The *Bacillus subtilis* *clpC* operon encodes DNA repair and competence proteins

Elke Krüger,¹ Tarek Msadek,² Steffen Ohlmeier¹ and Michael Hecker¹

Author for correspondence: Michael Hecker. Tel: +49 3834 864200. Fax: 3834 864202.
e-mail: hecker@microbio7.biologie.uni-greifswald.de

¹ Institut für Mikrobiologie und Molekularbiologie, Ernst-Moritz-Arndt-Universität, D-17487 Greifswald, Germany

² Unité de Biochimie Microbienne, Unité de Recherche Associée 1300 du Centre National de la Recherche Scientifique, Institut Pasteur, 25 Rue du Dr Roux, 75724 Paris Cedex 15, France

ClpC of *Bacillus subtilis*, controlling competence gene expression and survival under stress conditions, is encoded by the fourth gene of a six-gene operon. The product of *orf1* contains a potential helix–turn–helix motif, but shows no significant similarities with known protein sequences. The second and third genes encode proteins with similarities to zinc-finger proteins (*orf2*) and arginine kinases (*orf3*), respectively. The product of *orf5* contains a zinc-finger motif and an ATP-binding domain, and is highly similar to the product of the *Escherichia coli* *sms* gene. A strain bearing a disruption of *orf5* showed increased sensitivity to the alkylating agent methyl methanesulfonate. Furthermore, this mutant strain displayed decreased capacity for genetic recombination as measured by transformation experiments. The last open reading frame, *orf6*, encodes a protein with limited similarity in its C-terminal part to the *B. subtilis* *comEA* gene product and to the UvrC DNA repair excinuclease. Inactivation of *orf5* resulted in strongly diminished transformation with all types of DNA. Mutations affecting either *orf5* or *orf6* resulted in strains with decreased resistance to UV-irradiation in the stationary phase, indicating that these proteins play a role in the development of a non-specific stationary-phase resistance to UV-irradiation. Moreover, these results suggest an involvement of both proteins in transformation and presumably in DNA repair.

Keywords: stress proteins, *Bacillus subtilis*, ClpC, DNA repair, competence

INTRODUCTION

For soil bacteria such as *Bacillus subtilis* the ability to survive unfavourable environmental conditions is crucial. In order to respond to stressful situations or starvation, the cell has developed an adaptive network including stress responses and post-exponential-phase phenomena such as the development of genetic competence, motility, synthesis of degradative enzymes and finally sporulation. These processes are controlled by a complex and incompletely understood regulatory network accompanied by interactions and overlaps between the components (Msadek *et al.*, 1995).

Genetic competence of *B. subtilis*, the ability to take up exogenous DNA molecules, is controlled by many regulatory genes, such as *spo0A*, *comP*, *comA*, *degU*, *comS*, *mecA*, *clpC/mecB* and *comK*. These genes are required for appropriate expression of the late com-

petence genes, encoding DNA-binding and -uptake enzymes (Dubnau *et al.*, 1994). Moreover, the SOS system is also part of the competence response, and is subject to a dual regulation: a damage-inducible pathway and a competence-dependent induction (Dubnau, 1991; Cheo *et al.*, 1993).

Synthesis of bacterial stress proteins is induced by a wide variety of factors including heat shock, ethanol stress, pH and osmolarity changes, UV-irradiation, hydrogen peroxide, DNA-damaging agents and starvation (Bukau, 1993; Hecker *et al.*, 1996). ClpC, a member of the stress-response-related Clp-ATPase family, is a pleiotropic regulator involved in stress tolerance and cell division (Krüger *et al.*, 1994). The ClpC protein was also identified as MecB, controlling competence and synthesis of degradative enzymes (Msadek *et al.*, 1994). Therefore, ClpC may provide links between the competence pathway and the stress response. Originally, the *mecA* and *mecB* mutations were isolated as allowing competence gene expression in complex media (Dubnau & Roggiani, 1990). Interaction of MecB with MecA

Abbreviation: MMS, methyl methanesulfonate.

negatively regulates the synthesis of ComK, a transcriptional activator required for the expression of late competence genes (Kong & Dubnau, 1994; Msadek *et al.*, 1994). Expression of the *clpC* gene, encoding the general stress protein GSP12, was previously shown to be induced by various stress conditions (Krüger *et al.*, 1994). The nucleotide sequence of a 180 kb region containing *clpC* was reported as part of the *B. subtilis* genome sequencing project, suggesting that *clpC* is the fourth gene of an operon containing six open reading frames (Ogasawara *et al.*, 1994). We recently showed that the six genes are cotranscribed as an operon, preceded by two promoters. One resembles promoters recognized by the vegetative RNA polymerase $E\sigma^A$. The other promoter was shown to be stress-inducible and dependent upon σ^B , the general stress σ -factor in *B. subtilis* (Haldenwang, 1995). However, in a *sigB* mutant, the σ^A -like promoter became inducible, completely compensating for σ^B deficiency (Krüger *et al.*, 1996). Even in the absence of σ^B this promoter switch ensures a similar rate of protein synthesis as in the wild-type. In addition, we found conditions which exclusively induce the vegetative σ^A promoter such as amino acid limitation or hydrogen peroxide (Krüger *et al.*, 1996).

Two genes of the *clpC* operon, *orf3* and *orf5*, were cloned previously and designated *orfX* and *orfY* (Msadek *et al.*, 1994). Orf5 shares significant similarities with the *Escherichia coli* *sms* gene product (sensitivity to methyl methanesulfonate; Neuwald *et al.*, 1994). Potential functions of the *orf5* and *orf6* gene products encoded by this operon were investigated in this study.

METHODS

Bacterial strains, plasmids and culture conditions. Bacterial strains are listed in Table 1. *E. coli* strains DH5 α and RR1 were routinely grown in LB medium and used as hosts for DNA manipulation. *B. subtilis* was cultivated with vigorous aeration at 37 °C in LB medium or a previously described synthetic medium (Stülke *et al.*, 1993). Media were supplemented with the following antibiotics when necessary: ampicillin (100 μ g ml⁻¹), chloramphenicol (5 μ g ml⁻¹), spectinomycin (100 μ g ml⁻¹), or erythromycin (1 μ g ml⁻¹) plus lincomycin (25 μ g ml⁻¹). The *SPAC* promoter (*Pspac*) (Yansura & Henner, 1984) was induced by treatment with 1 mM IPTG.

Sensitivity to methyl methanesulfonate (MMS) was tested according to Neuwald *et al.* (1992) by growing the cells in LB into the exponential growth phase and plating them in soft agar onto LB plates. A paper disc with 10 μ l MMS was placed in the centre of the plate, giving a final concentration of 0.05 % MMS. To investigate survival after UV-irradiation, appropriate dilutions of cells from the exponential phase, the transition phase or the stationary phase were plated onto LB agar and exposed to UV light for 10 s (8 W cm⁻², 312 nm, Vilbert Lourmat transilluminator) and incubated overnight at 37 °C. The distance between the agar surface and the UV source was 1 cm.

General methods. Plasmid isolation, restriction enzyme analysis, transformation of *E. coli*, ligation of DNA fragments and filling in the recessed 3' termini using the Klenow fragment of DNA polymerase I were performed according to standard

protocols (Sambrook *et al.*, 1989). Recombinant plasmids were sequenced by the dideoxy chain-termination method of Sanger *et al.* (1977). DNA fragments were amplified by PCR as described earlier (Krüger *et al.*, 1994). Some oligonucleotides used for PCR included mismatches allowing creation of *EcoRI* and *BamHI* restriction sites. Chromosomal DNA from *B. subtilis* was isolated as described by Msadek *et al.* (1991). Transformation of *B. subtilis* with plasmid or chromosomal DNA was carried out using a two-step protocol (Hoch, 1991).

Construction of plasmids for generating *orf5* and *orf6* mutant strains. pMEC32 was constructed by deleting a 1826 bp *HindIII* fragment from pMEC28 (Msadek *et al.*, 1994), removing the last 111 codons of *orf3* and the first 497 codons of *clpC*. A 1238 bp *SmaI* fragment carrying the spectinomycin resistance gene from *Staphylococcus aureus* (Murphy, 1985) was then cloned into the unique *PstI* site within *orf5* (codon 109) in plasmid pMEC32, to give pMEC39. Plasmid pMEC39 was linearized by *EcoRI* digestion and transformed into *B. subtilis*, selecting for spectinomycin resistance, indicating a double crossover event (strain BEK9). To exclude transformants carrying the mutation as a Campbell-type integration, spectinomycin-resistant transformants were tested for erythromycin sensitivity.

The *orf5* disruption in strain BEK9 was verified by PCR using chromosomal DNA of the transformant as a template. Strain BEO1 was constructed by cloning the 531 bp *ScaI*-*BamHI* *orf5* fragment of pMEC28 into pDH88 (Henner, 1990) and transforming IS58 with the resulting plasmid pDSMS. In strain BEO1 *orf5* is disrupted by a single crossover recombination event allowing reinitiation of transcription by *Pspac* downstream of *orf5*.

pMEC60 was constructed by cloning a 200 bp *BamHI* PCR fragment internal to *orf6* using oligonucleotides TM-116 (5'-GGAGGATCCGATGCCGGATGCCACAATTTC-3') and TM-117 (5'-GGAGGATCCAGTGTTCGAATGGCTTGGTTC-3') into plasmid pHT181 (Lereclus & Arantes, 1993). When integrated by a Campbell-type event, this internal fragment gave rise to two truncated copies of *orf6* (strain BEK60). All Campbell-type disruptions were verified by Southern-blot analysis using digoxigenin-labelled PCR fragments as probes according to the manufacturer's recommendation (Boehringer Mannheim).

RESULTS

Predicted protein sequence analysis

The predicted amino acid sequences of the five other open reading frames cotranscribed with *clpC* (Fig. 1) were compared with the GenBank database using the BLAST (Altschul *et al.*, 1990) network service at the National Center for Biotechnology Information, with the default parameter values provided. Only matches with a probability lower than e^{-7} were considered significant, except in cases noted below.

A chromosomal locus of the facultative pathogen *Listeria monocytogenes* was identified recently, displaying a genetic organization similar to that of the *clpC* locus in *B. subtilis* (Rouquette *et al.*, 1996). Strong similarities were noted between Orf1, Orf2, Orf3, ClpC and Orf5 of *B. subtilis* and *L. monocytogenes*, with amino acid sequence identities of 64%, 29%, 50%, 73% and 70%, respectively (Rouquette *et al.*, 1996).

Table 1. Strains and plasmids used in this study

Strain/plasmid	Relevant genotype	Source/reference
<i>E. coli</i>		
RR1	F ⁻ <i>mcrB mrr hsdS20</i> (r _B ⁻ m _B ⁻) <i>ara-14 proA2 lacY1 leu galK2 rpsL20</i> (Sm ^r) <i>xyl5 mtl-1 supE44</i>	Bolivar <i>et al.</i> (1977)
DH5α	F ⁻ ϕ 80 <i>dlacZΔ</i> M15 Δ(<i>lacZYA-argF</i>)U169 <i>deoR recA1 endA1 hsdR17</i> (r _K ⁻ m _K ⁺) <i>supE44 thi-1 gyrA96</i>	Hanahan (1985)
<i>B. subtilis</i>		
IS58	<i>trpC2 lys-3</i>	Smith <i>et al.</i> (1980)
BEO1	<i>trpC2 lys-3 sms::pDH88</i>	pDSMS → IS58
BEK9	<i>trpC2 lys-3 sms::spc</i>	pMEC39 → IS58
BEK56	<i>trpC2 lys-3 amyE::(orf1-lacZ aphA3)</i>	Krüger <i>et al.</i> (1996)
BEK60	<i>trpC2 lys-3 orf6::pHT181</i>	pMEC60 → IS58
Plasmids		
pAC7	Ap ^r Km ^r <i>lacZ</i>	Krüger <i>et al.</i> (1996)
pDH88	Ap ^r Cm ^r <i>Pspac</i>	Henner (1990)
pHT181	Ap ^r Em ^r	Lereclus & Arantes (1993)
pWH331	Neo ^r Cm ^r	Gärtner <i>et al.</i> (1988)
pSPEC	Ap ^r Spc ^r	P. Trieu-Cuot, unpublished
pDSMS	pDH88 derivative with a 531 bp fragment of <i>orf5</i>	This work
pMEC28	Ap ^r Em ^r <i>E. coli</i> / <i>B. subtilis</i> shuttle vector containing part of <i>orf3</i> , <i>clpC</i> and part of <i>orf5</i>	Msadek <i>et al.</i> (1994)
pMEC32	pMEC28 derivative constructed by deleting a 1826 bp <i>Hind</i> III fragment	This work
pMEC39	pMEC32 derivative carrying the spectinomycin resistance gene in the unique <i>Pst</i> I site	This work
pMEC60	pHT181 derivative containing a 200 bp <i>Bam</i> HI PCR fragment internal to <i>orf6</i>	This work

Fig. 1. Schematic representation of the *clpC* operon structure. Similarities noted in domains of the corresponding gene products are indicated.

However, an equivalent of *orf6* of *B. subtilis* is not present in the reported *L. monocytogenes* nucleotide sequence (GenBank accession number U40604), where instead *orf5* is followed by an open reading frame whose product shows similarities with that of a gene lying downstream from *orf6* in *B. subtilis*. Except for ClpC, no functional role has been established for the products of *orf1*, 2, 3 and 5 in *L. monocytogenes* (Rouquette *et al.*, 1996).

The product of *orf1* (154 aa) of the *B. subtilis* *clpC* operon displays no significant similarities to proteins in the database. However, a predicted helix–turn–helix DNA-binding motif was detected in Orf1 using the weight matrix of Dodd & Egan (1990), beginning at amino acid 29 (RSEIADKFQCVPSQINYVIN, SD score 4.12. HTH probability > 90%). This suggests that Orf1 may have a regulatory role. Interestingly, this potential helix–turn–helix domain is highly conserved in the *orf1* product of *L. monocytogenes*.

Similarities between Orf2 (185 aa) of *B. subtilis* and proteins in the database were noted with a large family of eukaryotic regulatory proteins, characterized by a zinc-finger nucleic-acid-binding domain (Klug & Rhodes, 1987; Vallee *et al.*, 1991). Indeed, Orf2 contains two potential Cys₂–Cys₂ zinc-finger motifs, at positions C₃–C₆ to C₂₉–C₃₂ and C₈₁–C₈₄ to C₉₉–C₁₀₂.

orf3 encodes a putative protein (363 aa) with a domain that is highly conserved among ATP:guanidino phosphotransferases such as arginine kinases (e.g. *Homarus vulgaris*; Dumas & Camonis, 1993) or creatine kinases (e.g. *Schistosoma mansoni*; Stein *et al.*, 1990). This domain extends from amino acids 119 to 251 of Orf3, presenting 35% sequence identity on average with members of this family of kinases.

As previously reported (Msadek *et al.*, 1994; Ogasawara *et al.*, 1994), the deduced amino acid sequence of *orf5* (458 aa) shows 46% identity with that of the *E. coli* *sms* gene, whose absence confers sensitivity to MMS

Orf5	MAKT-KSKPL	CQSCGYESPK	WMGKCPGCGA	WNTMVEEMIK	KAP---ANR
Sms	MAKAPKRAEV	CNECCADYPR	WQGQCSACHA	WNTTTEVRLA	ASPMVARNER
Orf5	RAAFSHSVQT	VQKPSPTSIL	ETSEEPVKKT	QLGEFNRLVG	GGVVKGSVLV
Sms	LSGYAGSA-G	VAKVQKLSDI	SLEELPRFST	GFKEFORVLG	GGVVPSAIL
A Box					
Orf5	IGGD ¹⁰ PGIGKS	TLLQLVSAQL	SGSSNSVLYI	SGEESVKQTK	LRADRLGINN
Sms	IGGNEGAGKS	TLLQLTLCCK	AQQMKT-LYV	TGEESLQQVA	MRAHRLGLPT
B Box					
Orf5	PS ¹¹ EVLSSETD	MEYISSAIQD	MNPSFVVVDS	IQTVYQSDIT	SAPGSVSQVR
Sms	DNENMLSETS	IEQICLIAEE	EQPKLMVIDS	IQVMHMADVQ	SSPGSVAQVR
Orf5	ECTAELMKIA	KTKGIPFIV	GHVTKGSSIA	GPRLLEHMVD	TVLYFEGERH
Sms	ETAAYLTRFA	KTRGVALLMV	GHVTKGSSLA	GPVLEHCID	CSVLLDSQAD
Orf5	HTFRIILRAVK	NRFGSTNEMG	IFEMREGLT	EVLNPSIFL	EERSAGSAGS
Sms	SRFRLRSEK	NRFCAVNELG	VEAMTEGLR	EVSNPSTIFL	SRGDEVTSGS
Orf5	SITASMEGTR	PILVEIQALI	SPTSFGNPRR	MATGIDENRV	SLLMAVLEKR
Sms	SVMVVWEGTR	PILVEIQALV	DHSMMA ¹² NPRR	VAVGLEQNR	AILLAVLRRH
Orf5	VGLLLQNQDA	YLVVAGGVKL	DEPAIDLAIIV	ISIASFPRDT	PENPADCFIG
Sms	GSLQMAQDQV	FVNVVSGVKV	TETSAQLALL	LAMVSSLRDR	ELPQDLVVFG
Orf5	EVGLTGEVRR	VSRIEQRVKE	AAKLGFKRMI	IPAANLDGWT	KPKGLEVIGV
Sms	EVGLAGEIRP	VPSGQERISE	AAKGEFRAI	VPAANVPK-K	APEGMQIFGV
Orf5	ANVAEALRT-	-SLGG----			
Sms	KKLSDALSVF	DDLASECNT			

Fig. 2. Alignment of the *B. subtilis* Orf5 amino acid sequence with that of the *E. coli* sms gene product (Neuwald *et al.*, 1992). Identical residues are shaded. ATP-binding A and B motifs (Walker *et al.*, 1982) are boxed, and a potential zinc-finger nucleic-acid-binding domain is underlined.

(Neuwald *et al.*, 1992) (Fig. 2). The *orf5* and *sms* gene products both contain conserved ATP-binding A and B motifs (Walker *et al.*, 1982) as well as a potential zinc-finger nucleic-acid-binding domain (positions C₁₀–C₁₃ to C₂₄–C₂₇, Fig. 2). The *sms* gene appears to be highly conserved among prokaryotes and is also present in *Salmonella typhimurium*, *Haemophilus influenzae* and *Synechocystis* sp. Sms proteins also show similarities with RecA, in the vicinity of the ATP-binding site, and the carboxy-terminal region shows similarities with Lon proteases (Neuwald *et al.*, 1992).

Although the potential *orf6* gene product (360 aa, formerly *orf360*; Ogasawara *et al.*, 1994) shows no strong sequence similarities with proteins in the database, we noted some slight similarities with several proteins that interact with nucleic acids, such as DNA ligase of *Thermus thermophilus* and ATP-dependent RNA helicase of *Methanococcus jannaschii*. Indeed, a short carboxy-terminal region of Orf6 (amino acids 300–358) shows approximately 30% sequence identity with a corresponding stretch in these proteins as well as the *E. coli* UvrC DNA repair excinuclease (Sancar *et al.*, 1984) and ComEA of *B. subtilis* (Inamine & Dubnau, 1995).

Hydropathy profiles of the products of the six genes in the *clpC* operon, determined by the method of Kyte & Doolittle (1982), suggested that these proteins are all

cytoplasmic since no significantly hydrophobic potential transmembrane regions were noted (cutoff value: 2.6).

The *sms* phenotype of an *orf5* mutant

As indicated above, the deduced product of the fifth open reading frame shows about 50% amino acid sequence identity with the *E. coli* *sms* gene product. The *orf5* gene was disrupted, generating mutant BEK9.

To examine the possibility that *orf5* is indeed an *sms* homologue in *B. subtilis*, sensitivity to the alkylating agent MMS was tested using a paper disc method as described by Neuwald *et al.* (1990). Several independent experiments were performed by plating exponentially growing cultures of the BEK9 (*orf5*) and BEK60 (*orf6*) mutants in parallel with the corresponding wild-type strain IS58 on agar plates with a paper disc containing MMS (see Methods). Diameters of clear inhibition zones were measured. For a given strain, the diameters varied by less than ± 2 mm. Treatment with MMS (0.05%) resulted in average inhibition zones that were reproducibly larger in diameter for the mutant strain BEK9 (46 mm) than for the wild-type strain (40 mm), which is comparable to results obtained with the *E. coli* *sms* mutant. Strain BEK60 gave results nearly identical to the wild-type. These results suggest that a mutation in *orf5* leads to increased sensitivity of the mutant cells to

Table 2. Survival after UV-irradiation of *B. subtilis* wild-type strain in comparison to *orf5* and *orf6* mutants in different growth phases in competence medium

Growth phase	Survival of*:		
	Wild-type IS58	<i>orf5</i> mutant BEO1†	<i>orf6</i> mutant BEK60
Exponential (OD ₅₀₀ 0.4)	1.3 × 10 ⁻² (± 0.32 × 10 ⁻²)	0.9 × 10 ⁻² [69.2 %] (± 0.28 × 10 ⁻²)	1.1 × 10 ⁻² [84.6 %] (± 0.31 × 10 ⁻²)
Transition (OD ₅₀₀ 1.0)	7.1 × 10 ⁻² (± 1.0 × 10 ⁻²)	1.04 × 10 ⁻² [14.6 %] (± 0.5 × 10 ⁻²)	1.25 × 10 ⁻² [17.6 %] (± 0.6 × 10 ⁻²)
Stationary (OD ₅₀₀ 1.3)	4.95 × 10 ⁻² (± 1.0 × 10 ⁻²)	0.9 × 10 ⁻² [18.2 %] (± 0.15 × 10 ⁻²)	1.02 × 10 ⁻² [20.6 %] (± 0.5 × 10 ⁻²)

* Survival after UV-irradiation is indicated as the ratio of colony-forming units with and without UV treatment. Means of five independent experiments are shown, with standard deviations indicated in parentheses. For the *orf5* and *orf6* mutants, percentage survival in comparison to the wild-type is shown in square brackets.

† Cells of strain BEO1 were cultivated with the addition of 1 mM IPTG to induce *Pspac*.

MMS, as described for *E. coli*. We therefore propose that *orf5* be designated *sms*.

Survival of *orf5* and *orf6* mutants after UV-irradiation

Stationary-phase cells of *E. coli* are more resistant to UV-irradiation than exponentially growing cells, in a σ^S -dependent manner (for a review see Henнге-Aronis, 1993). Functional similarities between the σ^S regulon in *E. coli* and the σ^B regulon in *B. subtilis* have been described for the *katE* gene (Engelmann *et al.*, 1995; Engelmann & Hecker, 1996). Furthermore, the *clpC* operon is partly σ^B -dependent.

To investigate the UV survival response of mutants affected in the *clpC* operon, we first examined the survival of *B. subtilis* wild-type IS58 cells following exposure to UV-irradiation at different growth phases in glucose limitation medium (GLM) and under amino acid limitation (competence medium; CM). Samples were taken during exponential growth at an OD₅₀₀ of 0.4, in the transition phase (OD₅₀₀ 0.8 for GLM; OD₅₀₀ 1.0 for CM) or 1 h after entry into stationary phase (OD₅₀₀ 1.0 for GLM; OD₅₀₀ 1.3 for CM). Only amino-acid-starved cells showed an approximately five-fold increased resistance to UV-irradiation; glucose-starved cells showed no increase in resistance (data not shown). Therefore, competence medium was used for further experiments.

Since Orf5 and Orf6 have similarities with DNA-repair enzymes, we investigated mutants in both genes for survival after UV-irradiation. To avoid polar effects of the insertional mutation within *orf5* another mutant was constructed using plasmid pDSMS, allowing reinitiation of transcription by *Pspac*. This mutant, BEO1, also shows the *sms* phenotype. In all experiments IPTG was added to the culture of strain BEO1 to a final concentration of 1 mM. Several independent UV-irradiation experiments were performed, resulting in

approximately 1 % survival for the wild-type during the exponential growth phase. However, the number of viable wild-type cells increased to 7 % and 5 % during the transition and stationary phases, respectively. Setting survival of the wild-type to 100 %, only 15–20 % of the mutants BEO1 and BEK60 cells survived this procedure in transition and stationary phase, whereas in the exponential growth phase the sensitivity of the mutants was less pronounced (Table 2). These results suggest the involvement of Orf5 as well as Orf6 in the development of a non-specific stationary-phase resistance to UV-irradiation of amino-acid-starved *B. subtilis* cells. Results obtained with the *orf5* mutant BEK9 after UV-irradiation were similar to those with the BEO1 mutant, indicating that polar effects do not play a role (data not shown).

Orf5 and Orf6 are involved in genetic competence

We had previously noted that a *clpC* deletion mutant showed a decrease in transformability compared to a mutant with a point mutation in *clpC* (T. Msadek, unpublished). Given the slight similarities of the carboxy-terminal domain of Orf6 with ComEA, which is essential for competence development in *B. subtilis*, we investigated the possible role of Orf6 in competence. Since the SOS response and the development of genetic competence overlap (Cheo *et al.*, 1993), it was interesting to test whether competence is also influenced in an *orf5* mutant. Transformation experiments were carried out by preparing competent cells of strains BEO1, BEK60 and the corresponding wild-type strain IS58 in parallel. Cells were transformed with the replicative plasmid pWH331 (Gärtner *et al.*, 1988), with pAC7, a plasmid which cannot replicate in *B. subtilis* but integrates via a single crossover event in the *amyE* locus, or with chromosomal DNA of strain BEK56 requiring a double crossover event (Krüger *et al.*, 1996), with selection for the corresponding antibiotic resistance. Transformation

Table 3. Transformability of *orf5* and *orf6* mutants in comparison to the wild-type

DNA type	Frequency of transformation (%)*		
	Wild-type IS58	<i>orf5</i> mutant BEO1†	<i>orf6</i> mutant BEK60
Replicating plasmid (pWH331)	0.81	0.58 (1.4×)	0.18×10^{-1} (45×)
Non-replicating plasmid (pAC7)	0.12	0.52×10^{-2} (23×)	0.32×10^{-2} (37×)
single crossover			
Chromosomal (BEK56)	0.18	0.49×10^{-2} (37×)	0.51×10^{-2} (35×)

* Frequency of transformation is indicated as (kanamycin-resistant transformants/viable cells) $\times$ 100. The means of four independent experiments are shown; standard deviations were less than 10% of the mean. The numbers in parentheses indicate *x*-fold decrease of transformation frequency of the mutant strains in comparison to the wild-type.

† Cells of strain BEO1 were cultivated with the addition of 1 mM IPTG to induce *Pspac*.

experiments are summarized in Table 3. Transformation with a replicative plasmid was not strongly influenced in the *sms* mutant BEO1 in comparison to the wild-type. However, transformation rates dropped significantly with chromosomal DNA, requiring recombination events. The *orf6* disruption led to a strong decrease of competence in every case, indicating that *orf6* might be a novel competence gene. We therefore suggest that *orf6* be renamed *comY*.

DISCUSSION

To study the functions of the other five unknown gene products encoded by the *clpC* operon, database analysis was performed and phenotypes of selected mutants were investigated. The high probability of a helix–turn–helix motif within the amino acid sequence of Orf1 suggests a possible regulatory role for this protein. Preliminary results of our group indeed show that Orf1 may act as a repressor of the *clpC* operon expression under non-stressed conditions (E. Krüger & M. Hecker, unpublished).

For the fifth gene, *orf5*, whose product is similar to that of the *E. coli sms* gene (Neuwald *et al.*, 1992), similar phenotypes were observed for the mutant, leading to mild sensitivity to the alkylating agent MMS during exponential growth phase. Hence, the gene product may participate in repair of DNA damage by alkylation or DNA repair in general. This assumption is underlined by the increased sensitivity of *sms* (*orf5*) mutants to UV-irradiation. The increased sensitivity was also observed for mutants in *orf6*, suggesting that Orf6, which shows some weak similarities to nucleases, is also involved in DNA repair. Recently, we found that a σ^B - and a presumably σ^A -dependent promoter direct stress-induced transcription of the *clpC* operon. Expression from the σ^A -like promoter alone could be induced in response to stress conditions that do not induce σ^B , such as hydrogen peroxide treatment or amino acid starvation (Krüger *et al.*, 1996). Increased stationary-phase resistance to UV-irradiation was shown for amino-acid-starved but not for glucose-starved cells, indicating that this phenomenon is mediated by a σ^B -independent

mechanism. Our results suggest that Sms (Orf5) as well as ComY (Orf6) are involved in this mechanism, emphasizing an important function for σ^A -dependent induction of the *clpC* operon. However, the function of both Sms (Orf5) and ComY (Orf6) might only be important for the development of a non-specific stationary-phase resistance to UV-irradiation in addition to the constitutive excision and recombination repair systems involving the *recA* and the *uvrA* loci (Friedman & Yasbin, 1983).

Disruption of the sixth open reading frame, *orf6*, resulted in a significant decrease in competence for DNA transformation. Although there is no indication of the membrane association of Orf6, according to the hydropathy calculation method of Kyte & Doolittle (1982), one cannot exclude a role for this protein in DNA uptake, as observed for mutants in the *comEA* locus (Albano *et al.*, 1987; Hahn *et al.*, 1993; Inamine & Dubnau, 1995). An *sms* (*orf5*) mutation led to decreased transformability with DNA requiring homologous recombination. In view of the results discussed above and the inducibility of the *clpC* operon by DNA-damage-inducing treatment with hydrogen peroxide (Krüger *et al.*, 1996), an involvement of the *sms* and *comY* (*orf5* and *orf6*) gene products in either DNA-damage repair or competence, or both, could explain the phenotypes of these mutants. The link between the two mechanisms is an apparent overlap in the regulatory mode of the competence-dependent induction of the SOS system and the regulation of the competence regulon itself (Cheo *et al.*, 1993). However, previous data suggest that mutations in the regulatory genes of the competence pathway do not significantly affect vegetative-phase expression of a *clpC*–*lacZ* fusion (Msadek *et al.*, 1994). Furthermore, the consensus sequence found upstream of DNA-damage-inducible genes (GAAC-N₄-GTTC; Cheo *et al.*, 1993) is not present in the *clpC* operon. This suggests that DNA-damage induction of the *clpC* operon may involve a different mechanism from that of the *din* regulon. Protection of DNA or proteins against stress damage is essential for bacteria to keep existing cellular structures in a functional state. *B. subtilis* has developed efficient systems for detoxification and repair of DNA

under stress conditions. Interestingly, these include σ^B -dependent and σ^B -independent general stress proteins. The H_2O_2 -inducible genes *katA* and *mrgA*, the *din* genes, the alkyl hydroperoxide reductase operon *ahpC/ahpF* and the superoxide dismutase gene *sod* encode enzymes responsible for a specific oxidative stress resistance (Antelmann *et al.*, 1996; Bol & Yasbin, 1994; Cheo *et al.*, 1993; Chen *et al.*, 1995). However, genes of the σ^B regulon of *B. subtilis* seem to encode proteins that provide a non-specific protection of the cell against oxidative stress during starvation (Engelmann & Hecker, 1996). Our results suggest that Sms and ComY (Orf5 and Orf6) might belong to such non-specific systems. In this context, an overlap between the σ^B -dependent and σ^B -independent general stress responses, the SOS response and competence for DNA transformation seems plausible.

ACKNOWLEDGEMENTS

We thank Uwe Völker for critical reading of the manuscript and P. Trieu-Cuot for plasmid pSPEC. Susanne Engelmann is acknowledged for helpful discussions on oxidative stress response. Furthermore, we are grateful to Georges Rapoport in whose laboratory part of this work was carried out.

This work was supported by grants from the Deutsche Forschungsgemeinschaft and the Fonds der Chemischen Industrie to M.H.

REFERENCES

- Albano, M., Hahn, J. & Dubnau, D. (1987). Expression of competence genes in *Bacillus subtilis*. *J Bacteriol* **169**, 3110–3117.
- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Antelmann, H., Engelmann, S., Schmid, R. & Hecker, M. (1996). General and oxidative stress responses in *Bacillus subtilis*: cloning, expression, and mutation of the alkyl hydroperoxide reductase operon. *J Bacteriol* **178**, 6571–6578.
- Bol, D. & Yasbin, R. (1994). Analysis of the dual regulatory mechanisms controlling expression of the vegetative catalase gene of *Bacillus subtilis*. *J Bacteriol* **175**, 6744–6748.
- Bolivar, F., Rodrigues, R. L., Greener, P. J., Betlach, M. C., Heyneker, H. L., Boyer, H. W., Crosa, J. H. & Falkow, S. (1977). Construction and characterization of new cloning vehicles. II. A multipurpose cloning system. *Gene* **2**, 95–133.
- Bukau, B. (1993). Regulation of the *Escherichia coli* heat shock response. *Mol Microbiol* **9**, 671–680.
- Chen, L., Keramati, L. & Hellman, J. D. (1995). Coordinate regulation of *Bacillus subtilis* peroxide stress genes by hydrogen peroxide and metal ions. *Proc Natl Acad Sci USA* **92**, 8190–8194.
- Cheo, D. L., Bayles, K. W. & Yasbin, R. E. (1993). Elucidation of regulatory elements that control damage induction and competence induction of the *Bacillus subtilis* SOS system. *J Bacteriol* **175**, 5907–5915.
- Dodd, I. B. & Egan, J. B. (1990). Improved detection of helix–turn–helix DNA-binding motifs in protein sequences. *Nucleic Acids Res* **18**, 5019–5026.
- Dubnau, D. (1991). Genetic competence in *Bacillus subtilis*. *Microbiol Rev* **55**, 395–424.
- Dubnau, D. & Roggiani, M. (1990). Growth medium-independent genetic competence mutants of *Bacillus subtilis*. *J Bacteriol* **172**, 4048–4055.
- Dubnau, D., Hahn, J., Roggiani, M., Piazza, F. & Weihrauch, Y. (1994). Two-component regulators and genetic competence in *Bacillus subtilis*. *Res Microbiol* **145**, 403–411.
- Dumas, C. & Camonis, J. (1993). Cloning and sequence analysis of the cDNA for arginine kinase of lobster muscle. *J Biol Chem* **268**, 21599–21605.
- Engelmann, S. & Hecker, M. (1996). Impaired oxidative stress resistance of *Bacillus subtilis* *sigB* mutants and the role of *katA* and *katE*. *FEMS Microbiol Lett* **145**, 63–69.
- Engelmann, S., Lindner, C. & Hecker, M. (1995). Cloning, nucleotide sequence, and regulation of *katE* encoding a σ^B -dependent catalase in *Bacillus subtilis*. *J Bacteriol* **177**, 5598–5605.
- Friedman, B. M. & Yasbin, R. E. (1983). The genetics and specificity of the constitutive excision repair system of *Bacillus subtilis*. *Mol Gen Genet* **190**, 481–486.
- Gärtner, D., Geißendörfer, M. & Hillen, W. (1988). Expression of the *B. subtilis* *xyl* operons repressed at the level of transcription and is induced by xylose. *J Bacteriol* **170**, 3102–3109.
- Hahn, J., Inamine, G., Kozlov, Y. & Dubnau, D. (1993). Characterization of *comE*, a late competence operon of *Bacillus subtilis* required for the binding and uptake of transforming DNA. *Mol Microbiol* **10**, 99–111.
- Haldenwang, W. G. (1995). The sigma factors of *Bacillus subtilis*. *Microbiol Rev* **59**, 1–30.
- Hanahan, D. (1985). Techniques for transformation of *Escherichia coli*. In *DNA Cloning: a Practical Approach*, vol. 1, pp. 109–135. Edited by D. M. Glover. Oxford: IRL Press.
- Hecker, M., Schumann, W. & Völker, U. (1996). Heat shock and general stress response in *Bacillus subtilis*. *Mol Microbiol* **19**, 417–428.
- Hengge-Aronis, R. (1993). Survival of hunger and stress: the role of *rpoS* in early stationary phase gene regulation in *E. coli* K12. *Cell* **72**, 165–168.
- Henner, D. J. (1990). Inducible expression of regulatory genes in *Bacillus subtilis*. *Methods Enzymol* **185**, 223–228.
- Hoch, J. A. (1991). Genetic analysis in *Bacillus subtilis*. *Methods Enzymol* **204**, 305–320.
- Inamine, G. S. & Dubnau, D. (1995). ComEA, a *Bacillus subtilis* integral membrane protein required for genetic transformation, is needed for both DNA binding and transport. *J Bacteriol* **177**, 3045–3051.
- Klug, A. & Rhodes, D. (1987). 'Zinc fingers': a novel protein motif for nucleic acid recognition. *Trends Biochem Sci* **12**, 464–469.
- Kong, L. & Dubnau, D. (1994). Regulation of competence-specific gene expression by Mec-mediated protein–protein interaction in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **91**, 5793–5797.
- Krüger, E., Völker, U. & Hecker, M. (1994). Stress induction of *clpC* in *Bacillus subtilis* and its involvement in stress tolerance. *J Bacteriol* **176**, 3360–3367.
- Krüger, E., Msadek, T. & Hecker, M. (1996). Alternate promoters direct stress induced transcription of the *Bacillus subtilis* *clpC* operon. *Mol Microbiol* **20**, 713–723.
- Kyte, J. & Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. *J Mol Biol* **157**, 105–132.
- Lereclus, D. & Arantes, O. (1992). *spbA* locus ensures the segregational stability of pHT1030, a novel type of Gram-positive replicon. *Mol Microbiol* **6**, 35–46.
- Msadek, T., Kunst, F., Klier, A. & Rapoport, G. (1991). DegS–DegU

and ComP–ComA modulator–effector pairs control expression of the *Bacillus subtilis* pleiotropic regulatory gene *degQ*. *J Bacteriol* **173**, 2366–2377.

Msadek, T., Kunst, F. & Rapoport, G. (1994). MecB of *Bacillus subtilis*, a member of the ClpC ATPase family, is a pleiotropic regulator controlling competence gene expression and survival at high temperature. *Proc Natl Acad Sci USA* **91**, 5788–5792.

Msadek, T., Kunst, F. & Rapoport, G. (1995). A signal transduction network in *Bacillus subtilis* includes the DegS/DegU and ComP/ComA two-component systems. In *Two-component Signal Transduction*, pp. 447–471. Edited by J. A. Hoch & T. J. Silhavy. Washington, DC: American Society for Microbiology.

Murphy, E. (1985). Nucleotide sequence of a spectinomycin adenylyl transferase AAD(9) determinant from *Staphylococcus aureus* and its relationship to AAD(3'') (9). *Mol Gen Genet* **200**, 33–39.

Neuwald, A. F., Berg, D. E. & Stauffer, G. V. (1992). Mutational analysis of the *Escherichia coli* *serB* promoter region reveals transcriptional linkage to a downstream gene. *Gene* **120**, 1–9.

Ogasawara, N., Nakai, S. & Yoshikawa, H. (1994). Systematic sequencing of the 180 kilobases region of the *Bacillus subtilis* chromosome containing the replication origin. *DNA Res* **1**, 1–14.

Rouquette, C., Ripio, M.-T., Pellegrini, E., Bolla, J.-M., Tascon, R. I., Vázquez-Boland, J.-A. & Berche, P. (1996). Identification of a ClpC ATPase required for stress tolerance and *in vivo* survival of *Listeria monocytogenes*. *Mol Microbiol* **21**, 977–987.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.

Sancar, G. B., Sancar, A. & Rupp, W. D. (1984). Sequence of the *E. coli* *uvrC* gene and protein. *Nucleic Acids Res* **12**, 4593–4608.

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

Smith, I., Paress, P., Cabane, K. & Dubnau, E. (1980). Genetics and physiology of the *rel* system of *Bacillus subtilis*. *Mol Gen Genet* **178**, 271–279.

Stein, L. D., Harn, D. A. & David, J. R. (1990). A cloned ATP:guanidino kinase in the trematode *Schistosoma mansoni* has a novel duplicated structure. *J Biol Chem* **265**, 6582–6588.

Stülke, J., Hanschke, R. & Hecker, M. (1993). Temporal activation of β -glucanase synthesis in *Bacillus subtilis* is mediated by the GTP pool. *J Gen Microbiol* **139**, 2041–2045.

Vallee, B. L., Coleman, J. & Auld, D. S. (1991). Zinc fingers, zinc clusters, and zinc twists in DNA-binding protein domains. *Proc Natl Acad Sci USA* **88**, 999–1003.

Walker, J. E., Saraste, M., Runswick, M. J. & Gay, N. J. (1982). Distantly related sequences in the α - and β -subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. *EMBO J* **1**, 945–951.

Yansura, D. G. & Henner, D. J. (1984). Use of the *Escherichia coli* *lac* repressor and operator to control gene expression in *Bacillus subtilis*. *Proc Natl Acad Sci USA* **81**, 439–443.

Received 10 September 1996; revised 22 November 1996; accepted 5 December 1996.