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ORIGINAL RESEARCH PAPER

Over-expression of stress protein-encoding genes helps *Clostridium acetobutylicum* to rapidly adapt to butanol stress

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Abstract The toxicity of *n*-butanol in microbial fermentations limits its formation. The stress response of *Clostridium acetobutylicum* involves various stress proteins and therefore, over-expression of genes encoding stress proteins constitutes an option to improve solvent tolerance. Over-expression of *groESL*, *grpE* and *htpG*, significantly improved butanol tolerance of *C. acetobutylicum*. Whereas the wild type and vector control strain did not survive 2 % (v/v) butanol for 2 h, the recombinant strains showed 45 % (*groESL*), 25 % (*grpE*) and 56 % (*htpG*), respectively, of the initial c.f.u. after 2 h of butanol exposure. As previously, over-expression of *groESL* led to higher butanol production rates, but the novel strains over-expressing *grpE* or *htpG* produced only 51 and 68 %, respectively, of the wild type butanol concentrations after 72 h clearly differentiating butanol tolerance and production. Not only butanol tolerance but also the adaptation to butanol in successive stress experiments was significantly facilitated by increased levels of GroESL, GrpE and HtpG. Re-transformation and sequence analyses of the plasmids confirmed that not the plasmids, but the host cells evolved to a more robust phenotype.

Keywords Butanol stress · Solvent tolerance · Heat shock protein · GroESL · GrpE · HtpG

Introduction

Clostridial acetone/butanol/ethanol (ABE) fermentation has regained much interest in the past years because butanol represents a superior biofuel with several advantages over ethanol: butanol has a higher energy content, is less hydroscopic, less volatile and less corrosive, which makes it perfectly compatible with the current gasoline infrastructure (Lee et al. 2008; Green 2011). Since the maximum butanol tolerance is 20 g l^{-1} or less for clostridial strains, product removal by different techniques, such as gas stripping or pervoration, have been considered to economically improve ABE fermentation (Ezeji et al. 2005, 2007). The major cellular impact of high butanol

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concentrations is the destruction of the cytoplasmic membrane and various macromolecules, accompanied by the loss of vital functions (Bowles and Ellefson 1985; Taylor et al. 2008; Liu and Qureshi 2010). On the molecular level, butanol causes a stress response which is not yet fully understood; the most important issues and experimental approaches have been reviewed in detail recently (Ezeji et al. 2010; Nicolaou et al. 2010).

Referring to the development of suitable genomic tools, clostridial metabolite and solvent stresses have been studied in a global approach using DNA microarrays (Alsaker et al. 2004, 2010; Tomas et al. 2004; Alsa; Heluane et al. 2011; Hönicke et al. 2012) and proteome analyses (Terracciano et al. 1988; Mao et al. 2010, 2011). The results clearly showed that different heat shock proteins (HSPs) are involved in *Clostridium acetobutylicum*'s stress response to butanol. These molecular chaperones play important roles in protein biosynthesis and degradation and are highly upregulated under various stress conditions (Narberhaus and Bahl 1992; Narberhaus et al. 1992; Bahl et al. 1995; Rüngeling et al. 1999). Transcriptome data revealed that *dnaK*, *dnaJ*, *grpE*, *groES*, *groEL*, *clpP*, *clpC*, *htpG* and *hsp18* were significantly induced after butanol stress (Tomas et al. 2004). Interestingly, the same HSPs were generally upregulated in *C. acetobutylicum* Rh8, a mutant strain with improved butanol tolerance and production (Mao et al. 2010). An increased abundance of HSPs was not only detected after external butanol exposure, but also during the life cycle-associated solventogenic metabolism (Alsaker and Papoutsakis 2005; Jones et al. 2008). Taking high ATP consumption rates into account, the protective function of multiple HSPs against solvent stress is primarily based on fast refolding and/or degradation of denaturated proteins, although the regulatory circuits are not well understood (Nicolaou et al. 2010). The second known defense mechanism of *C. acetobutylicum* is a higher content of saturated fatty acids in the cell membrane to counteract the increasing fluidity caused by butanol (Vollherbst-Schneck et al. 1984; Baer et al. 1987, 1989; Taylor et al. 2008; Ezeji et al. 2010).

Regarding microbial solvent tolerance, very few bacteria exhibit a similar or better performance than species of *Clostridium*, e.g., *Pseudomonas putida* or lactic acid bacteria (Fischer et al. 2008; Knoshaug and Zhang 2009; Rühl et al. 2009; Li et al. 2010; Winkler et al. 2010). Therefore, it seems more reasonable to engineer solventogenic clostridia with naturally high butanol production capacities, although a major metabolic breakthrough for recombinant butanol production was described recently (Bond-Watts et al. 2011; Shen et al. 2011).

Apart from traditional random mutagenesis, only two examples of targeted genetic engineering to increase butanol tolerance of *C. acetobutylicum* and other solventogenic clostridia have been published thus far. Based on early DNA microarray results which indicated the importance of stress proteins, the bicistronic *groESL* operon, coding for the class I (Hsp60) chaperonin GroEL and its co-chaperonin GroES, was homologously overexpressed. The recombinant *C. acetobutylicum* strain exhibited a significantly improved tolerance and also higher butanol production rates as compared to the wild type (Tomas et al. 2003). More recently, the *gshAB* genes from *Escherichia coli* were expressed in *C. acetobutylicum* to establish a heterologous glutathione biosynthetic pathway. The resulting strain exhibited an improved butanol tolerance and a slightly increased butanol titer as compared to the vector control (Zhu et al. 2011). In this study, two novel over-expression strains of *C. acetobutylicum* with enhanced butanol tolerance properties were generated. Interestingly, we observed that the HSP gene overexpressing strains revealed an enhanced adaptation to high butanol concentrations.

Materials and methods

Bacterial strains and cultivation conditions

Strains, plasmids and oligonucleotides for PCR used in this study are listed in Supplementary Table 1. General recombinant DNA techniques were performed according to standard. Chromosomal DNA of *C. acetobutylicum* ATCC 824 (Fischer et al. 2006) was used as template and the oligonucleotides listed in Table 1 as PCR primers to amplify *groESL* (CAC-2703-2704), *grpE* (CAC1281) and *htpG* (CAC3315). The DNA fragments were cloned via *Bam*HI or *Bcl*I, respectively, and *Kas*I restriction sites into the pT*HydA* plasmid as described by Girbal et al. (2005) and Hillmann et al. (2009). To generate the vector control pT, the *hydA* gene was excised from pT*HydA* by *Bam*HI/*Kas*I restriction and re-ligated after

Table 1 Growth rates and fermentation products of *C. acetobutylicum*-strains

Strain	Wildtype	pT	pT::groESL	pT::grpE	pT::htpG
Growth rate (h^{-1})	0.26 ± 0.06	0.26 ± 0.04	0.16 ± 0.03	0.04 ± 0.02	0.13 ± 0.04
Acetate (mM)	22 ± 3.7	21 ± 3.4	21 ± 3.9	33 ± 1.2	21 ± 1.1
Butyrate (mM)	7 ± 2.3	9 ± 1.8	11 ± 0.3	14 ± 0.9	12 ± 1
Acetone (mM)	53 ± 2.9	52 ± 3.7	38 ± 3.6	35 ± 1.5	42 ± 2.2
Ethanol (mM)[a]	17 ± 1.5	35 ± 4	28 ± 1.5	23 ± 2.2	26 ± 1.8
Butanol (mM)	90 ± 4.7	85 ± 4.9	117 ± 10.8	46 ± 3.9	61 ± 2.5

Cultivations were conducted in 200 ml MS-MES medium and monitored by the OD_{600} values. Fermentation products were determined by GC; representative values after 72 h of cultivation are shown. Three independent replicates per strain were used

[a] Except for the wild type, erythromycin was added to the medium for plasmid maintenance, which led to an initial ethanol concentration of 18–20 mM

treatment with the Klenow fragment of DNA polymerase I (Fermentas GmbH, Germany). The resulting plasmids pT, pT::*groESL*, pT::*grpE* and pT::*htpG*, respectively, were transformed into *E. coli* DH5α and validated by DNA sequencing (LGC Genomics GmbH, Berlin, Germany). After in vivo methylation in *E.coli* ER2275 pAN2 (Mermelstein and Papoutsakis 1993), the plasmids were transformed into *C. acetobutylicum* ATCC 824 and selected on reinforced clostridial agar containing 40 μg erythromycin ml^{-1}.

Clostridial strains were cultivated in clostridial growth medium (CGM) or mineral salts medium (MS-MES) with 40 μg erythromycin ml^{-1} for recombinant *C. acetobutylicum* under anaerobic conditions at 37 °C; media, conditions and fermentation product analyses were performed as described previously (Lehmann and Lütke-Eversloh 2011; Lehmann et al. 2012).

Butanol stress experiments

For butanol stress and adaptation experiments, 200 ml CGM in serum bottles were inoculated with 10 % (v/v) CGM precultures. When the cultures were grown to OD_{600} of 1, they were divided into 10 ml aliquots in Hungate tubes and subjected to 1 or 2 % (v/v) *n*-butanol. Respective controls were treated similarly but without butanol addition. To determine the c.f.u. values, samples were drawn regularly and serial dilutions were plated on RCA and incubated anaerobically for 48 h at 37 °C prior to colony counting.

Plasmid maintenance was controlled by colony PCR using plasmid- and gene-specific oligonucleotides (Supplementary Table 1). In addition, plasmids from three successive stress experiments were isolated and re-transformed into *C. acetobutylicum* ATCC 824 (Harris et al. 2000); furthermore, DNA sequence analyses of each plasmid was conducted (LGC Genomics GmbH, Berlin, Germany).

Results and discussion

Butanol tolerance

Homologous over-expression of the *groESL* operon in *C. acetobutylicum* leads to a significantly improved phenotype regarding butanol resistance and production (Tomas et al. 2003). In this study, two additional HSP encoding genes, i. e. *grpE* and *htpG*, were over-expressed in *C. acetobutylicum* and as a positive control, *groESL* over-expression was included. As shown in Fig. 1, all three over-expression strains exhibited enhanced butanol tolerance in comparison to the wild type and the vector control strain in the presence of 1 and 2 % (v/v) butanol. Tomas et al. (2003) reported that the growth inhibition by butanol of the *groESL* overexpressing strain was up to 85 % less than the vector control. This value is not directly comparable to the results presented here, because higher butanol concentrations and not the growth rates of challenged cultures, but survival rates according to c.f.u. (c.f.u.) counts after the butanol exposure were preferentially chosen as parameters. However, the highly improved butanol tolerance of *C. acetobutylicum* pT::*groESL* is in good agreement. Regarding the novel strains *C. acetobutylicum* pT::*grpE* and *C. acetobutylicum* pT::*htpG*, significantly increased butanol resistance can also be mediated by values of the stress proteins GrpE and HtpG. Whereas the wild type and

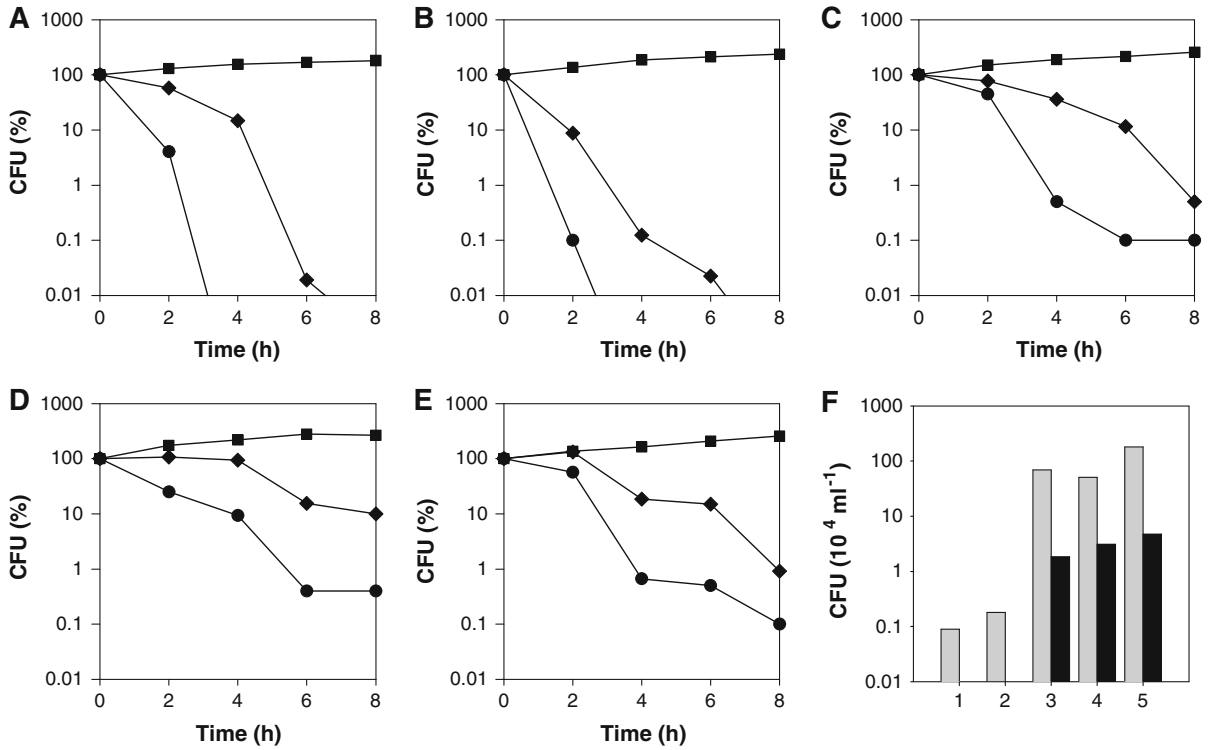

Fig. 1 Increased butanol tolerance of *C. acetobutylicum* pT::*groESL* (**c**), pT::*grpE* (**d**) and pT::*htpG* (**e**) in comparison to the wild type (**a**) and vector control (**b**). The respective strains were subjected to 1 % (v/v) (*diamonds*) and 2 % (v/v) (*circles*) *n*-butanol for 8 h. Samples were drawn every 2 h and serial dilutions were plated onto RCA to determine the c.f.u. with respect to the controls without butanol (*squares*). The relative c.f.u. values are shown in percent of the initial sample (**a–e**). Panel (**f**) depicts the total c.f.u. counts of the 6 h samples of 1 % (v/v) (*grey*) and 2 % (v/v) (*black*) butanol: *1 C. acetobutylicum* wild type, *2* vector control, *3* pT::*groESL*, *4* pT::*grpE*, *5* pT::*htpG*

the vector control had c.f.u. < 0.1 % after 6 h exposure to 1 % (v/v) butanol, referring to the initial c.f.u., the over-expressing strains still showed 12 % (*groESL*), 16 % (*grpE*) and 15 % (*htpG*), respectively, of the initial c.f.u.. In addition, the control strains did not survive 2 % (v/v) butanol for more than 2 h, but the three recombinant strains revealed 45 % (*groESL*), 25 % (*grpE*) and 56 % (*htpG*), respectively, of the initial c.f.u. after 2 h at 2 % (v/v) butanol (Fig. 1).

Butanol production

Since genetic tools for clostridia became available only recently, traditional production strain-breeding was common (and quite successful) for a long time, using random mutagenesis and selection by survival of high butanol concentrations (Jones and Woods 1986; Papoutsakis 2008; Lütke-Eversloh and Bahl 2011). The phenotype of such production strains implements usually both enhanced butanol tolerance and production, justifying the relation between both features. This connection was confirmed by the *groESL* overexpressing *C. acetobutylicum*, which was reported to produce 40 % more butanol than the wild type (Tomas et al. 2003; Alsaker et al. 2010). Using mineral salts medium instead of CGM, *C. acetobutylicum* pT::*groESL* produced 130 % of the wild type butanol titer, validating the fact that higher GroES and GroEL protein levels improves solvent production.

Surprisingly, *grpE* and *htpG* over-expression did not enhanced butanol production, only 51 % (*grpE*) and 67 % (*htpG*) of the wild type butanol concentrations were detected in the culture supernatants (Table 1). Fermenation experiments in MS-MES medium further revealed clearly hampered growth of *C. acetobutylicum* pT::*grpE*, whereas the growth rates of *C. acetobutylicum* pT::*groESL* and *C. acetobutylicum* pT::*htpG* were

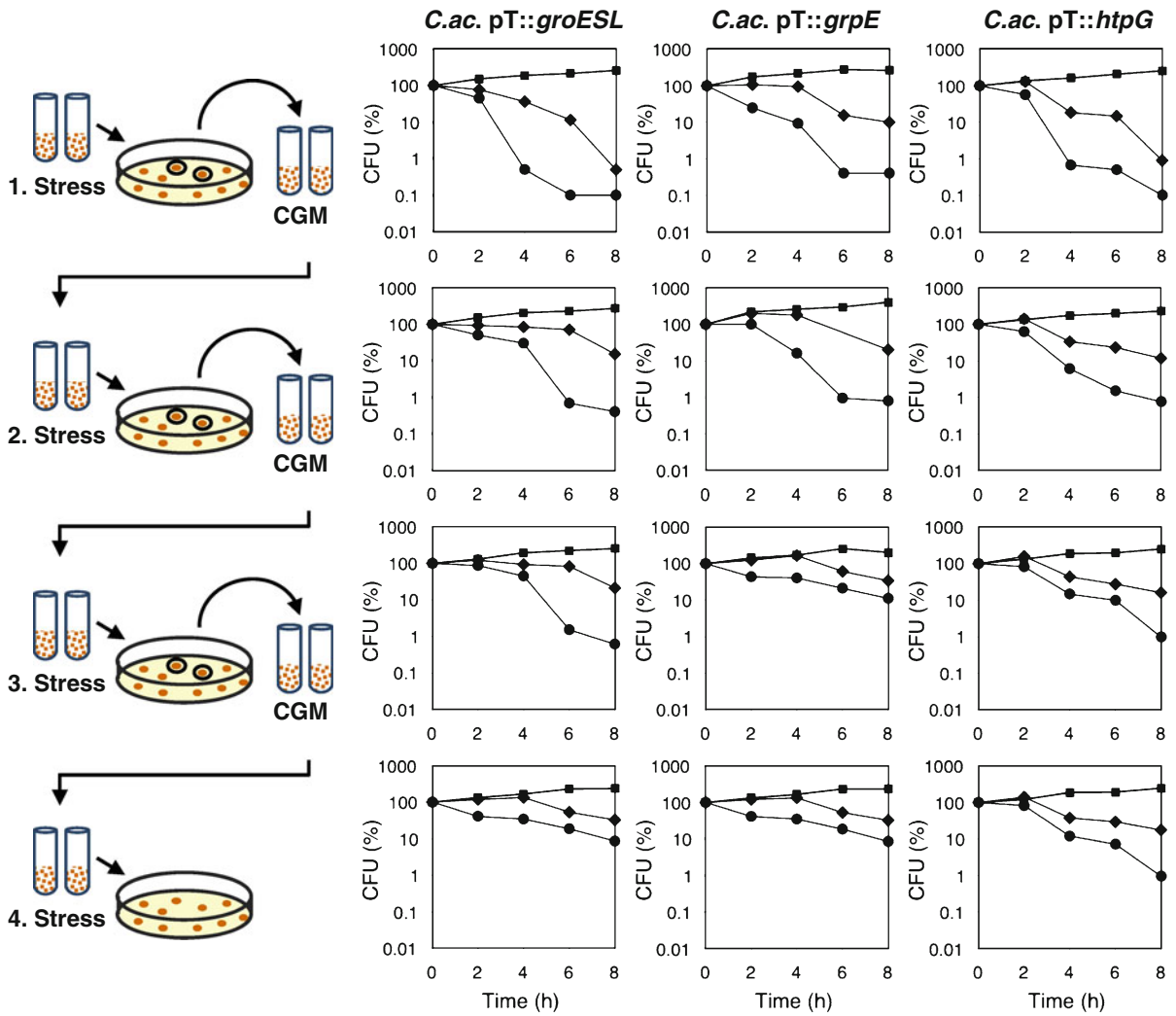

Fig. 2 Butanol stress adaptation of *C. acetobutylicum* pT::*groESL*, pT::*grpE* and pT::*htpG*. The experimental scheme is shown on the left: after each butanol stress experiment, two colonies of cultures exposed to 2 % (v/v) butanol for 6 h were used to inoculate fresh CGM, grown overnight and subjected again to 1 % (v/v) (*diamonds*) and 2 % (v/v) (*circles*) *n*-butanol for 8 h in comparison to the control without butanol (*squares*). The c.f.u. (c.f.u.) were determined as described in Fig. 1, the average data of two similar replicates each are shown

only moderately reduced as compared to the wild type and the vector control (Table 1).

Butanol adaptation

As depicted in Fig. 1, we observed an unexpected phenomenon in the repeatedly conducted butanol stress experiments: in contrast to the control strains, i. e. wild type and vector control, the stress protein overexpressing *C. acetobutylicum* strains harboring plasmids pT::*groESL*, pT::*grpE*, or pT::*htpG*, respectively, always revealed several colonies on the c.f.u. counting plates after exposure to high butanol concentrations (Fig. 1). This led to the hypothesis that the HSP overexpressing strains must have a mechanism to adapt to butanol stress more efficiently, and the question was whether the plasmids mutated or the whole cells evolved, enabled by improved tolerance mechanisms as well as a simple selection pressure. Therefore, consecutive butanol stress experiments were conducted and are summarized in Figs. 2 and 3. Plasmids were isolated after each stress experiment and

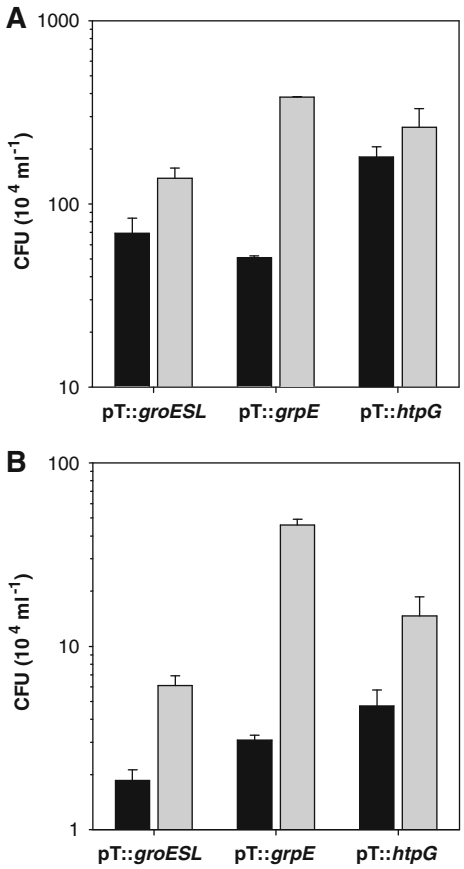

Fig. 3 Improved survival rates of *C. acetobutylicum* pT::groESL, pT::grpE and pT::htpG after butanol exposure. The strains were exposed to 2 % (v/v) butanol for 6 h and spread on RCA plates (*grey*), the respective controls (*black*) were treated similarly without butanol exposure prior to plating. From each strain, two randomly-picked colonies were used for a subsequent stress experiment employing 6 h of incubation in the presence of 1 % (v/v) (**a**) and 2 % (v/v) (**b**) butanol. The c.f.u. were determined from serial dilutions, average c.f.u. values from two independent experiments are shown

sequence analyses did not show any amino acid exchange of the respective stress proteins. Moreover, re-transformation of the isolated plasmids into *C. acetobutylicum* ATCC 824 did not alter the original phenotype (data not shown). Following the successive challenges at 2 % (v/v) butanol for 6 h, improved butanol resistance was noticed for all over-expression strains subsequently monitored for their resistance to both 1 and 2 % (v/v) butanol concentrations (Fig. 2). The absolute c.f.u. counts for the over-expression strains are shown in Fig. 3, exhibiting a better performance for all three overexpession strains, but particularly for *C. acetobutylicum* pT::*grpE*. The difference between butanol-stressed cells and the control cultures clearly demonstrated the positive influence of increased HSP abundance for enhanced butanol tolerance and adaptation.

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