



## Regulation of expression of *sodA* and *msrA* genes of *Corynebacterium glutamicum* in response to oxidative and radiative stress

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**ABSTRACT.** Promoters of genes encoding superoxide dismutase (*sodA*) and peptide methionine sulfoxide reductase (*msrA*) from *Corynebacterium glutamicum* were cloned and sequenced. Promoter region analysis of *sodA-msrA* was unable to identify putative sites of fixed eventual regulators except for possible sites of fixed OxyR and integration host factor. A study of the regulation of these genes was performed using the *lacZ* gene of *Escherichia coli* as a reporter placed under the control of sequences downstream of *sodA* and *msrA*. *In silico* analysis was used to identify regulators in the genome of *C. glutamicum*, which revealed the absence of homologs of *soxRS* and *arcA* and the presence of inactive *oxyR* and putative candidates of the homologs of *ahpC*, *ohrR*,

integration host factor, *furA*, *IdeR*, diphtheria toxin repressor, and *mntR*.

**Key words:** *Corynebacterium glutamicum*; Oxidative stress; Peptide methionine sulfoxide reductase; Superoxide dismutase

## INTRODUCTION

Little is known regarding bacterial superoxide dismutase (SOD) expression and regulation, particularly in Gram-positive bacteria. *Escherichia coli*, investigated in detail by Touati (1997), is an exception. This is because most studies have focused on pathogenic species to examine the relationship between antioxidant defenses and pathogenicity (Zhang et al., 1991; Alcendor et al., 1995; Battistoni et al., 2000; Ghanem, 2011). In comparison, little is known regarding their non-pathogenic relatives. Although the amino acid-producing, telluric and non-pathogenic, having moderate to high G+C content (Srivastava and Deb, 2005), corynebacterial species have been well-studied for decades. Most studies have focused on biochemical pathways or metabolic fluxes and not on their antioxidant defense mechanisms. Because of the high oxygen requirement of aerobic *Corynebacterium glutamicum* species (Cocaign-Bousquet et al., 1996), these organisms may use a hyper-antioxidant defense system including production of abundance SOD enzyme (El Shafey et al., 2009, 2010); thus, studying their antioxidant defenses is very important. SOD (EC 1.15.1.1) is considered to be a key enzyme in oxygen defense systems by catalyzing the dismutation of  $O_2^{\cdot -}$  into oxygen and  $H_2O_2$  (Fridovich, 1995), with the latter converted into water by catalase or peroxidase. SOD of *C. glutamicum* belongs to the Mn-dependent SOD family (El Shafey et al., 2008). In the promoter of this gene in *C. melassecola*, a phylogenetically related strain of *C. glutamicum* appears in a diverged orientation of another gene, *pmsr*, which may be involved in the response to oxidative stress. The open reading frames of the genes *sodA* (603 nucleotides) and *msrA* (654 nucleotides) of *C. melassecola* ATCC17965 are separated by 246 base pairs (bp) (GenBank accession No.: AF236111; Merkamm and Guyonvarch, 2001).

The *pmsrA* gene codes for a peptide methionine sulfoxide reductase (PMSR), an enzyme that catalyzes the reduction of the residues of sulfoxidated methionine [Met(o)]. The methionine sulfoxidated residues were obtained via oxidation of the sulfhydryl by reactive species such as hydrogen peroxide, hydroxyl radicals, and hypochlorite acid, but not by superoxide radicals. Oxidation of the SH group of the methionine residues by reactive oxygen species results inactivates many enzymes. Methionine sulfoxide reductases (MSRs) catalyze the reversal of these oxidation reactions, and thus restore proteins to their native and active state (Vogt, 1995). PMSRs demonstrated a general protective role against oxidative stress in *E. coli* (Moskovitz et al., 1995) and yeast (Moskovitz et al., 1998). PMSRs play the same role as SODs in response to oxidative stress. It was proposed that in addition to repairing damage caused by the sulfoxidation of methionine residues, PMSRs may play a regulatory role by modulating the oxidation level of some methionine residues (Vogt, 1995). The sulfoxidation of the methionine is one of the rare reversible oxidative reactions in proteins. For all reversible reactions, the passage from one form to another may be a mechanism of regulation; a regulatory role for PMSR was proposed in some pathogenic bacteria (Wizemann et al., 1996).

Thus, it is very important to determine whether PMSR in *C. glutamicum*, coded by *msrA*, plays a role in addition to repairing damage caused by oxidative stress. Divergent genes have not been described in *C. glutamicum*. In other organisms, genes interfering with the same

physiological mechanisms may be involved in a coordinated mechanism of regulation, meaning that their regulation is either identical (Escobar et al., 1998) or opposite (Dhandayuthapani et al., 1997). The interference of these genes in the protective mechanism against the reactive forms of oxygen indicates that concerted regulation of the *sodA* and *msrA* genes occurs.

The development of transcriptional fusions to a reporter gene enabled quantification of the level of expression of *sodA* under different conditions and determination of the relationship between the expression of these 2 genes.

## MATERIAL AND METHODS

### Bacterial strains and growth conditions

Tables 1 and 2 shows bacterial strains and vectors used in this study. *Corynebacte-*

**Table 1.** Bacterial strains used in this study.

Strains	Principal characteristics	Source or reference
<i>Corynebacterium glutamicum</i>		
CGL 1026	Wild-type strain	ATCC 13032
RES 167	CGL 1026 restriction deficient $\Delta$ ( <i>cglIM-cglIR-cglIIR</i> )	Kalinowski J, Universität Bielefeld
<i>Corynebacterium melassecola</i>		
CGL 1009	Wild-type strain	ATCC 17965
CGL 1019	CGL 1009 restriction deficient	DSM 12859
CGL 10016	<i>sod</i> :: Kan <sup>r</sup> (viMM12), derived from CGL1009	(Miller, 1972)
<i>Escherichia coli</i>		
DH5 $\alpha$	F <sup>-</sup> , $\Delta$ ( <i>lac-argF</i> )U169, <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> ( $\Gamma_K$ $\Gamma_K^{-}$ ), <i>supE44</i> , <i>gyrA1</i> , <i>relA1</i> , <i>deoR</i> , <i>thi-1</i> , ( $\phi$ 80 <i>dlacZ</i> $\Delta$ M15)	(Woodcock et al., 1989)

ATCC, American Type Culture Collection, Rockville, MD, USA. DSM, DSMZ - Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig - Germany.

**Table 2.** Vectors used in this study.

Vectors	Principal characteristics	Source or reference
pGEM-T (Easy)	Cloning vectors for the PCR products, origin of replication ColE1, Amp <sup>r</sup> . (pGEM-T and pGEM-T Easy different by the sites of restriction present around the site of insertion)	Promega
pCGL243	Shuttle vector <i>E. coli</i> - <i>corynebacteria</i> , with the origins of replication of pACYC184 for <i>E. coli</i> , and pBL1 for the <i>corynebacteria</i> , Kan <sup>r</sup> .	(Reyes et al., 1991)
pMF2	pMC1430 with <i>lacZYA</i> under the control of <i>P<sub>trc</sub></i> (Stratagene), in the genes <i>icd</i> of <i>C. melassecola</i> and <i>aphIII</i> , Amp <sup>r</sup> , Kan <sup>r</sup> inserted.	(Sanger et al., 1977)
pBC8	Plasmid derived from pMF2 cut by <i>EcoR</i> I/ <i>Bam</i> HI (T4 DNA polymerase), therefore it does not have a promoter before the <i>lac</i> anymore.	Guyonvarch A, Université Paris-Sud
pHTM1	pGEM-T Easy with the product of amplification by PCR on <i>C. glutamicum</i> with the primers S10 ( <i>Bam</i> HI upstream of ATG of <i>sodA</i> ) and M2 of 312 bp, Amp <sup>r</sup> .	This study
pHTM2	pGEM-T Easy with the product of amplification by PCR on <i>C. glutamicum</i> with the primers M6 ( <i>Bam</i> HI upstream of ATG of <i>msrA</i> ) and S8 of 327 bp, Amp <sup>r</sup> .	This study
pHTM3	Integrative vector for <i>corynebacteria</i> , with the fusion <i>sodA'-lacZ</i> under form of a fragment <i>EcoRI-BamHI</i> of pHTM1 (sequence downstream of <i>sodA</i> ) inserted in pMF2 <i>EcoRI-BamHI</i> , Kan <sup>r</sup> .	This study
pHTM4	Integrative vector for <i>corynebacteria</i> , with the fusion <i>msrA'-lacZ</i> under form of a fragment <i>EcoRI-BamHI</i> of pHTM2 (sequence downstream of <i>msrA</i> ) inserted in pMF2 <i>EcoRI-BamHI</i> , Kan <sup>r</sup> .	This study

Amp<sup>r</sup> = resistance to the ampicillin, Tet<sup>s</sup> = sensibility to the tetracycline, Tet<sup>r</sup> = resistance to the tetracycline, Kan<sup>r</sup> = resistance to the kanamycin, Cm<sup>r</sup> = resistance to the chloramphenicol. Promega Corporation, 2800 Woods Hollow Road, Madison, WI, USA.

*rium glutamicum* and *C. melassecola* strains were grown aerobically at 250 rpm and 34°C in brain heart infusion (Difco, BD Biosciences, Franklin Lakes, NJ, USA) rich medium, modified by addition of 0.1 mM MnSO<sub>4</sub> (El Shafey, 2004; El Shafey et al., 2008). Growth was followed by measuring the optical density at OD<sub>570 nm</sub> in a DU 7400 Beckman spectrophotometer (Brea, CA, USA). We added 25 µg/mL kanamycin and 15 µg/mL chloramphenicol to the medium when needed. *Escherichia coli* was grown aerobically at 37°C in Luria-Bertani medium (Difco). Growth was monitored at OD<sub>600 nm</sub>. We added 100 µg/mL ampicillin, 25 µg/mL kanamycin, and 30 µg/mL chloramphenicol to the medium when needed. All chemicals were purchased from Sigma (St. Louis, MO, USA).

### Determination of β-galactosidase activity

β-galactosidase activity was assayed using the standard colorimetric method described by Miller (1972). The absorbance data were collected and analyzed using Microsoft Excel.

### DNA manipulation

Standard molecular biology procedures were used according to Sambrook et al. (1989). Enzymes were obtained from BioLab, Inc. (Lawrenceville, GA, USA), Promega (Madison, WI, USA), and Boehringer Ingelheim (Mannheim, Germany). Plasmid DNA was prepared using the Wizard kit from Promega. DNA fragments were isolated from agarose gels using the Jetsorb kit (Genomed, Inc., Leesburg, FL, USA). *Corynebacterium glutamicum* chromosomal DNA was extracted as described by Ausubel et al. (1987). *Corynebacterium* strains were transformed by electroporation as described by Bonamy et al. (1990). Integration into the *Corynebacterium* chromosome was evaluated by Southern blotting as described by Labarre et al. (1993). DNA probes were labeled using the Digoxigenin DNA Labeling and Detection kit (Roche). The colonies kept for further characterization were purified once by single-colony isolation in selective solid medium. Stationary-phase cultures, grown from a single colony inoculated in 1 mL liquid medium, were mixed with 1 mL 80% glycerol and stored at -20°C.

### Determination and analysis of the nucleotide sequences

The determination of the nucleotide sequences was carried out by the society ESGS according to the method of Sanger et al. (1977). The GeneJockey program, DNA Strider, Blast (NCBI), and CLUSTAL W were used to analyze the nucleotide sequences.

### Polymerase chain reaction (PCR) amplification

The oligonucleotides described in Table 3 were used as primers. PCR was carried out with 2.5 U thermostable DNA polymerase (AmpliTaqGold from Perkin-Elmer, Waltham, MA, USA) in a reaction mixture containing 10-100 ng genomic *C. glutamicum* DNA, 0.2 mM of each deoxynucleotide triphosphate (Promega), 0.5 µM of each primer, 2 mM MgCl<sub>2</sub>, and 1x AmpliTaq Buffer in a final volume of 50 µL. For the amplification reaction, after 10 min at 94°C, 25 cycles (1 min of denaturation at 94°C, 1 min of hybridization at 50°C, 1 min of elongation at 72°C) were run, followed by a final elongation step of 5 min at 72°C. PCR experiments were carried out using the Crocodile II (Appligène, Cedex, France). The amplified

DNA fragment of the expected size was cloned into the pGEM-T vector (Promega).

**Table 3.** Oligonucleotides used in this study.

Name	Sequence (5'-3')	Site of restriction created
S8	AGCGGCGATGTGTGGCTCGAGAGCGTCGTA	
S10	TTCGGGATCCGCCATGGGTAAAAATCCT	<i>Bam</i> HI
M2	CGCCTCATCAGCGGTAACCATCACGGGTTCT	
M6	GAAAGGATCCGCATAACAGGAATGTTCTCT	<i>Bam</i> HI

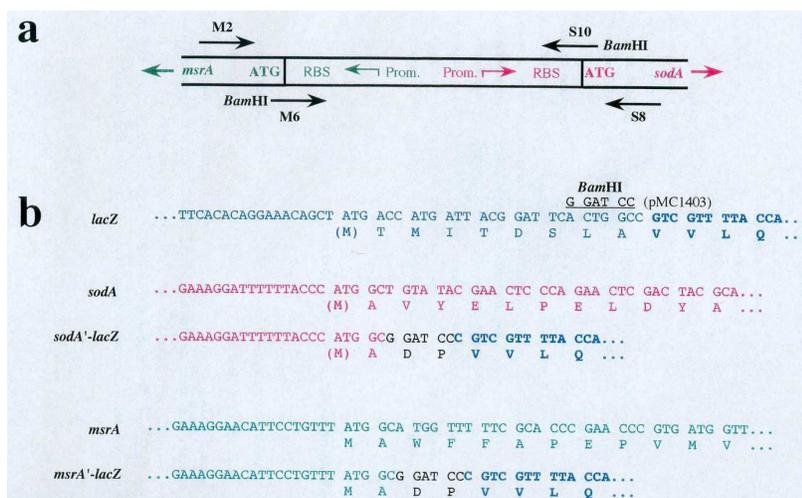
## RESULTS

Regulation of the expression of *sodA* and *msrA* genes was investigated using the *lacZ* gene of *E. coli* as a reporter gene placed under the control of the upstream sequences of *sodA* and *msrA*. The genes coding the SOD and PMSR were cloned and the chromosomal locus was studied.

### Amplification of the promoter region of *sodA-msrA* using PCR

Merkamm (2001) determined the locus integrity of the region *sodA-msrA* of *C. melassecola* and concluded that this region is a unique chromosomal DNA fragment, and that the organization of the locus is conserved between *C. melassecola* ATCC17965 and *C. glutamicum* ATCC13032.

Thus, 4 oligonucleotides were chosen based on the nucleotide sequence of the region of *sodA-msrA* of *C. melassecola* ATCC17965 (Figure 1).



**Figure 1.** Localization of PCR primers (a) and sequences of junctions obtained by cloning to site *Bam*HI of pMC1403 (b).

A *Bam*HI site was included between the third and fourth codons of *sodA* by PCR amplification using the oligonucleotide S10 as a primer, the *Bam*HI site, and the oligonucleotide M2 located 46 bp beyond the ATG of *msrA* (Figure 1). The 312-bp fragment after the first codons of *sodA* was followed by the *Bam*HI site.

In parallel, the sequence upstream of *msrA* was amplified by PCR using the oligonucleotides M6 as a primer to include the site *Bam*HI after the second codon of *msrA*, and the oligonucleotide S8 localized 62 bp downstream of the ATG of *sodA* (Figure 1). The 327-bp fragment obtained in this way included some codons of *sodA*, the sequences upstream *msrA*, and its first 2 codons followed by the *Bam*HI site.

A fragment obtained by PCR from *C. melassecola* of the expected size was 312 bp using the primers S10 and M2, and of 327 bp with the primers S8 and M6. PCR of the chromosomal DNA of RES167 amplified unique fragments of the expected sizes.

### **Cloning in the pGEM-T Easy vector**

Amplified fragments were cloned into the pGEM-T Easy vector. The plasmids pHTM1 and pHTM2 were obtained for the promoter regions of *sodA* and *msrA*, respectively.

### **Determination of the partial sequence of insert**

The nucleotide sequence of the fragments of the chromosomal DNA inserted in the pHTM1 and pHTM2 were determined by DNA walking up to the sites of insertion of the chromosomal DNA fragments in the pGEM-T Easy vector.

### **Nucleotide sequence accession number**

We assigned an accession number to the nucleotide sequence of the *msrA-sodA* promoter region in the GenBank nucleotide sequence database EF032882. Sequences corresponding to the degenerated oligonucleotides were identified. As expected, the nucleotide sequence and the deduced amino acid sequence showed complete similarity with the *C. melassecola* sequence.

### **Southern blot analysis of genomic DNA of RES167 and *E. coli***

The fragments resulting from amplification by S10 and M2 and by S8 and M6, cloned in the pGEM-T Easy vector to prepare plasmids pHTM1 and pHTM2, respectively, were isolated and purified. They served as probes in the Southern blot for hybridization on a membrane containing the chromosomal DNA of RES167 and *E. coli* subjected to electrophoresis after restriction enzyme digestion with *Pvu*II, *Pst*I, and *Hind*III. Autoradiography showed that clear signals were obtained with the DNA of RES167, while there were no signals were obtained with *E. coli*. This ensures that the origin of the amplification fragments for pHTM1 and pHTM2 included the DNA of RES167.

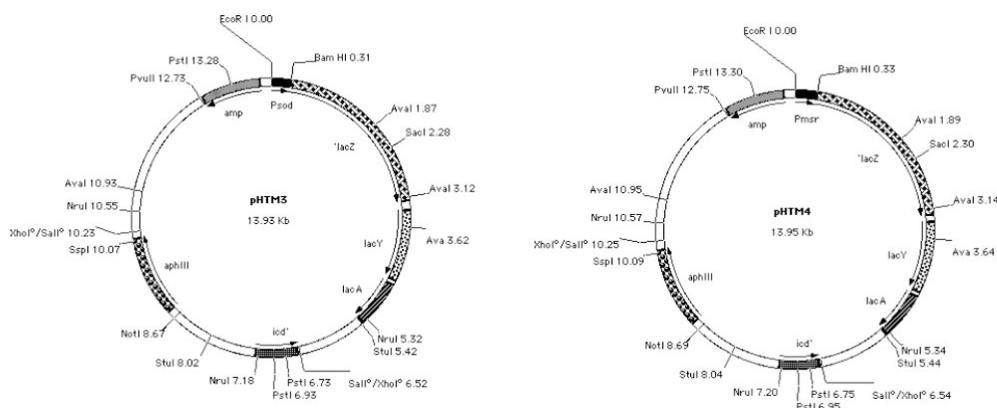
### **Construction of transcriptional fusions with *lacZ***

The *lacZ* gene of *E. coli*, which codes for  $\beta$ -galactosidase, was placed under the control of a sequences downstream the *sodA* and *msrA* genes. The *lacZ* gene was used as a reporter gene to examine the regulator sequences of many genes coding bacterial SODs, in addition to many studies of *Corynebacteria* (Labarre et al., 1993; Soual-Hoebeke et al., 1999; Battistoni et al., 2000).

## Fusions construction of integrative vectors of RES167

The *lacZ* gene originated from vector pMC1403 (Casadaban et al., 1980). The *Bam*HI restriction site was between codons 7-9 of *lacZ* for the construction of transcriptional fusions, in which the *lacZ* region is not amputated except the first 8 amino acids of the native protein, conserving all  $\beta$ -galactosidase activity (Casadaban et al., 1980).

Sequences downstream the first 2 codons of *sodA* and *msrA* were isolated from the vectors pHTM1 and pHTM2, respectively, using *Eco*RI (site of pGEM-T Easy, 10 bp upstream of the restriction site) and *Bam*HI (site sites introduced by mutagenesis downstream of the second codon). These fragments were inserted into the vector pMF2 (Soual-Hoebeke et al., 1999) and replaced the 350-bp fragment *Eco*RI-*Bam*HI for the promoter *trc* (*P*<sub>trc</sub>) upstream of *lacZYA*. The plasmid pMF2 is replicative in *E. coli*, but not in *Corynebacteria*. The *bla* and *aphIII* genes confer resistance to ampicillin and kanamycin, respectively. An approximately 2-kb DNA fragment of *C. melassecola* enabled integration of the vector by homologous recombination at the level of the chromosomal locus *icd* (isocitrate dehydrogenase). The plasmid pMF2 encoded *P*<sub>trc</sub> between *Eco*RI-*Bam*HI of pHTM1 and pHTM2, forming pHTM3 and pHTM4. *lacZ* was placed under control of sequences upstream of *sodA* and *msrA*, respectively. The *sodA* and *msrA* sequences, in addition to the junction with *lacZ*, were verified in the 2 vectors. The plasmids pHTM3 and pHTM4 are shown in Figure 2.



**Figure 2.** Integrative vectors pHTM3 and pHTM4 containing the fusions *sodA*'-*lacZYA* and *msrA*'-*lacZYA*, respectively.

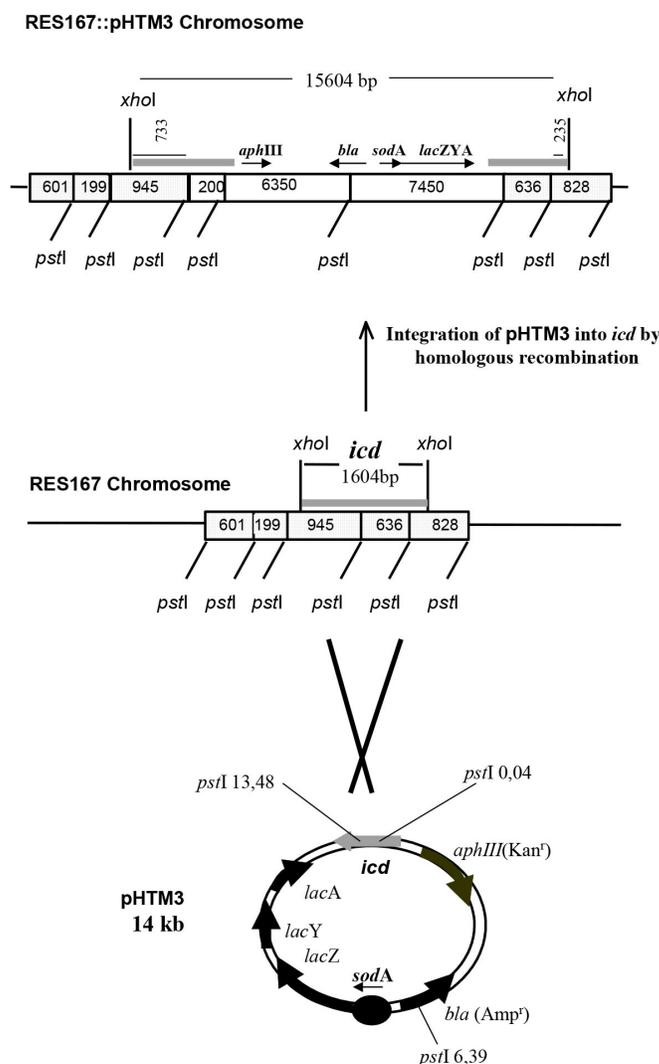
## Fusions transfer into RES167

The RRS167 strain was transformed with 8.5  $\mu$ g plasmid pHTM3 and pHTM4 extracted from DH5 $\alpha$ . The replicative plasmid in *Corynebacterium* pCGL243 extracted from DH5 $\alpha$  was used as a positive transformation control.

Two integrations for the plasmids pHTM3 and pHTM4 were possible in *C. glutamicum* through homologous recombination. The first by the DNA fragment of *C. glutamicum* of approximately 2 kb allowed integration of the vector by homologous recombination at the locus *icd*, and the second by homologous recombination at the intergenic *sodA*-*msrA* chromosomal region of *C. glutamicum*. To determine on which chromosomal locus the integration

had occurred, we conducted Southern blot analysis. RES167 chromosomal DNA and clones were extracted and digested with *Pst*I endonuclease. After gel electrophoresis and transfer to a membrane, DNAs were hybridized with plasmids pHTM3 and pHTM4 extracted from DH5 $\alpha$  and marked using the digoxigenin kit.

We expected that for RES167 signals of hybridization fragments, sizes would be 636, 945, and 1745 bp. If integration was evaluated by homologous recombination at the level of chromosomal locus *icd* of RES167, we observed bands of 200, 636, 945, 1745, 6350, and 7450 bp. All signals obtained were the expected sizes for the clones RES167 :: pHTM3#1 and RES167 :: pHTM4#1, indicating that the promoter regions of *sodA* and *msrA* were introduced in the genome of RES167 at the chromosomal locus *icd* by homologous recombination (Figure 3).



**Figure 3.** Integration of pHTM3 into the RES167 chromosome.

RES167 was transformed by the integrative plasmids in to *Corynebacteria*, with pMF2 following *lacZYA* under the control of the promoter *P<sub>trc</sub>*, and pBC8 containing the *lacZYA* operon without a promoter region. These plasmids served as positive and negative controls of expression in the tested promoter regions.

### Following regulation expression of *sodA* and *msrA* genes

To test the response to different environmental conditions, pre-cultures were grown overnight and inoculated to weak optical density in brain heart infusion + kanamycin media for RES167::pHTM3#1, RES167::pHTM4#1, RES167::pMF2 as a positive control, and RES167::pBC8 as a negative control. The growth of these cultures in addition to a culture of RES167 in brain heart infusion media were followed by measuring optical density at 570 nm, and aliquots were removed to determine  $\beta$ -galactosidase activity. The introduction of the stress factors was conducted at the time of entry into the stationary growth phase.

### Growth and expression of *sodA* and *msrA* genes without stress

Growth curves show no significant differences, while the expression curves of *msrA* was only one-third of *sodA*, and expression curves of *sodA* and *msrA* showed constant growth.

### Response to oxidative stress

The culture was exposed to different concentrations of oxidative agents at the end of the exponential phase of growth (after 2 h incubation,  $OD \approx 4$ ). Growth and  $\beta$ -galactosidase activity were followed 2 h 30 min, 3 h, 3 h 30 min, 4 h, 5 h, and 24 h starting at the addition of the stress agent.

Addition of hydrogen peroxide at the examined concentrations (0.1, 1.0, and 5.0 mM) had no effect on growth, and no regulation at the level of the expression of *sodA* and *msrA*. Dimethyl sulfoxide at the examined concentrations (1.0 and 5.0 mM) had a weak stimulatory effect on growth, which was stronger at 5 mM than at 1 mM, and no effect on the regulation of *sodA* and *msrA* expression. Addition of menadione, Paraquat (methyl viologen), plumbagin (5-hydroxy-2-methyl-1,4-naphtoquinone), and phenazine methosulfate had no effect on growth, except inhibition at 1.0 mM menadione was detected. No regulation at the level *sodA* expression was observed, while weak stimulation of *msrA* expression was observed at the late stationary phase of growth using menadione.

### Response to radiative stress

The UV lamp (254 nm) used was a VILBER LOURMAT VL-215G (Fisher Bioblock Scientific, Cedex, France). Study of viability revealed that weak doses (0.007-0.211 J/cm<sup>2</sup>) had no effect on viability, while 3.43 J/cm<sup>2</sup> decreased the colony-forming units from 10<sup>8</sup>-10<sup>5</sup>. These results indicate that the dose 3.43 J/cm<sup>2</sup> caused maximum stress because it decreased colony-forming units by a factor of 1000. Using stronger doses may also increase the number of mutations.

The results showed weak stimulation of growth at doses 0.007, 0.021, and 0.042 J/cm<sup>2</sup>. Inhibition of growth was observed at 3.43 J/cm<sup>2</sup>, and no effect was observed at doses 0.084 and 0.21 J/cm<sup>2</sup>. No effect on *sodA* expression was observed at all doses examined, although *msrA* was induced at 3.43 J/cm<sup>2</sup> after 0.5-3.0 h after irradiation, yet this induction had

disappeared after 24 h.

### Response to thermic stress

The method described by Muffler et al. (2002) was used to study effects of thermic stress at the end of the exponential growth phase.

Growth decreased for all clones after thermic shock, while  $\beta$ -galactosidase activity was inhibited at 5 and 15 min after thermic shock. Thus, no regulation of *sodA* and *msrA* expression was detected except for potential induction of *msrA* during the late stationary phase.

### Response to addition of metals *in vivo*

Addition of manganese ( $\text{MnSO}_4$ ) at concentrations of 0.1 and 1.0 mM had no effect on the growth of all clones and no effect on the expression of *sodA* and *msrA*. Addition of iron II [ $\text{Fe}(\text{NH}_4)_2(\text{SO}_4)_2$ ] at concentrations 1.0 and 5.0 mM stimulated the growth of all clones, and  $\beta$ -galactosidase activity was weakly inhibited in all clones. This indicated that this inhibition is not a specific effect of *sodA* or *msrA*. The addition of iron III ( $\text{FeCl}_3$ ) at concentrations 1.0 and 5.0 mM had no effect on either growth or expression of *sodA* and *msrA*.

### *In silico* analysis

To clarify this meaning of these results, *in silico* analysis was conducted to identify regulators in the intergenic *sodA-msrA* region and genes controlling the expression of *sodA* and *msrA* in the *C. glutamicum* genome.

### Analysis of the intergenic region *sodA-msrA* of RES167

Sites of binding of the regulators SoxRS, OxyR, AhpC, OhrR, Fur, MntR, ArcA, Fnr, c-AMP receptor protein (CRP), and integration host factor (IHF) were examined in the intergenic *sodA-msrA* region.

A consensus sequence for SoxRS, OhrR, Fur, MntR, ArcA, Fnr, and CRP binding could not be identified in the promoter region of *sodA-msrA* in *C. glutamicum* RES167, while a 50% consensus sequence was observed for OxyR in *E. coli* and 1 of the 2 possible sequences for fixing IHF may have been binding sites for OxyR and IHF, respectively, in the sequences upstream of *sodA* and *msrA* of RES167.

### Genome analysis of *C. glutamicum*

We searched for genes controlling the expression of *sodA* and *msrA* in the genome of *C. glutamicum* using BLASTX of NCBI (<http://www.ncbi.nlm.nih.gov/BLAST/>). Sequences showing weak alignments were excluded.

Although we did not identify a regulator of *soxSR* in the *C. glutamicum* genome, a transcriptional regulator was found that may be homolog of a regulator in mycobacteria. High identity and similarity exists between OxyR and OxyS of *M. tuberculosis* H37Rv. The AhpC/TSA family in the genome of *C. glutamicum* was represented by a hypothetical membrane protein, yet this region was absent in *Mycobacterium tuberculosis*. The genes *ahpC* (alkylhy-

droperoxydase C) - *oxyR* (OxyR pseudogene) in *M. tuberculosis* resembled a bacterioferritin comigratory protein or a transcriptional regulator with low identity and similarity, while the *ohrR* gene coding for organic hydroperoxide resistance protein of *Xanthomonas campestris* spp. *phaseoli* was a stress-induced protein with high identity and similarity. The *furA* gene in *M. tuberculosis* and *M. smegmatis* was a regulator for the acquisition of  $\text{Fe}^{2+}/\text{Zn}^{2+}$ , while a gene similar to the IdeR (DtxR) iron-dependent repressor in *M. bovis* spp. *bovis* and *C. diphtheriae* was found to be a transcriptional regulator that was Mn-dependent. The *mntR* genes of *B. subtilis* and *Staphylococcus aureus* were found to be Mn-dependent transcriptional regulators. The *fnr* gene of *M. tuberculosis*, *M. leprae*, *B. licheniformis*, and the possible pseudogene *fnr* of *M. leprae* were a protein containing c-AMP binding domain. No proteins aligned between the *arcA* gene of *M. tuberculosis* and the genome of *C. glutamicum*. The *mihF* gene of *C. diphtheriae* coding IHF and similar to *M. tuberculosis* and *M. smegmatis* genes coding IHF produced a hypothetical protein according to BLAST against the genome of *C. glutamicum*.

We examined the *C. glutamicum* genome to identify different factors responding to heat shock (chaperones, proteases, alternatives sigma factors), and identified chaperones in mycobacteria (*groE*, *dnaK*, and *dnaJ*) and proteases involved in the response to heat shock (Lon and Clp). The principal sigma factors *sigA* and *sigB* were found to be homologs of the *sigA* and *sigB* of *M. tuberculosis*, while the *sigE* gene of mycobacteria coding an alternative sigma factor SigE with extracytoplasmic functions was found to be represented by DNA-directed ARN polymerase specialized subunit sigma. Absence of the homologs of *sigF* (coding a stress response/stationary phase sigma factor of the mycobacteria) and *sigH* of *S. coelicolor* of the genome of *C. glutamicum* was observed.

## DISCUSSION

SOD of *C. glutamicum* is expressed during the exponential growth phase, which characterizes expression under control of the major RNA polymerase or vegetative RNA polymerase. The *sodA* region showed strong homology with sequences with other members of the Fe/MnSOD family.

Analysis of the intergenic sequence *sodA-msrA* revealed no fixed regulators in this region, although OxyR and IHF may be fixed. This may result from absence of regulation type searched for these genes, or recognition sequences of the regulators are not sufficiently conserved between species.

The absence of 1) regulation by all superoxide generators examined (menadione, Paraquat, plumbagin, phenazine methosulfate), 2) site of SoxRS binding to the promoter region of *sodA-msrA*, and 3) genes with high identity and similarity to *soxRS* in *E. coli* indicate that *C. glutamicum* either does not use a regulatory system during superoxide stress or that it uses a different system in *E. coli* and *S. typhimurium*. The system may also switched-on constitutively given its close relationship with pathogenic bacteria, requiring the maintenance of a constant of antioxidant enzymes during the infectious cycle.

The absence of regulatory genes *sodA* and *msrA* following hydrogen peroxide treatment despite the presence of gene *oxyR* and the possible presence of a consensus sequence for OxyR binding suggests that *C. glutamicum* lost some of its capacity to respond to oxidative stress during evolution.

Suggesting a role for the hypothetical membrane protein from the AhpC/TSA family in the *C. glutamicum* genome, but absent in *M. tuberculosis*, without isolating and identify-

ing the protein is difficult. The bacterioferritin comigratory protein found in the genome of *C. glutamicum* belongs to the ubiquitous family of thiol peroxide reductase, suggesting the presence of at least 1 member of this family in *C. glutamicum*. The presence of the *ohrR* gene in the genome of *C. glutamicum* represented by a stress-induced protein with high identity and similarity suggests the presence of OhrR at *C. glutamicum*, yet the absence may have been observed because the recognition sequences OhrR regulator was not sufficiently preserved between species.

The presence in the genome of *C. glutamicum* of a Fe<sup>2+</sup>/Zn<sup>2+</sup>-acquiring molecule resembling the *furA* gene in *M. tuberculosis* and *M. smegmatis* suggests the presence of FurA, while the absence of a binding site for FurA in the promoter region *sodA-msrA* of *C. glutamicum* RES167 may explain the absence of regulation at least under the environmental conditions used in this study. This may be because of 1) the absence of a binding site for FurA, 2) the absence of FurA, which was not previously isolated from *C. glutamicum*, or 3) *sodA* is not regulated by *furA* of *C. glutamicum*, if present.

The presence of an Mn-dependent transcriptional regulator in *C. glutamicum* resembling IdeR and the gene *mntR* was observed. The absence of a binding site for MntR in the promoter region suggests that the absence of regulation of *sodA* by manganese is because of either the absence of a binding site for MntR or the absence of a direct relationship between the increase in manganese concentration in the medium and *sodA*.

A complex interdependence exists between iron and manganese regulation and the response to oxidative stress. This relationship remains unclear. The absence of SOD regulation by manganese and iron, with increased SOD activity following the manganese addition, confirms that manganese acts on the post-transcriptional level. This suggests an autogenous repressor role for the apo-superoxide dismutase, and as PMSRs are not metalloproteins, and absence of regulation of *msrA* by the 2 metals tested is not surprising.

Under the aerobic conditions used in this study, no effect of the dimethyl sulfoxide was observed. Dimethyl sulfoxide reductase activity is more important in anaerobes than in aerobes. The Fnr binding site (total regulator anaerobic) could not be identified in the promoter region *sodA-msrA*, while the *fnr* gene of *M. tuberculosis*, *M. leprae*, *B. licheniformis*, and the possible *fnr* pseudogene of *M. leprae* were identified in the genome of *C. glutamicum* represented by the c-AMP protein containing domain. The c-AMP protein, known as CRP or catabolite activator protein, is the first member of the family of transcriptional regulators CRP-Fnr. Additionally, the CRP binding site could not be found in the promoter region of *sodA-msrA*, while a catabolite gene activator was found in the genome of *C. glutamicum*. Thus, the family of regulators CRP-Fnr is well-represented in *C. glutamicum*, and the absence of binding sites for CRP and Fnr may be because the recognition sequences for regulators were not sufficiently preserved between species as previously mentioned.

The *sodA* gene in *E. coli* is negatively regulated under anaerobic conditions by aerobic regulation control, fumarate nitrate reductase, and IHF, which also controls the expression of various aerobic metabolism genes.

The presence of IHF represented by a hypothetical protein with average identity and similarity, and the presence of 1 of the 2 possible sequences for IHF binding could neither confirm nor deny the presence of IHF in *C. glutamicum*. However, the purification and characterization of preceding hypothetical proteins may clarify this.

The absence of aerobic regulation control and its binding site suggest an alternative system to the aerobic regulation control in *C. glutamicum*.

The response of *C. glutamicum* to oxidative stress is similar to that of *M. tuberculosis*, a phylogenetically similar species to *C. glutamicum*, and differs from the known models of response to oxidative stress in enteric bacteria, including *E. coli* and *S. typhimurium*.

Studies on the regulation of *sodA* and *msrA* in *C. glutamicum* RES167 under the radiative stress suggest that the *sodA* is either not present in the response system to the radiative stress in *C. glutamicum*, or that the gene *sodA* does respond to the UVC light used, while the *msrA* gene appears to be induced by UV light.

The absence of *sodA* regulation by heat shock may result from the thermotolerance of the SOD enzyme in *C. glutamicum* RES167, which was stable for at least 1 month at 4°C. Induction of *msrA* during the late stationary phase agrees with the results of previous studies, indicating that a PMSR homolog exists in *Mycoplasma genitalium* based on the profile of transcriptional changes in the genome of *C. glutamicum* after heat shock (Muffler et al., 2002).

Our results cannot confirm or deny the presence of alternative sigma factors in *C. glutamicum*, and the only genes coding for sigma factors in *Corynebacteria* currently known, namely *sigA* and *sigB* of *Brevibacterium lactofermentum* coding primary vegetative sigma factors and *sigE* of *C. glutamicum* include a possible sigma factor ECF (*rpoE*), have not been characterized.

To better understand the defense system of oxidative stress in *C. glutamicum*, it is very important to examine the regulation of the *sodA* and *msrA* genes in response to 1) stress oxidative factors, 2) different concentrations of stress oxidative factors, 3) other types of radiation, 4) other heat shock temperatures, and 5) anaerobic growth conditions.

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