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Investigation into a stress-inducible promoter region from *Marinococcus halophilus* using green fluorescent protein

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Abstract A 480-bp *Marinococcus halophilus* DNA-fragment upstream of the ectoine genes *ect*ABC was linked to the reporter gene gfp_{uv} in the stress probe plasmid pBRGFP$_{uv}$. On the basis of green fluorescence emitted after application of different types of stress to recombinant *Escherichia coli*, the DNA sequence was characterized as a stress-responding promoter region. In *E. coli*, osmotic stress resulted in an increasing fluorescence intensity, whereas the effects of heat or cold were insignificant, suggesting that transcription and/or translation is controlled mainly by an osmotic stress/low-water stress response. Additionally, the stress-counteracting effects of the compatible solutes ectoine and betaine were demonstrated.

Key words Ectoine · Compatible solutes · Green fluorescent protein · Osmotic stress · Stress response

Introduction

Bacteria have to face several types of stress conditions, including physical stress such as high osmolarity. Of the adaptational strategies employed by halophilic and halotolerant bacteria, the production and accumulation of compatible solutes has to be considered the most flexible answer to the problem of limited availability of water (Galinski and Trüper 1994; Galinski 1995; Kempf and Bremer 1998). Compatible solutes are produced in response to elevated osmolarities to protect the cells against dehydration and to maintain the intracellular activity of biomolecules.

The compatible solute ectoine (1,4,5,6-tetrahydro-2-methyl-4-pyrimidine carboxylic acid) is a typical product of halophilic eubacteria, and it is of biotechnological interest because it provides a range of qualities beyond osmoprotection of bacteria. It has been shown to stabilize biomolecules in vitro against heating, freeze-thawing, drying, and chemical treatment (Lippert and Galinski 1992; Göller and Galinski 1999; Knapp et al. 1999; Barth et al. 2000). Additionally, ectoine can be used as a sun protectant and moisturizer for skin care products (Bünger 1999). Even though the genes of the ectoine biosynthetic pathway, *ect*ABC (*ect*A: L-2,4-diaminobutyric acid-N-γ-acetyltransferase; *ect*B: L-2,4-diaminobutyric acid transaminase; *ect*C: L-ectoine synthase), from the Gram-positive *Marinococcus halophilus* have already been sequenced and expressed in *E. coli*, resulting in osmoregulated ectoine production (Louis and Galinski 1997), our knowledge of the regulation of gene expression and ectoine production is still very insufficient. The most remarkable feature of the above ectoine gene cluster is that *E. coli* seems to recognize one or more specific promoter elements on the *M. halophilus* DNA sequence, because ectoine production appears to be osmoregulated even in the absence of specific *E. coli* promoters (Louis and Galinski 1997). Therefore, it would be particularly interesting to see if *E. coli* recognizes the same promoter sequences as *M. halophilus*, or if transcription is just due to a very fortunate coincidence. In this study, we examined the stress-responding putative promoter region upstream of the *ect*A gene, referred to as *ect*Up, in *E. coli* using the green fluorescent protein (GFP$_{uv}$) as a reporter molecule.

Materials and methods

Bacterial strains and plasmids

E. coli DH5α (Hanahan 1983) was used for the generation of a recombinant strain harboring the stress probe plasmid pBRGFP$_{uv}$-*ect*Up. Plasmid pBR322 was used as parent vec-

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tor, pGFPuv (Clontech, Palo Alto, CA, USA) as source of the gfp_{uv} gene, and pOSM12 (Louis and Galinski 1997) as source of the *M. halophilus* DNA region *ect*Up.

Construction of pBRGFP$_{uv}$-*ect*Up

The gene gfp_{uv} was excised from pGFP$_{uv}$ by digestion with *Sph*I and *Eag*I and inserted into the *Sph*I and *Eag*I sites of pBR322 to give the plasmid pBRGFP$_{uv}$ (Fig. 1). Adjustment of *ect*Up to the reading frame of gfp_{uv} was performed by amplification with modified polymerase chain reaction (PCR) primers 5′-ttt acc ggt gTA CCA CCT TGT AGA AGT AA-3′ (5′-overhang in lowercase letters; *Age*I recognition site underlined) and 5′-cga aag tgt cta gac gt-3′ (*Xba*I recognition site underlined) and subcloning into the plasmid pGEM-T (Promega, Mannheim, Germany), followed by cloning into the *Age*I and *Xba*I sites of pBRGFP$_{uv}$ within the previously inserted fragment carrying the gene gfp_{uv}. In this way the insertion of *ect*Up upstream of gfp_{uv} was not only in frame, but positioned in such a way that the gfp_{uv} start codon corresponded exactly with the start codon of *ect*A in the *M. halophilus* genome. The correct amplification was verified by sequencing of the fragment (GATC, Konstanz, Germany). A similar approach to the construction of stress probe plasmids for the heat shock stress protein promoter elements of *rpo*H, *clp*B, and *dna*K has been described by Joon Cha et al. (1999).

Growth media and conditions

E. coli precultures were grown overnight at 37° or 30°C (for heat shock experiments from 30° to 42°C) in minimal medium MM63 (Larsen et al. 1987) supplemented with 50 mg/l ampicillin (Sigma, Taufkirchen, Germany).

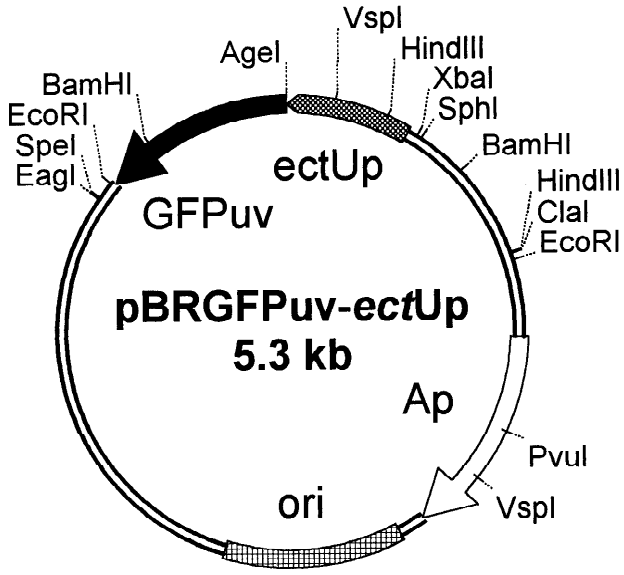

Fig. 1. Restriction map of the plasmid pBRGFP$_{uv}$-*ect*Up. Not all restriction sites are shown

Cultures of 100 ml minimal medium MM63 with 50 mg/l ampicillin were inoculated with 2% of the precultures and grown at 37° or 30°C (for heat shock experiments from 30° to 42°C) for 2 h before the stress induction experiments were performed.

Application of stress and fluorescence measurements

Osmotic stress was induced by addition of solid NaCl to 100-ml cultures containing a basic salt level between 1% and 3% NaCl. Every experiment included a control culture, which was not supplemented with NaCl. Directly before the osmotic upshock and subsequently every 2 h, culture samples were diluted with fresh media to an optical density (OD_{600}) of 0.05 (Novaspec II, Pharmacia Biotech, Freiburg, Germany) and fluorescence intensity was measured with a fluorescence spectrometer (LS-50B; Perkin-Elmer, Beaconsfield, Buckinghamshire, England) at an excitation wavelength of 395 nm and an emission wavelength of 509 nm relative to that of the nonstressed control.

Besides NaCl, the cell-penetrating osmolyte glycerol was used for comparison. Heat shock was induced by raising the growth temperature from 30° to 42°C or 44°C, and cold shock was induced by lowering the growth temperature to 15°C. The response to ethanol was studied by adding 2%, 4%, or 6% ethanol to the medium, and subsequent fluorescence measurements were performed as described above for the salt stress experiments. To investigate the stress-preventing effect of compatible solutes, the growth media were supplemented with 2 mM ectoine (Bitop, Witten, Germany) or betaine (Sigma, Taufkirchen, Germany). Neither substance showed any enhancing or quenching effect on fluorescence. All experiments were independently performed at least three times.

Analysis of intracellular betaine and ectoine

The extraction of intracellular solutes from freeze-dried cells was done by a modification of the method of Bligh and Dyer (1959) with methanol/chloroform/water (10:5:4) as described previously (Galinski and Herzog 1990). The cell extracts were analyzed using isocratic HPLC methods (Galinski and Herzog 1990).

Results and discussion

Rapid in vivo fluorescence measurements

The discovery and characterization of promoter sequences can be easily performed with green fluorescent protein (GFP) as a reporter molecule. Using the UV-optimized GFP$_{uv}$, which is efficiently expressed and very stable in *E. coli*, we established a direct fluorescence assay for liquid cultures without any necessity to pellet and resuspend the bacteria. In a series of optimization experiments using the

plasmid pGFP$_{uv}$ under isopropyl-β-D-thiogalactopyranoside (IPTG) induction (data not shown), we were able to demonstrate a linear increase in fluorescence upon addition of IPTG, whereas fluorescence remained unchanged in noninduced cells. However, only defined growth media with negligible background fluorescence such as the minimal medium MM63 used in this study are suitable for such liquid-culture assays. Because of the optimized fluorescence of GFP$_{uv}$ and the sensitivity of fluorescence measurements, the samples had to be diluted to an OD_{600} of about 0.05 with fresh medium. As the ratio of culture medium to fresh medium did not influence the data, possible quenching or enhancing effects due to variations in the growth medium appear to be negligible in this experimental setup. Furthermore, cells growing very slowly owing to the applied stress still produced GFP$_{uv}$ in response to this stress. The described in vivo fluorescence measurement provides a means of fast data collection without the requirement of long sample processing and seems to be particularly suitable for the determination of osmotic stress response.

Response to osmotic stress

Upon the addition of NaCl, an increase in the relative fluorescence could be detected (Fig. 2). Relative fluorescence increased over a period of 6 to 8 h and remained relatively constant for at least another 10 h. Within limits, the osmotic upshock can be considered the trigger of stress response. Osmotic shocks from 1% to 2% NaCl resulted in an only 1.5-fold increase in fluorescence, whereas upshocks to either 3% or 4% yielded approximately 3.5-fold higher values (Fig. 3). The relative increases after upshocks from 2% to 3% and from 2% to 4% NaCl agreed very well with the expected values (Fig. 3), with the strongest increase in relative fluorescence being between 2% and 3% NaCl (2.4-fold). From this we assumed a critical threshold for osmotic response of between 2% and 3% NaCl in *E. coli* harboring the *ect*Up region. The significant difference in fluorescence intensity between 2% and 3% NaCl, which was not increased upon further upshock to 4%, seems to indicate a limitation of cellular stress response in this host. In a control experiment with *E. coli* DH5α harboring the plasmid pBRGFP$_{uv}$, it was shown that without *ect*Up no change of fluorescence resulted from osmotic shock.

The freely permeant polyol glycerol is known to penetrate bacterial cells (Poirier et al. 1998). Therefore, elevated osmolarities generated by the addition of glycerol to the growth medium are not expected to cause osmotic gradients over the membrane as solutes excluded from the cytoplasm do. Upon addition of 1 M glycerol, which leads to approximately the same increase in osmolarity as the addition of 3 g NaCl to a final volume of 100 ml, relative fluorescence was elevated twofold, compared to a fourfold increase after the equivalent upshock from 1% to 4% NaCl. The addition of 1.67 M glycerol, causing the same shift of osmolarity as the addition of 5 g NaCl to a final volume of 100 ml, led to a threefold elevated fluorescence. Therefore, it can be said that, surprisingly, a shift in osmolarity by glycerol also induces *ect*Up, albeit at a lower level. This might be an indication that the sensing of low water activity plays an important role in the cellular stress response, which was further confirmed by the fact that neither ectoine nor betaine could counteract the glycerol effect, as they did salt stress (data not shown).

The presence of compatible solutes counteracts osmotic stress

For osmoprotection, *E. coli* accumulates compatible solutes from the medium (Jebbar et al. 1992; Poolman and Glaasker 1998; Kempf and Bremer 1998). The release of osmotic stress achieved in this way would make additional stress response mechanisms unnecessary. To investigate whether the presence of compatible solutes interferes with the induction of osmotically induced genes, ectoine or betaine (2 mM) were added to the growth medium. In contrast to the experiments without supplementation of compatible solutes, the fluorescence was only slightly elevated after

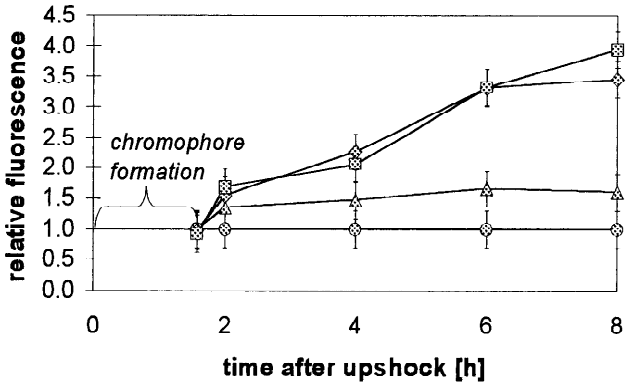

Fig. 2. Time course of relative fluorescence at 1% NaCl (*circles*) and after osmotic upshock to 2% (*triangles*), 3% (*diamonds*), and 4% (*squares*) NaCl. The starting point of the graph was set to 95 min in view of the time lag for GFP$_{uv}$ chromophore formation

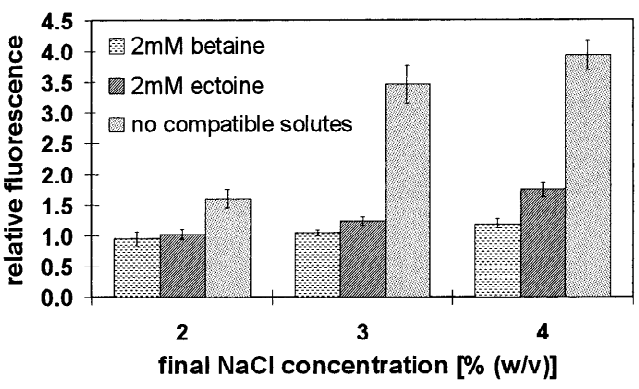

Fig. 3. Relative fluorescence 8 h after osmotic upshock from 1% NaCl to a final salt concentration of 2%, 3%, or 4% NaCl, in the absence of compatible solutes (*right bars*) and under supplementation with 2 mM betaine (*left bars*) or 2 mM ectoine (*middle bars*)

upshock to 3% and 4% NaCl with ectoine and not at all with betaine in the growth medium (Fig. 3). The insufficient stress-preventing effect of ectoine at high salinities may be explained by the fact that accumulation failed to reach intracellular concentrations reported for halotolerant ectoine-synthesizing organisms such as *Halomonas elongata* (Fig. 4).

Reversibility of stress induction

To investigate whether stress-induced expression of gfp_{uv} could be switched off by the addition of solutes, ectoine or betaine (2 mM) was added in a second experiment to cultures already exposed to elevated salt concentrations (9 h). Upon the addition of ectoine to a culture with 3% NaCl, which did not show significant growth but increased GFP_{uv} production, bacterial growth resumed and gfp_{uv} expression ceased, resulting in a decrease in relative fluorescence (Fig. 5). This decrease mainly resulted from a dilution of the initial GFP_{uv}-containing cells by the formation of new cells, which in the absence of stress did not produce any more green fluorescent protein. Similar results were obtained with betaine for cultures with both 3% and 4% NaCl (Fig. 5). Because of the long half-life of the very stable protein GFP_{uv} (>24 h), the direct observation of ceased gene expression and subsequent degradation of the protein was not possible. For such observations, a different approach using destabilized GFP mutants such as GFP(ASV), GFP(AAV), or GFP(LVA) would be necessary (Andersen et al. 1998). Even though ectoine and betaine have similar effects on *ect*Up, it is at present not possible to conclude whether these compounds act as repressors or whether altered physical conditions (water activity) are responsible for this "silencing" effect on gfp_{uv} expression.

Heat, cold, and ethanol

If the *E. coli* stress factors that trigger a promoter on the *M. halophilus* DNA sequence are induced not only by elevated osmolarities but also by stress in general, the application of physical and chemical stress other than salt should also induce the production of GFP_{uv} in our test system. However, neither heat shocks from 30° to 42°C (or even 44°C) nor lowering the growth temperature to 15°C induced any increases in fluorescence. Ethanol, on the other hand, which is already known to be an inducer of cellular heat stress and oxidative stress response in *E. coli* (Neidhardt and Van Bogelen 1987; Van Dyk et al. 1994; Belkin et al. 1996) proved to be a potent inducer molecule. Whereas the addition of 2% ethanol to the cultures had no effect on fluorescence, 4% ethanol increased the relative fluorescence by a factor of 2.0, and 6% ethanol by a factor of 2.6. The observation that ethanol induces fluorescence, whereas heat shock had no effect, points toward an induction mechanism different from a heat-shock response, possibly based on the osmotic effect of the added ethanol in the culture medium. The osmolarities of 2%, 4%, and 6% ethanol are similar to those of 1%, 2%, and 3% NaCl, respectively. From this one may speculate that the response of *ect*Up is specifically optimized for osmotic stress or, alternatively, that osmotic and oxidative stress may have a common basis for fluorescence induction.

Promoter analysis

Having demonstrated the promoter activity of *ect*Up, which appears to be triggered by osmotic stress and/or low water activity in *E. coli*, we analyzed *ect*Up for potential recognizable promoter sequences using a neural network promoter prediction algorithm (Reese and Eeckman 1995) based on 263 *E. coli* promoters published by Harley and Reynolds (1987). A *Bacillus subtilis* σ^B binding site has already been described by Louis and Galinski (1997). Because *M. halophilus* is related to the genus *Bacillus* (Farrow et al. 1992), this promoter is a promising candidate for transcription regulation in the donor. Our database search revealed additional promoter binding sites, which may also be recognized in *E. coli* (Fig. 6). The promoter binding site about 60 bp upstream of *ect*A showed the highest grade of homology in the database search. The –35 and –10 regions are

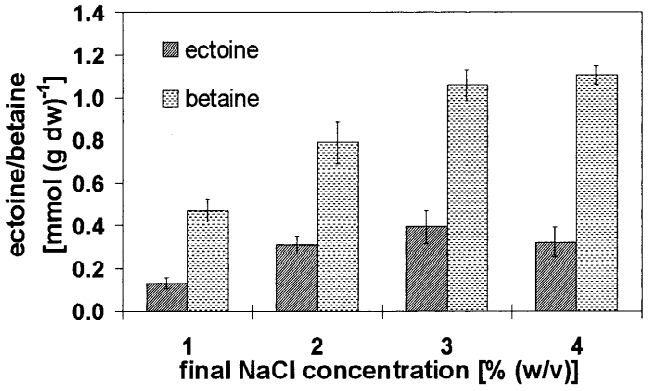

Fig. 4. Intracellular concentrations of ectoine and betaine in *E. coli* grown at various levels of salinity for 8 h (2 mM solutes in medium)

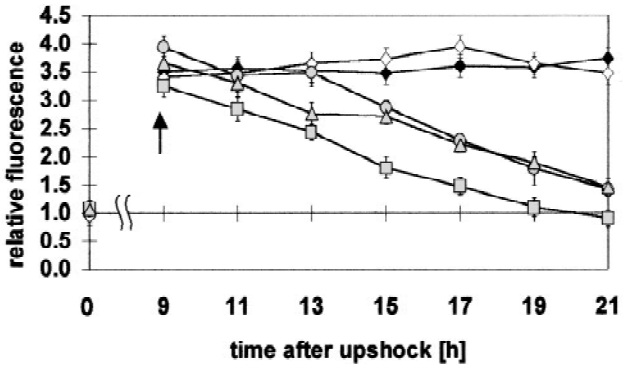

Fig. 5. Development of the relative fluorescence of upshocked *E. coli* upon additional supplementation with compatible solutes 9 h (*arrow*) after the osmotic upshock: 3% NaCl/no supplementation (*solid diamonds*); 4% NaCl/no supplementation (*open diamonds*); 3% NaCl/2 mM ectoine (*shaded circles*); 3% NaCl/2 mM betaine (*shaded squares*); 4% NaCl/2 mM betaine (*shaded triangles*)

Fig. 6. The sequence of *ect*Up from *M. halophilus*. *B. subtilis* σ^B binding site (*underlined*) as described by Louis and Galinski (1997). Potential *E. coli* binding sites with low scores are underlined (*dotted lines*). The binding sites with the highest scores are marked by boxes

most probably recognized by the primary *E. coli* σ-factor σ^{70} (for a review of the consensus sequences, see Wösten 1998). The alternative sigma factor, σ^S, which mediates the general stress response in *E. coli*, can generally recognize the same promoter sequences as σ^{70}, whereas the physiological differentiation is based on both interaction with other regulatory molecules (Hengge-Aronis 1999) and distinct preferences in promoter recognition (Wise et al. 1996). However, dependence of osmotically driven transcription activation is not restricted to a specific sigma factor, because both σ^{70}- or σ^S-mediated osmotic response is known (Mellies et al. 1995; Lamark et al. 1996; Wise et al. 1996). Significant similarities to promoter binding sites of typical osmoregulated genes such as *bet*T, *pro*P, or *pro*U could not be found within the *ect*Up region. The same was true for promoter sequences preferentially recognized by the heat-shock-specific factor σ^{32}. Considering the key role of σ^{32} in the heat-shock response (Arsène et al. 2000), this might explain the failure of heat stress to induce fluorescence.

Because of the complexity of the interplay of transcription regulation and stress response, it would be very speculative to define a regulatory mechanism by a simple promoter search. Nevertheless, this approach provides a good opportunity for discussion, critical review of experimental data, and, last but not least, a starting point for subsequent studies to localize the promoter region in detail.

In conclusion, we were able to demonstrate that: (1) promoter activity of *ect*Up was osmotically induced in *E. coli*; (2) the presence of suitable compatible solutes repressed promoter induction, even though water activity was still low; and (3) heat or cold had no effects on transcription activation. Our data are consistent with the finding that *ect*Up contains promoter binding sites that might potentially be recognized by the sigma factors σ^{70} or σ^S. The fact that glycerol and ethanol also trigger promoter activity points toward the possibility that induction is achieved by direct sensing of low water activity and/or indirectly via common oxidative stress induction. Based on our results from the fluorescence assay with green fluorescent protein, further research into the regulation of gene expression by RNA- and protein-based methods seems very promising.

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