Corynebacterium glutamicum σ^E is involved in responses to cell surface stresses and its activity is controlled by the anti- σ factor CseE

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In this study, we demonstrate that σ^E , an alternative σ factor of *Corynebacterium glutamicum*, is involved in cell surface stresses. Cells in which the *sigE* gene was deleted evidenced increased sensitivity to magnesium deficiency, as well as to SDS, lysozymes, EDTA and heat. We utilized physiological analyses to show that the downstream gene, designated *cseE*, encodes an anti- σ factor. The retarded growth of the *cseE* mutant cells under ordinary growth conditions could be recovered by an additional deletion of *sigE* encoding σ^E . Under stress conditions, the phenotype of the *cseE*-overexpressing cells mimicked that of the *sigE* mutant. The *sigE* and *cseE* genes were transcribed into a single transcript, and gene transcription was stimulated by heat. The SigE and CseE proteins interacted physically *in vitro*, in the form of glutathione S-transferase (GST) and maltose binding protein (MBP) fusion proteins, respectively. 2D-PAGE analysis of the wild-type and mutant crude extracts showed that the *sigE* mutant failed to synthesize a 34 kDa polypeptide that was normally induced in wild-type cells grown under heat (or SDS)-stressed conditions. The protein turned out to be expressed from ORF NCgl1070 and showed similarity to methyltransferases which may confer resistance to antibiotics. Accordingly, the *sigE* mutant evidenced extreme sensitivity to antibiotics, including nalidixic acid, penicillin and vancomycin. Finally, we present a discussion of the possible role of the *sigE* and *cseE* genes in the acclimation of *C. glutamicum* to cell surface stress conditions.

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INTRODUCTION

Corynebacterium glutamicum is a Gram-positive organism, and belongs to the order *Actinomycetales*, which also includes the genera *Mycobacterium* and *Streptomyces*. *C. glutamicum* is a remarkable organism, and has been shown to be capable of producing a variety of amino acids and nucleotides in large quantities. Due to the industrial importance of this organism, its genetic and biochemical features have been extensively characterized. Moreover, the

recent availability of genome sequence data (Ikeda & Nakagawa, 2003; Kalinowski *et al.*, 2003) has made it possible to characterize the genetic background of the organism in a new dimension.

The correct regulation of gene expression in a given organism is crucial for adaptation and survival under a variety of growth conditions. This is achieved, in part, by the reversible association of different σ factors with bacterial RNA polymerase to modulate the transcription of the target genes (Browning & Busby, 2004; Gruber & Gross, 2003). Bacterial genomes may encode a principal σ factor which is dedicated to the transcription of house-keeping genes, and may also harbour a variety of alternative σ factors which participate in the coordination of gene expression during a variety of stress responses as well as morphological development. The extracytoplasmic

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Abbreviations: ECF, extracytoplasmic function; ESI-MS, electrospray ionization MS; GST, glutathione S-transferase; MBP, maltose binding protein; SAM-MT, S-adenosylmethionine methyltransferase.

function (ECF) σ factors constitute a class of alternative σ factors (Helmann, 2002), and the genes that encode these ECF σ factors have been identified in a variety of organisms, although the roles and mechanisms inherent to regulation are only now coming to light. At least 10 putative genes that encode ECF σ factors have been identified within the genome of *Mycobacterium tuberculosis* (Rodrigue *et al.*, 2006), and 51 genes in *Streptomyces coelicolor* (Hahn *et al.*, 2003; Lee *et al.*, 2005).

In the *C. glutamicum* genome, a total of seven putative σ factor genes have been detected (Brinkrolf *et al.*, 2007; Engels *et al.*, 2004; Kalinowski *et al.*, 2003). The *sigA* gene encodes the essential primary σ factor of *C. glutamicum* and is also responsible for the promoter recognition of housekeeping genes (Halgasova *et al.*, 2001; Oguiza *et al.*, 1996). The alternative σ factor SigB replaces SigA during transition from exponential growth to stationary phase, and modulates the expression of a number of genes (Larisch *et al.*, 2007). In addition to *sigA* and *sigB*, the annotation of the genome sequence of *C. glutamicum* ATCC 13032 has revealed five ECF σ factor genes, *sigC*, *sigD*, *sigE*, *sigH* and *sigM* (Engels *et al.*, 2004; Kim *et al.*, 2005; Nakunst *et al.*, 2007), and several anti- σ factor genes (Pátek, 2005). Although σ factors have been shown to regulate gene transcription profiles in a simple mechanism involving the modulation of σ factor levels, the activity of certain σ factors can be regulated further by antagonistic proteins. Anti- σ factors interact directly with specific σ factors. The sequestration of σ factors blocks their association to RNA polymerases, thus preventing the transcription of target genes until an appropriate environmental stimulus is applied to the bacterium.

Among the ECF σ factor genes, only *sigH* and *sigM* have been studied to some extent thus far. The σ factors are involved in responses to thiol-oxidative stress and constitute a hierarchical regulatory network (Kim *et al.*, 2005; Nakunst *et al.*, 2007). In this study, we examined the *sigE* gene in detail and generated evidence to suggest that the *sigE* gene of *C. glutamicum* is involved in surface stress responses. In addition, we present evidence that SigE interacts with its cognate anti- σ factor, which is referred to as CseE. This is, to the best of our knowledge, the first report in *C. glutamicum* regarding the role of *sigE* as well as the presence and interaction of an anti- σ factor with its cognate σ factor.

METHODS

Growth conditions. All strains, plasmids, and primers employed in this study are listed in Table 1. *Escherichia coli* DH5 α F' was utilized for the construction and propagation of plasmids. *E. coli* BL21 DE3 was employed for the expression of *sigE* and *cseE* cloned into pMAL-c2 and pGEX-4T-3, respectively. Unless otherwise stated, *E. coli* and *C. glutamicum* cells were cultured at 37 °C in Luria broth (Sambrook & Russell, 2001) and at 30 °C in MB medium (Follettie *et al.*, 1993), respectively. Minimal media for *C. glutamicum* were based on MCGC (von der Osten *et al.*, 1989). Carbon source glucose was added to the minimal medium at a concentration of 1% (w/v). Antibiotics were

added at the following concentrations: 50 μ g ampicillin ml⁻¹; 20 μ g chloramphenicol ml⁻¹; and 20 μ g kanamycin ml⁻¹. X-Gal was added to the media at a concentration of 40 μ g ml⁻¹. For protein expression, IPTG was added to a final concentration of 1 mM.

DNA analysis. Standard molecular cloning, transformation and electrophoresis procedures were employed (Sambrook & Russell, 2001). Plasmids were introduced into *C. glutamicum* cells via electroporation (Follettie *et al.*, 1993), and mini-plasmid preparation for *C. glutamicum* cells was conducted as described by Yoshihama *et al.* (1985). PCR was carried out as previously described (Kim *et al.*, 2005).

Plasmid construction and gene inactivation. The pSL419 and pSL420 plasmids were constructed via the crossover PCR method (Hwang *et al.*, 2002; Link *et al.*, 1997). The primers utilized herein are listed in Table 1. The primary PCR products, AB and CD, were amplified with 600 nM of outer primers and 60 nM of inner primers. These products were then directly utilized as templates for secondary PCR with 600 nM of outer primers. The secondary PCR products were ligated into the *Bam*HI site of pK19*mobsacB*. The fragment was then introduced into *Sall*- and *Sma*I-digested pK19*mobsacB*, and the resultant plasmid was introduced into *C. glutamicum* AS019E12 in order to delete chromosomal *sigE* and/or *cseE*. Subsequent steps were conducted as described previously (Hwang *et al.*, 2002; Schäfer *et al.*, 1994), and the chromosomal deletion of *sigE* and/or *cseE* in *C. glutamicum* AS019E12 was confirmed via PCR (data not shown). The pSL418 plasmid was constructed via the amplification of the *cseE* gene using the *cseE*-oe1 and *cseE*-oe2 primers, and by subsequently ligating the amplified DNA into the *Pst*I site of pSL360. The pSL421 plasmid was constructed via the amplification of the *sigE* gene using the GSTSigE1 and GSTsigE2 primers, and by subsequently ligating the amplified DNA with the *Bam*HI/*Pst*I-digested pGEX-4T-3 vector. The pSL422 plasmid was constructed by the same scheme with the exception of the primers, which were MALCseE1 and MALCseE2.

RNA analysis. RNA was purified with TRIzol reagent (Invitrogen), followed by the RNeasy Mini kit (Qiagen) and RNase-Free DNase set (Qiagen). RT-PCR analysis for the *sigE* and *cseE* genes was conducted using the oligonucleotides listed in Table 1. First-strand cDNA was synthesized with the SuperScript III kit (Invitrogen) in accordance with the manufacturer's instructions. A 1 μ l aliquot of a first-strand cDNA reaction mixture was employed as a PCR template. Each of the 20 cycles was conducted for 20 s at 95 °C, 30 s at 58 °C, and 45 s at 72 °C. The reaction products were then electrophoretically separated on agarose gel and visualized upon ethidium bromide staining.

Protein purification and analysis. The glutathione S-transferase (GST)-SigE and maltose binding protein (MBP)-CseE fusion proteins were expressed and purified with the pGEX-4T-3 and pMAL-c2 vectors, respectively, in accordance with the manufacturer's instructions. The cell extracts were prepared as described by Follettie *et al.* (1993). The purified proteins were divided into aliquots and maintained at -70 °C. Proteins were measured via the Bradford method, with BSA as the standard (Bradford, 1976). SDS-PAGE and Western blot analysis were conducted as described elsewhere (Laemmli, 1970). 2D-PAGE was conducted as follows. First, IEF was conducted on an Ettan IPGphor II system (GE Healthcare), in accordance with the manufacturer's instructions. Second-dimension analyses were conducted on Protean II vi cells (Bio-Rad). The proteins were visualized via silver staining using a kit from Bio-Rad. Peptide analysis of the protein spots was performed by a commercial service (In2gen) via electrospray ionization MS (ESI-MS).

Interaction of GST-SigE and MBP-CseE. Partially purified proteins (30 μ g each) were incubated for 1 h at 25 °C in binding

Table 1. Bacterial strains, plasmids and primers used in this study

Restriction sites added artificially are underlined.

Strain, plasmid or primer	Relevant characteristics	Reference or source
<i>E. coli</i>		
DH5 α F'	Φ 80 Δ <i>lacZ</i> Δ M15, <i>recA1</i> , <i>gyrA96</i> , <i>thi1</i> , <i>hsdR17</i> , <i>supE44</i> , <i>relA1</i> , <i>deoR</i> , Δ (<i>lacZYA-argF</i>) U169, λ^-	Bethesda Research Laboratory
BL21 DE3	<i>hsdS gal</i> (λ <i>cl ts857 ind1 Sam7 nin5 lacUV5-T7 gene 1</i>)	Novagen
HL1153	BL21 DE3/pSL421	This study
HL1154	BL21 DE3/pSL422	This study
<i>C. glutamicum</i>		
AS019E12	Restriction-deficient mutant of AS019	Follettie <i>et al.</i> (1993)
HL1150	<i>C. glutamicum</i> AS019E12 derivative, Δ <i>sigE</i>	This study
HL1151	<i>C. glutamicum</i> AS019E12 derivative, Δ <i>cseE</i>	This study
HL1152	<i>C. glutamicum</i> AS019E12 derivative, carrying pSL418, Km ^R , Cm ^R	This study
HL1155	<i>C. glutamicum</i> AS019E12 derivative, Δ <i>sigE</i> / Δ <i>cseE</i>	This study
Plasmids		
pK19 <i>mobsacB</i>	Gene disruption vector carrying the <i>sacB</i> selection system, Km ^R	Schäfer <i>et al.</i> (1994)
pMAL-c2	Gene expression vector carrying MBP	New England Biolabs
pGEX-4T-3	GST fusion vector carrying <i>lacI</i> ^l	GE Healthcare
pSL360	Overexpression vector carrying P ₁₈₀ promoter	Park <i>et al.</i> (2004)
pSL418	Overexpression of <i>cseE</i> , P ₁₈₀ - <i>cseE</i> , Km ^R and Cm ^R	This study
pSL419	Gene disruption vector, pK19 <i>mobsacB</i> / Δ <i>sigE</i>	This study
pSL420	Gene disruption vector, pK19 <i>mobsacB</i> / Δ <i>cseE</i>	This study
pSL421	SigE overexpression vector, pGEX-4T-3/ <i>sigE</i>	This study
pSL422	CseE overexpression vector, pMALc2/ <i>cseE</i>	This study
Primers		
sigE_del_A	5'-CGGGATCCGCGAGGTCAGTGTGGTGTATCG-3'	This study
sigE_del_B	5'-CCCATCCACTAAACTTAAACAATCTCGGGACTTCTTTTTCATATAA-3'	This study
sigE_del_C	5'-TGTTTAAGTTTAGTGGATGGGGAGGAAGTTTCTTTGTTGGTTCCA-3'	This study
sigE_del_D	5'-CGGGATCCGCTGAAAGGTGATCAACAGAGG-3'	This study
cseE_del_A	5'-CGGGATCCAGTTGAGCCCAGACTTCCGCG-3'	This study
cseE_del_B	5'-CCCATCCACTAAACTTAAACAGGTGTCGGAATTGAAATATCTTC-3'	This study
cseE_del_C	5'-TGTTTAAGTTTAGTGGATGGGGTAGTGC GCGCAGTTGTAAG-3'	This study
cseE_del_D	5'-CGGGATCCCTTCGCCTTCTGTTCTTCGGC-3'	This study
cseE-oe1	5'-GCCTGCAGATGTTCAATTCGACACCACC-3'	This study
cseE-oe2	5'-GCCTGCAGTTAGCGGCCTTGGTTCTTACG-3'	This study
GSTsigE1	5'-CGGGATCCATATGAAAAAGAAGTCCCGAGATGA-3'	This study
GSTsigE2	5'-CGGAATTCCTTAGTGGGTGGAACCAACAAAGA-3'	This study
MALCseE1	5'-CGGAATTCATGTTCAATTCGACACCACC-3'	This study
MALCseE2	5'-GCCTGCAGTTAGCGGCCTTGGTTCTTACG-3'	This study
SAM-MT-A	5'-CCTTCCTTTCTGGCGGTCACTTC-3'	This study
SAM-MT-B	5'-GGTGGCCTGCCGCTTGTTTC-3'	This study
16s-rDNA-F	5'-GCATAACTTGAGTGCTGTAGG-3'	This study
16s-rDNA-R	5'-GCTGGCAACATAAGACAAGG-3'	This study
Pull-sigE-F	5'-CGCATATGAAAAAGAAGTCCCGAGATGAC-3'	This study
Pull-cseE-R	5'-CGGAATTCCTTAGCGGCCTTGGTTCTTACG-3'	This study

buffer [50 mM Tris/HCl, 0.1 M NaCl, 1 mM DTT, 10 % (v/v) glycerol, 1 % (v/v) Triton X-100, pH 7.5] and loaded onto a Glutathione Sepharose 4B column (GE Healthcare). The column was washed with 10 volumes washing buffer [50 mM HEPES, 0.15 M NaCl, 0.1 % (v/v) Tween 20, pH 7.5]. Finally, the bound proteins were eluted with elution buffer (0.1 M Tris/HCl, 0.1 M NaCl, 20 mM glutathione, pH 8.0). Aliquots (20 μ l) were subjected to 12 % (v/v) SDS-PAGE. The protein bands were visualized by Coomassie Blue staining.

RESULTS

Growth properties of the Δ *sigE*, Δ *cseE*, and Δ *sigE*/ Δ *cseE* mutants

Engels *et al.* (2004) have reported that ORF NCgl1075 (cg1271) of *C. glutamicum* encodes an ECF σ factor, σ^E . The encoded SigE protein is composed of 213 aa, and

appears to be a member of the Group 4 proteins, which harbour the conserved regions 2 and 4 (Gruber & Gross, 2003). σ factors in Group 4, which are also often referred to as ECF σ factors, are often involved in response to stress conditions, including oxidative stress and surface stress (Alba & Gross, 2004). The amino acid sequence identities of the *C. glutamicum* SigE with those of the known SigE proteins from the closely related organisms *M. tuberculosis* and *S. coelicolor* were 63 and 42 %, respectively. Downstream of the *sigE* gene, the ORF NCgl1076 (cg1272), encoding a 164 aa polypeptide, was located. The ORF harboured an HX₃CX₂C motif that is found in many anti- σ factors (data not shown). In a variety of organisms, the *sigE* locus is conserved and the anti- σ factor gene is located immediately downstream of its cognate σ factor gene. Therefore, ORF NCgl1076 was designated *cseE* (control of *sigE*). Downstream of the *cseE* gene, the *tatB* gene encoding the twin arginine translocase protein was located.

In order to determine the possible functions of and relationships between SigE and CseE, the corresponding genes of *C. glutamicum* were deleted via gene disruption, as described in Methods. The constructed mutant strains were grown on minimal media, and the phenotypic changes of *C. glutamicum* mutants were assessed. As shown in Fig. 1, the growth of the Δ *sigE* mutant was comparable to that of the parental strain, evidencing a doubling time of ~3 h. This suggests that the gene plays a non-essential role under ordinary growth conditions. However, the Δ *cseE* mutant strain evidenced retarded growth, manifesting a doubling time of approximately 6 h. To our surprise, when we additionally deleted the *sigE* gene of the Δ *cseE* mutant strain, the growth rate of the constructed Δ *sigE*/ Δ *cseE*

double mutant strain recovered to almost the parental level (Fig. 1). This indicates an antagonistic relationship between *cseE* and *sigE*, and suggests a possible interaction of the *sigE* and *cseE* gene products in the cell as a σ factor and its cognate anti- σ factor (see below).

Effect of cell surface stresses on the growth of Δ *sigE*, Δ *cseE* and Δ *sigE*/ Δ *cseE* mutants

As the ECF σ factors are known to be involved in responses to environmental stress conditions, we challenged the parental strain and the constructed mutants with a variety of environmental stresses and compared the growth properties. As the *sigE* genes of other organisms are known to be involved in cell surface stress responses, we initially administered heat challenge to the constructed mutants. In the case of the parental AS019E12 strain, 77 and 72 % cells survived after 3 and 5 h of heat treatment at 42 °C (Fig. 2), respectively. However, in the case of the Δ *sigE* mutant, only 53 and 42 % of the cells survived after the same treatment. Mutants harbouring the deleted *cseE* gene evidenced growth comparable to that of the parental strain, with 76 % survival after 3 h. In the case of the Δ *sigE*/ Δ *cseE* double mutant strain, the survival rate was 52 %. Surprisingly, when the *cseE* gene was overexpressed via the introduction of pSL418 into the wild-type strain, the sensitivity of the recombinant cells to heat increased to levels comparable to those observed with the Δ *sigE* mutant strain. Comparable results were observed when the cells were challenged with anionic detergent, such as 0.01 % SDS (Table 2). No increased sensitivities of the mutants were observed in conjunction with other types of stress, including diamide oxidation stress, NaCl or KCl for salt stress, and pH stress (data not shown). These data show that the *sigE* gene is involved in responses to detergents and heat, and that the *cseE* gene likely antagonizes the activity of *sigE*.

Assuming that heat and detergents affect cell surface integrity, we conducted further analyses of the mutants with other agents that affect cell surface structures. In a magnesium-depleted medium, the Δ *sigE* and Δ *sigE*/ Δ *cseE* mutants evidenced only 20 % of the growth seen in the parental strain (Table 2). The addition of 4 mM EDTA to the ordinary minimal medium also resulted in a retardation of the growth of the Δ *sigE* mutant strain, but not the parental strain (Table 2). We also evaluated the effects of lysozymes on the growth of the mutant cells. As shown in Table 2, the growth of the Δ *sigE* and Δ *sigE*/ Δ *cseE* mutants was substantially affected by $<0.5 \mu\text{g lysozyme ml}^{-1}$, whereas the growth of the parental strain was marginally affected. For an unknown reason, the Δ *cseE* mutant strain survived to a greater degree than the parental strain under these conditions. Similar survival patterns were observed when the cells were challenged with the cell wall lytic enzyme mutanolysin (data not shown). Consequently, these data show not only the involvement of the *sigE* gene in the maintenance of cell envelope integrity, but also the role of *cseE* in antagonizing the action of the *sigE* gene.

Fig. 1. Growth of *C. glutamicum* AS019E12 wild-type, Δ *sigE*, Δ *cseE* and Δ *sigE*/ Δ *cseE* mutants in MCGC minimal medium with 1 % (w/v) glucose as a carbon source. Symbols: ●, wild-type; □, Δ *sigE*; △, Δ *cseE*; ○, Δ *sigE*/ Δ *cseE* double mutant. Mean values of three independent experiments are shown.

Fig. 2. Effects of heat stress on *C. glutamicum* AS019E12 wild-type, $\Delta sigE$, $\Delta cseE$ and $\Delta sigE/\Delta cseE$ mutants. Cells harbouring the appropriate deletions were grown to late-exponential phase in MCGC minimal medium, heat-stressed at 42 °C for 3 or 5 h, and plated on MB media to determine c.f.u. c.f.u. measured immediately before the heat stress were arbitrarily set as 100 %. Symbols: W, wild-type; S, $\Delta sigE$; C, $\Delta cseE$; D, $\Delta sigE/\Delta cseE$ double mutant; O, *C. glutamicum* AS019E12 carrying pSL418, which overexpresses CseE. Error bars indicate SD from at least three independent measurements.

Expression of the *sigE* and *cseE* genes

Knowing the interaction of the *sigE* and *cseE* genes, we assessed the possible organization of the genes in an operon. As expected, a single transcript of 1325 bp was detected in an RT-PCR experiment (Fig. 3), thereby indicating that the two genes are transcribed in a single transcription unit. Within the normal cellular propagation period, the *sigE-cseE* transcripts were expressed only marginally in the wild-type strain. However, upon heat treatment, the quantity of the *sigE-cseE* transcripts was increased significantly.

Interaction of SigE and CseE *in vitro*

Thus far, the data we obtained using the mutant cells indicated that the SigE and CseE proteins might interact with one another as a σ factor and its cognate anti- σ factor, respectively. As shown in Fig. 1, under ordinary growth

conditions, the deletion of the *cseE* gene induced a retardation of cellular growth, and the phenotype was recovered via the introduction of an additional *sigE* deletion. Furthermore, under stress conditions, cells that overexpressed the *cseE* gene mimicked the phenotype of the *sigE* mutant strain, thereby suggesting the possible titration of SigE by CseE. In order to analyse directly a possible interaction of the two proteins, we partially purified the proteins as MBP (42 kDa) or GST (27 kDa) fusion proteins, and then analysed the protein–protein interactions *in vitro*. As is shown in Fig. 4 (lanes 3 and 4), the two proteins of GST–SigE (27 kDa + 24 kDa = 51 kDa) and MBP–CseE (42 kDa + 17 kDa = 59 kDa) formed protein–protein complexes, as is evidenced by the coelution of the two proteins. The presence or absence of DTT or diamide had no effect on the interaction (data not shown). These data indicate that the antagonistic activity of CseE against SigE (Fig. 2, Table 2) is exerted via protein–protein interaction. It also indicates that the $\Delta sigE$ -mimicking

Table 2. Effects of magnesium deficiency and various cell envelope stresses on the growth of *C. glutamicum* cells

ND, Not determined.

<i>C. glutamicum</i> strain	Phenotype	Stress			
		Mg deficiency*†	SDS (0.01 %)†	EDTA (4 mM)†	Lysozyme concentration (µg ml ⁻¹)‡
AS019E12	Wild-type	100 ± 10	100 ± 10	100 ± 9	0.5
HL1150	<i>sigE</i> mutant	20 ± 9	63 ± 9	37 ± 5	<0.5
HL1151	<i>cseE</i> mutant	90 ± 10	110 ± 20	100 ± 10	5
HL1152	CseE overexpression	20 ± 4	68 ± 10	ND	ND
HL1155	<i>sigE</i> , <i>cseE</i> mutant	20 ± 6	52 ± 10	36 ± 7	<0.5

*The cells were grown for 40 h in magnesium-depleted MCGC media. Mean ± SD of three independent experiments is shown.

†Relative growth (%). The values shown are means ± SD of at least three independent experiments.

‡A tenfold dilution series of lysozyme was applied to MCGC plates spread with a lawn of the indicated *C. glutamicum* cells. The means of three independent experiments are shown. The plates were incubated for 20 h at 30 °C prior to the measurement of growth-inhibitory zones.

Fig. 3. Detection of *sigE-cseE* transcripts by RT-PCR. *C. glutamicum* AS019E12 cells were grown in MCGC media to late-exponential phase and subjected to heat stress at 42 °C (Heat) for 3 h. Subsequently, total RNA isolated from the *C. glutamicum* cells was subjected to RT-PCR analysis using oligonucleotides specific for 16S rDNA or *sigE-cseE*. RT-PCR was performed at least three times with similar results.

phenotype of the *cseE*-overexpressing cells was induced by the titration of SigE by CseE proteins.

Identification of a *sigE*-dependent gene

Knowing that the *sigE* and *cseE* genes likely function as a σ factor and its cognate anti- σ factor, we attempted to identify genes under the control of SigE via analyses of the total cell proteins using 2D gel electrophoresis. We searched for proteins present in the wild-type cells but not in the Δ *sigE* mutant strain, after treating cells with heat or SDS. After careful scrutiny of the silver-stained gels, we identified a protein spot consistent with our guidelines. As shown in Fig. 5(a), the protein with a pI of ~5.4 and M_r of 34 000 was detected in the heat- or SDS-treated wild-type cells but not in the Δ *sigE* mutant, regardless of heat or SDS treatment. In accordance with our expectations, the protein was not detected in a *cseE*-overexpressing strain (data not shown). The amino acid sequences of the internal peptides of the protein (QGYVTLGGAGLR, VGAVIADAWAR, DLVEFEMLLDQK), which were obtained via ESI-MS, matched that of the ORF

NCgl1070-encoded protein of *C. glutamicum* ATCC 13032. The ORF (also referred to as cg1266) encoded a protein with an estimated molecular mass of 30 805 Da and a pI of 5.27, which are quite consistent with our 2D-PAGE data. In addition, the encoded protein showed similarity to a putative methyltransferase of *Corynebacterium jeikeium* (YP 251177), a putative mycinamicin-resistant protein of *Corynebacterium efficiens* (NP 737782) and a putative macrolide-resistance protein of *Corynebacterium diphtheriae* (NP 939350). Interestingly, the ORF was located in the close vicinity of the *sigE* and *cseE* loci (data not shown). We also analysed the expression profiles of NCgl1070 by monitoring the mRNA level. Consistent with the 2D-PAGE data, the mRNA of the ORF NCgl1070 was only detected in the heat-stressed wild-type cells (Fig. 5b). We also identified several other protein spots which appeared to be under the control of *sigE*. However, these proteins were not inducible by heat or SDS; therefore, the proteins were excluded from the present study (data not shown).

Increased sensitivity of the Δ *sigE* mutant to antibiotics

Methyltransferases often confer resistance to antibiotics, either by modifying the cell wall permeability (Boissier *et al.*, 2006; Dubnau *et al.*, 2000) or by methylating the ribosomal content (Wachino *et al.*, 2006). Knowing that the ORF NCgl1070, which may encode methyltransferases, is under the control of *sigE*, we assessed possible sensitivity of the Δ *sigE* mutants to various antibiotics. As shown in Fig. 6, in the presence of antibiotics such as nalidixic acid, penicillin and vancomycin, the Δ *sigE* mutants evidenced poor growth compared to the wild-type strain, whereas the growth of the Δ *cseE* mutant strain was affected only marginally, considering the intrinsic slow growth of the Δ *cseE* mutant strain. In addition, compared to the wild-type strain, the Δ *sigE* mutant showed increased sensitivity to antibiotics such as tetracycline, ampicillin, chloramphenicol and ethambutol (data not shown). These data may indicate that the increased sensitivity of the *sigE* mutant cells to such antibiotics with different roles is attributable to the increased permeability of the cell envelope.

Fig. 4. Interaction of SigE and CseE *in vitro*. The physical interaction of SigE with CseE was monitored by affinity chromatography. GST-tagged SigE and MBP-tagged CseE were mixed, incubated, and applied to a Glutathione Sepharose 4B column. The bound proteins were eluted with reduced glutathione. The proteins were visualized via Coomassie staining and marked by size markers. Lanes: 1, partially purified GST-SigE (51 kDa) and MBP-CseE (59 kDa); 2, size markers; 3, wash fraction; 4–5, column eluents.

Fig. 5. Identification of a protein by (a) 2D-PAGE and (b) transcription of the corresponding gene by RT-PCR. *C. glutamicum* AS019E12 or $\Delta sigE$ mutants were grown in MCGC media to late-exponential phase and subjected to heat stress for 3 h at 42 °C. The cell extracts were prepared and subjected to 2D-PAGE analysis. Proteins were visualized via silver staining. For RT-PCR analysis, cells were cultivated as described above. RT-PCR was performed as described in Methods. All experiments were repeated three times with independent extracts and representative results are shown.

DISCUSSION

In this work, we have presented evidence that the *sigE* gene of *C. glutamicum* is involved in responses to cell surface stresses, including heat, detergents and antibiotics. In this regard, the σ^E of *C. glutamicum* evidences functional similarity to that of *M. tuberculosis*, the *sigE* of which is probably induced after exposure to heat shock, the detergent SDS, and vancomycin (Rodrigue *et al.*, 2006). In this bacterium, the disruption of the *sigE* gene results in an increased sensitivity of the cells to those agents. In *S.*

coelicolor, SigE is also required for normal cell integrity, although an anti- σ factor is not involved in controlling the activity (Hutchings *et al.*, 2006; Paget *et al.*, 2001). The sensitivity of the *C. glutamicum* $\Delta sigE$ mutant cells to antibiotics and detergents may be attributable to the absence of methyltransferases, the expression of which is controlled by *sigE*, as shown in this study. In accordance with our assumption, in the related species *M. tuberculosis*, the S-adenosylmethionine methyltransferase (SAM-MT) protein has been demonstrated to catalyse key chemical modifications in defined positions of mycolic acids, thereby modifying the permeability of the cell envelope and conferring increased resistance to environmental agents, including antibiotics (Boissier *et al.*, 2006; Dubnau *et al.*, 2000). Ikeda and Nakagawa (2003) and Kalinowski *et al.* (2003) annotated NCgl1070 (cg1266) as encoding SAM-MT and rRNA guanine-N₁-methyltransferase, respectively. Since the *sigE* mutant strain showed sensitivity to antibiotics that not only affect cell wall integrity but also inhibit cellular translation processes, it appears more likely that cg1266 encodes a methyltransferase, such as SAM-MT, that affects cellular permeability. The location of cg1266, in close proximity to the *sigE/cseE* locus, also supports this notion. In addition, other genes clustered within this region appear to be involved in cell envelope biosynthesis.

As shown in this study, the σ^E of *C. glutamicum* appears to play roles distinctly different from those of σ^H . The σ^H and σ^M of *C. glutamicum* are involved in oxidative and heat-stress responses (Engels *et al.*, 2004; Kim *et al.*, 2005; Nakunst *et al.*, 2007), whereas σ^E is involved in cell envelope stresses. In this regard, the sensitivity of the $\Delta sigE$ mutants to heat may be attributed to increased membrane fluidity, thus allowing the entry of harmful extracellular substances. In addition, although SDS generally causes damage to membrane structures, it has also been suggested that SDS induces lipid peroxidation and the induction of

Fig. 6. Effects of antibiotics on the growth of *C. glutamicum* AS019E12 wild-type, $\Delta sigE$ and $\Delta cseE$ mutants. The cells were cultivated in MB for 20–24 h in the presence of 100 μ g nalidixic acid ml^{-1} , 0.2 μ g penicillin ml^{-1} or 0.2 μ g vancomycin ml^{-1} . Error bars indicate SD from at least three independent measurements.

the oxidative stress response, thus inducing genes associated with thioredoxin, glutathione and glutaredoxin (Nickerson & Aspedon, 1992; Singer & Tjeerdema, 1993; Sirisattha *et al.*, 2004). However, this does not appear to be the case for the *sigE* gene of *C. glutamicum*, as the *C. glutamicum sigE* mutant did not evidence sensitivity to thiol-oxidative agents, including diamide. In *C. glutamicum*, the *sigH* and *sigM* genes participate in responses to oxidative stress (Kim *et al.*, 2005; Nakunst *et al.*, 2007).

The SigE protein of *C. glutamicum* is likely antagonized by its cognate anti- σ factor CseE. In this study, we have presented evidence that the *cseE* gene product of *C. glutamicum* functions as an anti- σ factor for σ^E . This was demonstrated by the following: (1) the *cseE* mutant phenotype could be recovered by the introduction of an additional deletion of *sigE*; (2) the *cseE*-overexpressing cells evidenced a $\Delta sigE$ mutant phenotype under stress conditions; (3) the SigE and CseE proteins interacted physically *in vitro*; and (4) there was physical linkage of the genes, which were expressed as a single transcription unit. The basis of the anti- σ factor inhibition of the function of the σ factor is the reversible protein-protein interaction of the anti- σ factor with its cognate σ factor (Duncan & Losick, 1993; Hughes & Mathee, 1998). CseE of *C. glutamicum*, a protein of 164 aa, harbours four conserved cysteine residues, all of which might be essential for CseE to inhibit the activity of SigE. As suggested for *S. coelicolor* (Bae *et al.*, 2004; Kang *et al.*, 1999; Paget *et al.*, 2001) and *M. tuberculosis* (Rodrigue *et al.*, 2006; Song *et al.*, 2003), the cysteine may perform important functions in controlling the activity of CseE. In *S. coelicolor*, the cysteine residues of the RsrA protein, an anti- σ factor for SigR, are coordinated under reducing conditions by zinc. Upon oxidation, disulfide bonds are formed among the cysteine residues to induce conformational changes in the protein, thus releasing the σ factor. In this bacterium, the HCC motif contributes to zinc coordination. It will be interesting to determine whether or not the CseE protein of *C. glutamicum*, which also harbours the HCC motif, also participates in the coordination of zinc. Furthermore, the signal and the potential effectors involved in the release of SigE by CseE are currently unknown, and remain to be elucidated.

C. glutamicum evidences intrinsic resistance to certain antibiotics, including nalidixic acid. The presence of long-chain α -alkyl, β -hydroxy fatty acids, the so-called mycolic acids, in the cell wall contributes significantly to the permeability of the cell wall (Daffé, 2005; Liu *et al.*, 1996). The $\Delta sigE$ mutant strain evidenced elevated sensitivity to a variety of antibiotics, including nalidixic acid, penicillin and vancomycin. Penicillin inhibits the transpeptidation reaction by binding to the transpeptidase enzymes involved in peptidoglycan biosynthesis, and vancomycin inhibits transpeptidation by binding to the terminal D-Ala-D-Ala residues of uncross-linked peptides present in the peptidoglycan (Hubbard & Walsh, 2003). In addition, the *sigE* mutant cells were more sensitive to lysozymes, which attack

the peptidoglycan layer. These data may indicate the disruption of the peptidoglycan sacculus in the *sigE* mutant strain, thus indicating a role for the *sigE* gene in both peptidoglycan biosynthesis and osmoprotection. However, we were able to dismiss the possibility of an osmoprotective role for *sigE*, as the growth properties of the *sigE* mutant cells were indistinguishable from those of the parental strain when exposed to high-salt conditions (data not shown). Considering that the mutant cells were also sensitive to nalidixic acid, which affects DNA synthesis via the inhibition of DNA gyrase, a topoisomerase that induces the negative supercoiling of DNA (Cozzarelli, 1980; Gellert, 1981), we can conclude that the increased sensitivity of the *sigE* mutant cells to such antibiotics with different roles appears to be attributable to the increased permeability of the cell envelope, thus defining the role of *sigE*-controlled genes in the synthesis of cell surface structures to protect cells against cell surface stresses.

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