionic osmolytes in rich and minimal media and no special *E. coli* host strains are required for the use of the pOSEX expression vectors.

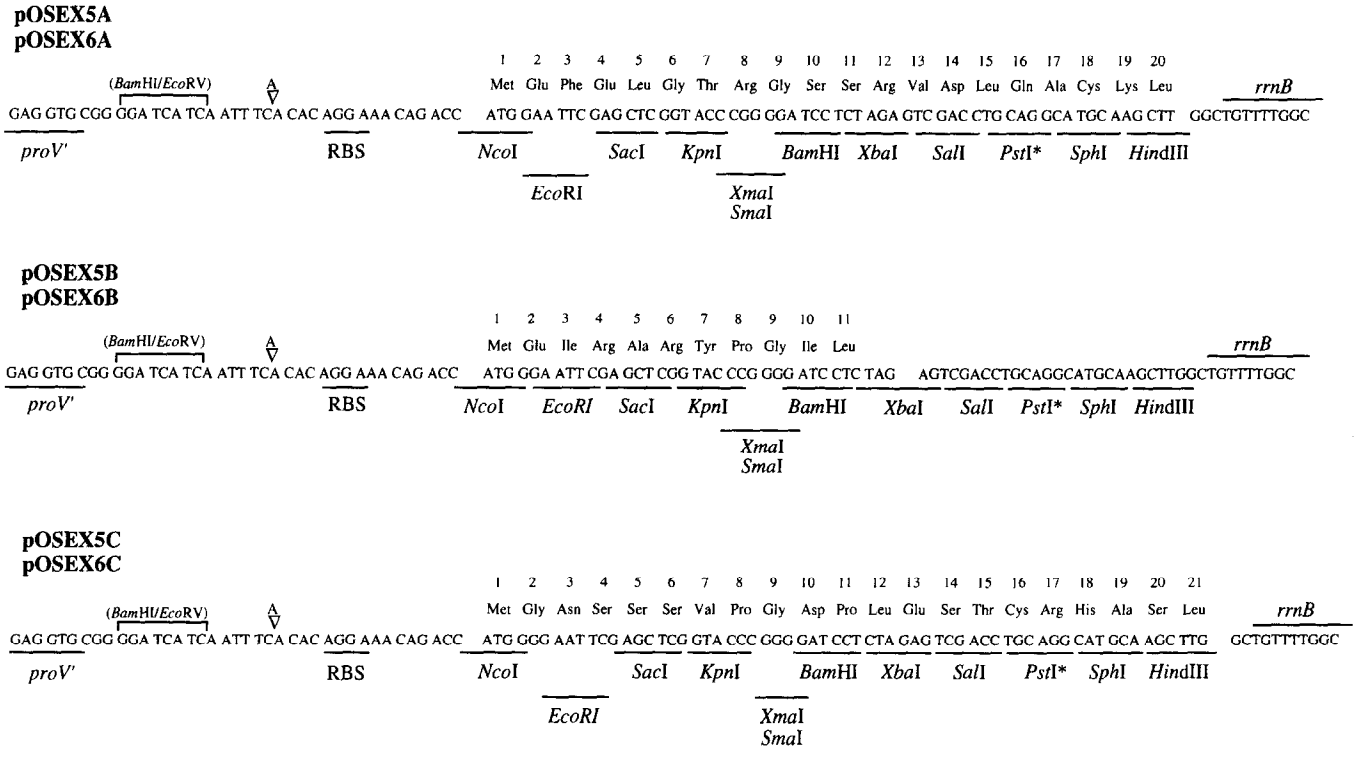

Fig. 3. The nt sequence of MCS and the translation start elements present in the expression vectors pOSEX5 and pOSEX6. The RBS is underlined and the start codons and reading frames in the expression vectors are indicated. Restriction sites that are not unique are marked with asterisks (*) and restriction sites destroyed during the in vitro manipulations are shown in brackets. The reading frame of the *proV'* gene (103 codons) is indicated and the position of the TAA stop codon introduced by site-directed mutagenesis into the pOSEX5 vectors is indicated by an arrow head. **Methods:** Plasmid pOSEX3 (Fig. 1A) was linearized with *Eco*RI, the overhanging ends were filled in with dNTP and PolIk and the DNA molecule was then religated to yield plasmid pOSEX3.1. DNA fragments carrying the translation initiation elements (the RBS and the ATG start codon) and MCS were amplified by PCR from plasmids pTrc99A, pTrc99B and pTrc99C (Amann et al., 1986) using two primers complementary to sequences upstream from the MCS present in the pTrc vectors (5'-GTGAGCGGATATCAATTTCACACAGG; primer 1 generates an *EcoRV* site; underlined) and the *bla* gene (5'-GCGGTTAGCTCCTTCGGTCC; primer 2). These DNA segments were then cut with *Eco*RV + *Sca*I and the resulting restriction fragments were ligated into plasmid pOSEX3.1. For this purpose, plasmid pOSEX3.1 was cleaved with *Bam*HI + *Sca*I, the overhanging ends were filled in with dNTP and PolIk and the resulting molecule was then ligated with the *Eco*RV-*Sca*I fragment originating from the PCR DNA amplification. These in vitro manipulations resulted in the construction of plasmids pOSEX5A (4443 bp), pOSEX5B (4444 bp) and pOSEX5C (4445 bp). For the isolation of plasmids pOSEX6A (3351 bp), pOSEX6B (3352 bp) and pOSEX6C (3353 bp), an analogous set of plasmid constructions was made except that plasmid pOSEX4 (Fig. 1A) was used as the starting material. To introduce a TAA stop codon (underlined) between the *proV'* gene and the ATG start codon in the MCS, a derivative of primer 1 (5'-GTGAGCGGATATCAATTTAACACAGG) was employed for the PCR amplification reaction; the in vitro manipulations yielded ultimately plasmids pOSEX7A, pOSEX7B and pOSEX7C.

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REFERENCES

Amann, E., Ochs, B. and Abel, K.-J.: Tightly regulated *tac* promoter vectors useful for the expression of unfused proteins in *Escherichia coli*. Gene 69 (1988) 301–315.

Arakawa, T. and Timasheff, S.N.: The stabilization of proteins by osmolytes. Biophys. J. 47 (1985) 411–414.

Balbás, P., Soberón, X., Merino, E., Zurita, M., Lomeli, H., Valle, F., Flores, N. and Bolivar, F.: Plasmid vector pBR322 and its special-purpose derivatives – a review. Gene 50 (1986) 3–40.

Barron, A., May, G., Bremer, E. and Villarejo, M.: Regulation of envelope protein composition during adaptation to osmotic stress in *Escherichia coli*. J. Bacteriol. 167 (1986) 433–438.

Bendrat, K. and Buckel, W.: Cloning, sequencing and expression of the gene encoding the carboxytransferase subunit of the biotin-dependent Na^+ pump glutaconyl-CoA decarboxylase from *Acidaminococcus fermentans* in *Escherichia coli*. Eur. J. Biochem. 211 (1993) 697–702.

Casadaban, M.J.: Transposition and fusion of the *lac* genes to selected promoters in *Escherichia coli* using bacteriophage lambda and Mu. J. Mol. Biol. 104 (1976) 541–555.

Csonka, L.N. and Hanson, A.D.: Prokaryotic osmoregulation: genetics and physiology. Annu. Rev. Microbiol. 45 (1991) 569–606.

Culham, D.E., Lasby, B., Marangomi, A.G., Milner, J.L., Steer, B.A., van Nues, R.W. and Wood, J.M.: Isolation and sequencing of

Escherichia coli gene *proP* reveals unusual structural features of the osmoregulatory proline/glycine betaine transporter, ProP. J. Mol. Biol. 229 (1993) 268–276

Dattananda, C.S., Rajkumari, K. and Gowrishankar, J.: Multiple mechanisms contribute to osmotic inducibility of *proU* operon expression in *Escherichia coli*: demonstration of two osmoresponsive promoters and of a negative regulatory element within the first structural gene. J. Bacteriol. 173 (1991) 7481–7490.

Gowrishankar, J.: Nucleotide sequence of the osmoregulated *proU* operon of *Escherichia coli*. J. Bacteriol. 171 (1989) 1923–1931.

Lucht, J.M. and Bremer, E.: Characterization of mutations affecting the osmoregulated *proU* promoter of *Escherichia coli* and identification of 5′ sequences required for high-level expression. J. Bacteriol. 173 (1991) 801–809.

Lucht, J.M. and Bremer, E.: Adaptation of *Escherichia coli* to high osmolarity environments: osmoregulation of the high-affinity glycine betaine transport system ProU. FEMS Microbiol. Rev. 14 (1994) 3–20.

Lucht, J.M., Dersch, P., Kempf, B. and Bremer, E.: Interactions of the nucleoid-associated DNA-binding protein H-NS with the regulatory region of the osmotically controlled *proU* operon of *Escherichia coli*. J. Biol. Chem. 269 (1994) 6578–6586.

May, G., Faatz, E., Villarejo, M and Bremer, E.: Binding protein-dependent transport of glycine betaine and its osmotic regulation in *Escherichia coli* K-12. Mol. Gen. Genet. 205 (1986) 225–233.

Minton, N.P.: Improved vectors for the isolation of translational *lac* gene fusions. Gene 31 (1984) 269–273.

Sambrook, J., Fritsch, E.F. and Maniatis, T.: Molecular Cloning. A Laboratory Manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.

Sanger, F., Nicklen, S. and Coulson, A.R.: DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA 74 (1977) 5463–5467.

Silhavy, T.J., Berman, M.L. and Enquist, L.W.: Experiments with Gene Fusions. Cold Spring Habor Laboratory, Cold Spring Harbor, NY, 1984.

Ueguchi, C. and Mizuno, T.: The *Escherichia coli* nucleoid protein H-NS functions directly as a transcriptional repressor. EMBO J. 12 (1993) 1039–1046.