

## Sulfur-limitation-regulated proteins in *Bacillus subtilis*: a two-dimensional gel electrophoresis study

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**Little is known about the genes and enzymes involved in sulfur assimilation in *Bacillus subtilis*, or about the regulation of their expression or activity. To identify genes regulated by sulfur limitation, the authors used two-dimensional (2D) gel electrophoresis to compare the proteome of a wild-type strain grown with either sulfate or glutathione as sole sulfur source. A total of 15 proteins whose synthesis is modified under these two conditions were identified by matrix-assisted laser desorption/ionization time of flight (MALDI TOF) mass spectrometry. In the presence of sulfate, an increased amount of proteins involved in the metabolism of C<sub>1</sub> units (SerA, GlyA, FOLD) and in the biosynthesis of purines (PurQ, Xpt) and pyrimidines (Upp, PyrAA, PyrF) was observed. In the presence of glutathione, the synthesis of two uptake systems (DppE, SsuA), an oxygenase (SsuD), cysteine synthase (CysK) and two proteins of unknown function (Ytml, YurL) was increased. The changes in expression of the corresponding genes, in the presence of sulfate and glutathione, were monitored using slot-blot analyses and *lacZ* fusions. The *ytml* gene is part of a locus of 12 genes which are co-regulated in response to sulfur availability. This putative operon is activated by a LysR-like regulator, Ytll. This is the first regulator involved in the control of expression in response to sulfur availability to be identified in *B. subtilis*.**

Keywords: sulfur metabolism, proteome analysis, regulation

### INTRODUCTION

All living organisms require sulfur for the synthesis of proteins and essential cofactors. Sulfur can be assimilated either from inorganic sources like sulfate and thiosulfate, or from organic ones like sulfate esters, sulfamates and sulfonates. In *Escherichia coli*, sulfate is transported into the cell and subsequently reduced to

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**Abbreviations:** 2D, two-dimensional; ABC, ATP-binding cassette; DIG, digoxigenin; MALDI-TOF, matrix-assisted laser desorption/ionization time of flight.

sulfide, which reacts with O-acetylserine to form cysteine. More than 20 genes involved in this process have been identified (Kredich, 1996). Most of them are positively regulated by CysB, a transcriptional activator belonging to the LysR family of regulators. Full expression of the cysteine biosynthetic pathway requires the CysB activator, the inducer N-acetylserine and reduced-sulfur limitation. CysB binds upstream from the –35 region of the positively regulated promoters and facilitates the formation of a transcription initiation complex in the presence of N-acetylserine in an as yet unknown way. L-Cysteine, sulfide and thiosulfate down-regulate L-cysteine biosynthesis. Sulfide and thiosulfate appear to act as anti-inducers of N-acetylserine, while L-cysteine feedback inhibits the synthesis of O-acetylserine (Kredich, 1996).

Under sulfate-starvation conditions, *E. coli* synthesizes a

set of proteins which are absent during growth in the presence of sulfate. These proteins are involved in the biosynthesis of cysteine from sulfide and in the utilization of alternative sulfur sources such as sulfonates (Uria-Nickelsen *et al.*, 1993). Random *lacZ* fusions and 2D gel electrophoresis revealed a specific increase in the synthesis of several proteins, such as oxygenases (TauD, SsuD), high-affinity uptake systems (ATP-binding cassette – ABC – transport systems, sulfate- and cystine-binding proteins), alkyl hydroperoxide reductase (AhpC), O-acetylserine lyase A (CysK), and of several unidentified proteins (Kertesz *et al.*, 1993; Quadroni *et al.*, 1996; van der Ploeg *et al.*, 1996, 1999). Several proteins induced under sulfate-starvation conditions have also been identified in *Pseudomonas aeruginosa* (Hummerjohann *et al.*, 1998; Kertesz *et al.*, 1999; Quadroni *et al.*, 1999).

In *Bacillus subtilis*, sulfate assimilation and cysteine biosynthesis may proceed via an *E. coli*-like pathway. The enzymes leading to the conversion of sulfate into sulfide, and to its incorporation into cysteine, are present in *B. subtilis* (Kunst *et al.*, 1997; Pasternak *et al.*, 1965). The expression of the *cysH* gene, which encodes 3'-phosphoadenosine 5'-phosphosulfate sulfotransferase, was found to be repressed by both cysteine and sulfide (Mansilla & de Mendoza, 1997). The *cysH* gene is the first gene of an operon which encodes a sulfate permease (CysP) and enzymes catalysing the first steps of the sulfate assimilation pathway (Mansilla & de Mendoza, 2000). Little is known about the regulation of sulfur metabolism. In Gram-positive bacteria, a highly conserved sequence, the S-box, is located in the leader region of several operons including genes involved in the biosynthesis of methionine or cysteine. These genes are proposed to form a new regulon controlled by a global transcription-termination control system (Grundy & Henkin, 1998).

Only a few *B. subtilis* genes necessary for the assimilation of sulfur from sulfonates have been identified (van der Ploeg *et al.*, 1998). Three of them encode an ABC transport system (*ssuBAC*) and another one encodes a monooxygenase (*ssuD*).

To get insight into the global regulation of sulfur-related gene expression in *B. subtilis*, we associated the recent knowledge of the whole genome sequence (Kunst *et al.*, 1997), the highly sensitive technique of 2D gel electrophoresis (O'Farrell, 1975) and mass spectrometry. 2D gel electrophoresis now allows the separation of more than 1000 proteins (Bernhardt *et al.*, 1999) and many can be identified by MALDI TOF spectrometry (Shevchenko *et al.*, 1996). We took advantage of this technique to identify proteins whose synthesis is regulated by sulfur availability in *B. subtilis*. The protein synthesis patterns of a wild-type strain grown with either sulfate or glutathione as a sulfur source were compared. Synthesis of 15 proteins was up- or down-regulated. These proteins were identified unambiguously by MALDI TOF spectrometry. For most of them, the alteration in expression of the corresponding genes under these two

conditions was confirmed by slot-blot analyses or *lacZ* fusions.

## METHODS

**Bacterial strains and culture conditions.** The *B. subtilis* strains used in this work are listed in Table 1. Experiments were carried out using *B. subtilis* strains grown at 37 °C with agitation in minimal medium (6 mM K<sub>2</sub>HPO<sub>4</sub>, 4.5 mM KH<sub>2</sub>PO<sub>4</sub>, 0.3 mM trisodium citrate, 5 mM MgCl<sub>2</sub>, 22 mg ferric ammonium citrate l<sup>-1</sup>, 0.5% glucose, 0.1% glutamine, 100 mg l<sup>-1</sup> of auxotrophic requirements). Solid medium was supplemented with 1.5% agar. The sulfur source varied in the different experiments as stated: 5 mM taurine, 4 mM MgSO<sub>4</sub>, 2 mM reduced glutathione, 1 mM methionine, 1 mM thio-sulfate or 0.5 mM cysteine. Antibiotics were added to the following concentrations: ampicillin, 100 µg ml<sup>-1</sup>; chloramphenicol, 5 µg ml<sup>-1</sup>; kanamycin, 5 µg ml<sup>-1</sup>; erythromycin 1 µg ml<sup>-1</sup> plus lincomycin 25 µg ml<sup>-1</sup>. The transformation procedures used for *E. coli* and *B. subtilis* were as described previously (Sambrook *et al.*, 1989; Kunst & Rapoport, 1995). The integration of DNA fragments into the *amyE* locus by double cross-over was assessed by monitoring the loss of amylase activity (Stülke *et al.*, 1997). All experiments were performed in accordance with the European regulation requirements concerning the use of genetically modified organisms (level 1 containment, agreement no. 2735).

**Analytical 2D gel electrophoresis.** *B. subtilis* strain 168 was grown in minimal medium using either sulfate or glutathione as sulfur source. Exponentially growing cells (50 ml) were harvested by centrifugation and washed twice in TE buffer (10 mM Tris pH 7.5, 1 mM EDTA). The pellet was then resuspended in 1 ml buffer (10 mM Tris pH 7.5, 1 mM EDTA, 8 M urea, 100 mM DTT, 1% Triton X-100, 4% CHAPS, 2 mM PMSF) with 15 µl of a DNase (1 mg ml<sup>-1</sup>)/RNase (0.5 mg ml<sup>-1</sup>) solution. After sonication, the cell debris was removed by ultracentrifugation (60 min, 90000 g). Proteins (100 µg) were resuspended in sample buffer (0.5% Pharmalyte 3–10, 8 M urea, 100 mM DTT, 2% Nonidet P40). Isoelectric focusing was performed on Immobiline DryStrips (pH 4–7) with the IPGPhor Isoelectric System (Pharmacia). Strips rehydrated for 4 h at 20 °C were focused for 45000 Vh. Separation in the second dimension was performed with 11.5% SDS-polyacrylamide gels. The gels were fixed in 40% ethanol/10% acetic acid and silver-stained. The gels were digitized using a JX-330 scanner (Sharp). Spot detection and quantification were performed using the PDQUEST software package (Bio-Rad).

**MALDI-TOF spectrometry identification.** Protein spots were cut off and digested in gel slices with trypsin (Roche) as described previously (Shevchenko *et al.*, 1996). The matrix used for the desalted digestions was a saturated solution of 2,5-dihydroxybenzoic acid in 0.1% trifluoroacetic acid. MALDI-TOF spectra of the peptides were obtained with a Voyager-DE STR Biospectrometry Workstation mass spectrometer (PE Biosystems). The analysis was performed in positive ion reflector mode. The trypsin autoprolysis products were used as internal calibrants. Data mining was performed using ProFound and MS-FIT software against non-redundant databases. A mass deviation of 0.1–0.3 Da was allowed in the database searches.

**Slot-blot analyses.** Digoxigenin (DIG)-labelled probes corresponding to *ytml*, *dppE*, *pyrE*, *serA*, *purQ*, *glyA*, *aroF* and *upp* were obtained using the PCR DIG probe synthesis kit

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

Strain	Genotype	Source*
168	<i>trpC2</i>	Laboratory stock
BSIP1207	<i>trpC2 amyE::cysK'-lacZ cat</i>	pDIA5566 → 168
BSIP1214	<i>trpC2 ytlI::aphA3</i>	PDIA5577 → 168
BSIP1223	<i>trpC2 ytmI'-lacZ erm ΔytmI ytlI::aphA3</i>	DNA BSIP1214 → BFS71
BSIP1248	<i>trpC2 amyE::serA'-lacZ cat</i>	pDIA5593 → 168
BSIP1249	<i>trpC2 ytlI::aphA3 amyE::serA'-lacZ cat</i>	pDIA5593 → BSIP1214
BFS68†	<i>trpC2 ytnM'-lacZ erm ΔytnM</i>	V. Vagner
BFS70†	<i>trpC2 ytnJ'-lacZ erm ΔytnJ</i>	V. Vagner
BFS71†	<i>trpC2 ytmI'-lacZerm ΔytmI</i>	V. Vagner
BFS85†	<i>trpC2 ytmJ'-lacZ erm ΔytmJ</i>	J. Chapuis
BFS86†	<i>trpC2 ytmK'-lacZ erm ΔytmK</i>	J. Chapuis
BFS88†	<i>trpC2 ytmM'-lacZ erm ΔytmM</i>	J. Chapuis
BFS424†	<i>trpC2 ssuA'-lacZ erm ΔssuA</i>	L. A. Rivas
BFS1249†	<i>trpC2 yurL'-lacZ erm ΔyurL</i>	M. F. Hullo
LCC1	<i>amyE::xpt'-lacZ cat</i>	H. Saxild

\* Arrows indicate construction by transformation; *cat* is the pC194 chloramphenicol acetyltransferase gene; *aphA3* is the *Enterococcus faecalis* kanamycin-resistance gene and *erm* is an erythromycin-resistance gene. M. F. Hullo, Institut Pasteur, Paris, France; V. Vagner and J. Chapuis, INRA, Jouy en Josas, France; L. A. Rivas, CSIC, Cantoblanco, Madrid, Spain; H. Saxild, Technical University, Lyngby, Denmark.

† These strains were constructed within the framework of the EC project for functional characterization of the genome of *B. subtilis* (<http://locus.jouy.inra.fr/cgi-bin/genmic/madbase/progs/madbase.operl>).

from Roche. Total RNA was extracted from exponentially growing cells using the High Pure RNA Isolation Kit (Roche). Increasing amounts (50, 100, 200 and 400 ng) of total RNA were transferred to a positively charged nylon membrane and hybridized with the DIG-labelled probes. Chemiluminescent detection was performed with anti-DIG-AP and CDP-Star (Roche). The chemiluminescent signal was detected on X-ray film. Slot-blots were analysed and quantified using the Image Master 1D software from Pharmacia.

**Plasmid constructs.** A 1055 bp DNA fragment containing part of the *ytlI* gene from position -209 to +846 (numbering is relative to the translational start site) was amplified by PCR using chromosomal DNA as template and two specific primers containing an *EcoRI* or a *BamHI* restriction site. The *EcoRI*-*BamHI* fragment was cloned into the pJH101 vector (Ferrari *et al.*, 1983) to give pDIA5576. A 1.5 kb *Clal* DNA fragment corresponding to the kanamycin-resistance gene *aphA3* was inserted at the unique *Clal* site of pDIA5576. The resulting plasmid pDIA5577 linearized with *ScaI* was used to transform *B. subtilis* 168, leading to the disruption of the *ytlI* gene by insertion of a kanamycin cassette (Table 1).

A 336 bp and a 474 bp DNA fragment corresponding to the *serA* promoter (from position -270 to +66 relative to the translational start site) and to the *cysK* promoter (from position -210 to +264 relative to the translational start site) was amplified by PCR. Oligonucleotides were used to create an *EcoRI* restriction site at the 5' end and a *BamHI* restriction site at the 3' end of the fragments. After digestion of the PCR products with *BamHI* and *EcoRI*, they were inserted into plasmid pAC6 (Stülke *et al.*, 1997). The resulting *serA-lacZ* and *cysK-lacZ* fusions were subsequently integrated at the *amyE* locus (Table 1).

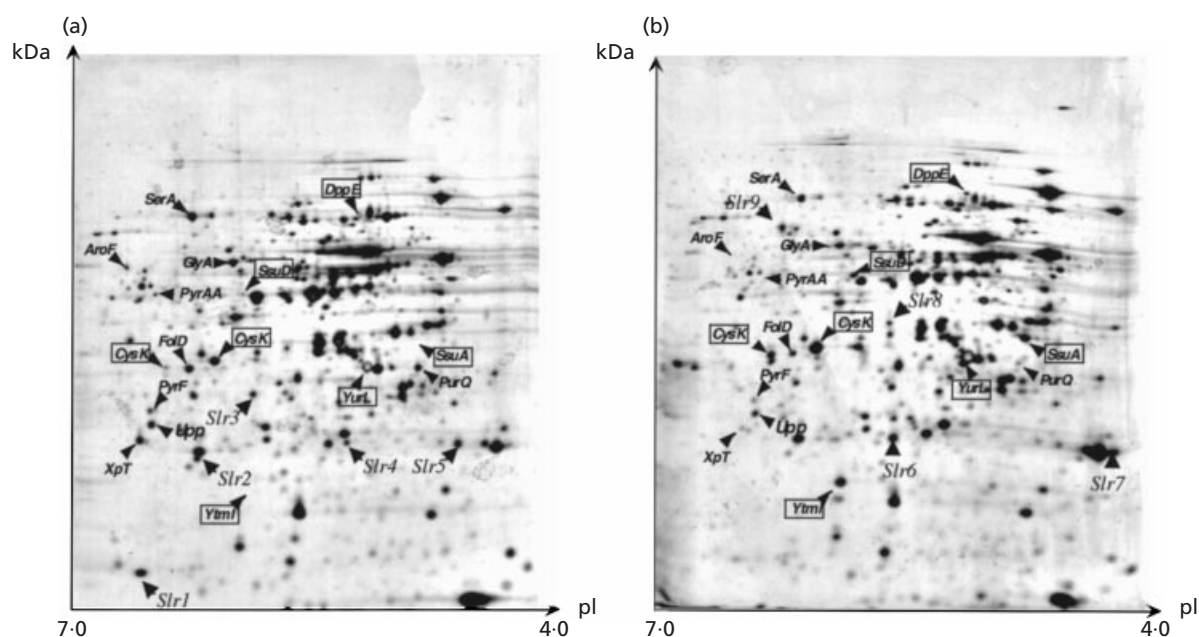
Within the framework of a European project on the functional analysis of the genome of *B. subtilis*, more than 1100 genes have been disrupted by fusion with the *lacZ* reporter gene (see <http://locus.jouy.inra.fr/cgi-bin/genmic/madbase/progs/madbase.operl>). An internal fragment of each gene was amplified by PCR using primers creating *HindIII* and *BamHI* restriction sites at the 5' and 3' ends of the PCR product, respectively. After digestion with *HindIII* and *BamHI*, these PCR products were cloned into the pMUTIN4 vector (Vagner *et al.*, 1998). The pMUTIN derivatives were then integrated into the *B. subtilis* chromosome by homologous recombination at the target locus. This strategy was used to inactivate *yurL*, *ssuA* and six genes of the *ytmI* locus while creating a *lacZ* fusion (see Table 1).

**β-Galactosidase assays.** Cells were grown in minimal medium in the presence of different sulfur sources. Samples of the cultures were taken during exponential growth and harvested by centrifugation. The β-galactosidase activity was measured using the Miller assay (Miller, 1972) with cell extracts obtained by lysozyme treatment. All the assays were repeated at least twice. One unit (U) of β-galactosidase is defined as the amount of enzyme which produces 1 nmol *o*-nitrophenol min<sup>-1</sup> at 28 °C.

## RESULTS

### Comparison of the protein synthesis patterns of *B. subtilis* cells grown with sulfate or glutathione as sulfur source

Using 2D electrophoresis, the protein synthesis pattern of a wild-type *B. subtilis* strain grown on minimal medium in the presence of different sulfur sources was



**Fig. 1.** Comparison of the protein synthesis patterns of *B. subtilis* 168 after growth in the presence of glutathione or sulfate. Cells were grown exponentially in minimal medium supplemented with  $50 \mu\text{g}$  tryptophan  $\text{ml}^{-1}$  and either (a) 4 mM sulfate or (b) 2 mM glutathione at  $37^\circ\text{C}$ . Proteins were separated with IPG pH 4–7 in the first dimension and with 11.5% polyacrylamide gel in the second dimension. After silver staining, proteins regulated by the sulfur source were submitted to MALDI TOF spectrometry. The proteins identified as being more abundant after growth with glutathione are boxed. The protein names are according to SubtiList (<http://genolist.pasteur.fr/SubtiList>). The proteins which could not be identified by MALDI spectrometry are named Slr (sulfur-limitation-regulated). The CysK protein is identified at two different positions on the gel. Post-translational modification of this protein is possible.

**Table 2.** Sulfur-regulated proteins identified by 2D gel analysis

Proteins were selected by computer-assisted comparison of silver-stained gels and identified by MALDI TOF spectrometry. The results are representative of four independent experiments. G, glutathione; S, sulfate.

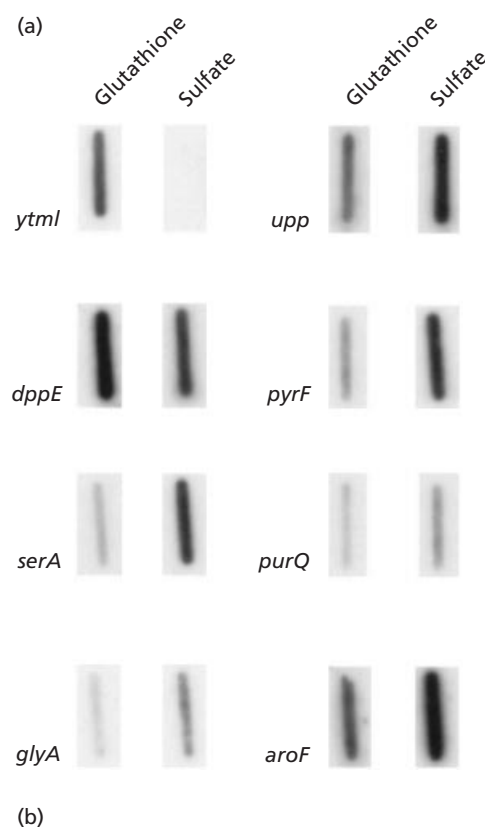
Glutathione			Sulfate		
Gene	Protein	Synthesis	Gene	Protein	Synthesis
<i>ytml</i>	Unknown	Induced on G/absent on S	<i>xpt</i>	Xanthine phosphoribosyltransferase	6-fold higher on S than on G
<i>yurL</i>	Unknown	Induced on G/absent on S	<i>purQ</i>	Phosphoribosylformylglycinamide synthase I	5-fold higher on S than on G
<i>ssuA</i>	Aliphatic sulfonates ABC transporter	Induced on G/absent on S	<i>upp</i>	Uracil phosphoribosyltransferase	2-fold higher on S than on G
<i>ssuD</i>	Aliphatic sulfonates monooxygenase	Induced on G/absent on S	<i>pyrAA</i>	Carbamoyl-phosphate synthase	Induced on S/absent on G
<i>dppE</i>	Dipeptide ABC transporter	Induced on G/absent on S	<i>pyrF</i>	Orotidylate decarboxylase	Induced on S/absent on G
<i>cysK</i>	Cysteine synthase	5-fold higher on G than on S	<i>serA</i>	Phosphoglycerate dehydrogenase	2-fold higher on S than on G
			<i>glyA</i>	Serine hydroxymethyltransferase	2-fold higher on S than on G
			<i>fold</i>	Methylenetetrahydrofolate dehydrogenase	2-fold higher on S than on G
			<i>aroF</i>	3-Dehydroquinate dehydratase	Induced on S/absent on G

analysed. Significant differences were observed in the growth of the strain in media with the different sulfur sources: the generation time was 50 min with sulfate, 55 min with methionine, 155 min with taurine and 180 min with glutathione. The utilization of glutathione as sulfur source corresponds to sulfur-limitation conditions. Furthermore, it alleviates the problems linked to the use of taurine, which behaves like an ampholyte and is therefore difficult to use in 2D experiments.

To create a 2D reference map, the experimental conditions optimizing gel resolution and MALDI TOF spectrometry identification rates were established (See Methods). We identified 47 spots which were used as markers allowing the comparison of our gels with other *B. subtilis* 2D gels available on the internet (<http://microbio2.biologie.uni-greifswald.de:8880/>). Furthermore, these markers facilitate the prediction of the molecular mass and the isoelectric point of a spot according to its position on the gel. These reference spots represent proteins involved mainly in the metabolism of carbohydrates, lipids or C<sub>1</sub> units, in the biosynthesis of nucleotides, or in response to stress and in oxidative phosphorylations (data not shown).

We then compared the protein synthesis patterns (proteome) of the wild-type strain grown on sulfate or glutathione. For both sulfur sources, two independent protein preparations were made and for each preparation at least two gels were run, silver-stained and analysed. This allowed us to identify 24 proteins whose synthesis was up- or down-regulated (by at least a factor of 2) in the presence of one of these sulfur sources (Fig. 1). The synthesis of 10 proteins was increased in the presence of glutathione while 14 others were more abundant in the presence of sulfate (Fig. 1). These 24 spots were cut out of the gel, digested with trypsin and submitted to MALDI TOF spectrometry. In these conditions, 15 spots were unambiguously identified (Table 2) while 9 others remained unidentified. In the presence of glutathione, a significant increase in the synthesis of uptake systems (DppE, SsuA), of an oxygenase (SsuD), of two proteins of unknown function (YtmI, YurL) and of cysteine synthase (CysK) was observed. After growth with sulfate, the amount of proteins involved in the metabolism of C<sub>1</sub> units (SerA, GlyA, FodD), in the biosynthesis of purines (PurQ, Xpt) and pyrimidines (Upp, PyrAA, PyrF) and in the biosynthesis of chorismate (AroF) was increased (Table 2). However, the alteration in synthesis of these proteins could be due either to the regulation in response to sulfur limitation or to a more general effect related to the reduction of growth rate.

Slot-blot analysis was used to determine if the changes in the quantities of GlyA, SerA, FodD, PurQ, Upp, PyrAA and PyrF observed by 2D gel analysis resulted from altered transcription of the corresponding genes. Total RNA was extracted after growth of strain 168 in minimal medium containing glutathione or sulfate. Increasing amounts of these RNAs were hybridized with DIG-labelled probes covering *serA*, *glyA*, *fodD*, *purQ*,



**Fig. 2.** Quantitative analyses of the expression of the genes regulated by the sulfur source. (a) Slot-blot analyses. Total RNAs were extracted after growth on minimal medium supplemented with sulfate or glutathione. Various amounts (50 ng for *purQ*; 100 ng for *glyA*; 200 ng for *serA*, *pyrF*; 400 ng for *ytml*, *dppE*, *upp* and *aroF*) of RNAs were blotted onto nylon membranes and hybridized with DIG-labelled probes specific for the genes indicated. (b) Quantification of the transcription levels. The chemiluminescent signals of the slot-blot were quantified using the Image Master 1D software. The ratios between means from two independent experiments were calculated for each gene and each growth condition.

*upp*, *pyrF* and *aroF* (Fig. 2a and data not shown). In the presence of sulfate, the expression of the *serA*, *glyA*, *purQ*, *upp*, *pyrF* and *aroF* genes was increased 5-, 3.5-, 1.7-, 2-, 2- and 1.8-fold, respectively, as compared to their expression in the presence of glutathione (Fig. 2b). No detectable variation was observed in the *fodD* transcription level (data not shown). The  $\beta$ -galactosidase activity of a strain carrying an *xpt-lacZ* fusion was also 3-fold higher during growth in the presence of

**Table 3.** Effect of different sulfur sources on the growth rates of the wild-type and  $\Delta ytmI$  mutant and on the expression of a  $ytmI-lacZ$  fusion in different backgrounds

IPTG was added to the medium. The *pspac* promoter controls the transcription of the genes downstream of *ytmI* in an IPTG-dependent fashion (Vagner *et al.*, 1998). The  $\beta$ -galactosidase activities were obtained from cultures in the middle of exponential growth. These values represent means of at least two independent experiments.

Sulfur source	Generation time (min)		$\beta$ -Galactosidase activity [U (mg protein) <sup>-1</sup> ]	
	Wild-type (168)	$\Delta ytmI$ (BFS71)	<i>ytmI-lacZ</i> (BFS71)	<i>ytmI-lacZ</i> $\Delta ytmI$ (BSIP1223)
Glutathione	180	190	2481	27
Taurine	155	> 500	2170	12
Methionine	55	55	2065	30
Sulfate	50	50	7	1.6
Cysteine	80	80	3	1.3
Thiosulfate	55	55	3.2	1.3

sulfate when compared to the activity measured in the presence of glutathione [300 U (mg protein)<sup>-1</sup> and 95 U (mg protein)<sup>-1</sup>, respectively]. The modification of expression of these genes in the two conditions tested seems to occur at the transcriptional level.

#### Increased synthesis of proteins involved in sulfur metabolism in the presence of glutathione

In *B. subtilis*, the synthesis of three proteins clearly involved in sulfur metabolism (CysK, SsuA and SsuD) was enhanced in the presence of glutathione.

Cysteine synthase, CysK, catalyses the synthesis of L-cysteine from sulfide and O-acetylserine (Kredich, 1996). The amount of this protein was increased fivefold in the presence of glutathione as revealed by 2D gel analysis (Table 2). To test the regulation of expression of the *cysK* gene, strain BSIP1207, carrying a *cysK-lacZ* transcriptional fusion, was constructed. This strain was grown in minimal medium in the presence of sulfate or glutathione as sulfur source.  $\beta$ -Galactosidase activity was 265 U (mg protein)<sup>-1</sup> after growth in the presence of glutathione compared to 50 U (mg protein)<sup>-1</sup> in the presence of sulfate. A comparable repression was observed after growth in the presence of cysteine [the  $\beta$ -galactosidase activity was 28 U (mg protein)<sup>-1</sup>].

The SsuA and SsuD proteins are involved in the utilization of sulfonates in *B. subtilis* (van der Ploeg *et al.*, 1998). SsuA is the binding protein of an ABC permease system for aliphatic sulfonates and SsuD is likely to be a monooxygenase (Eichhorn *et al.*, 1999). These two proteins were not detected after growth with sulfate (Fig. 1, Table 2). The expression of an *ssuA-lacZ* fusion appeared to be upregulated 600-fold in the presence of glutathione and 1000-fold in the presence of methionine when compared with its expression level in the presence of sulfate. The  $\beta$ -galactosidase activities measured in the presence of these sulfur sources were 1109, 1646 and 1.6 U (mg protein)<sup>-1</sup>, respectively. The regulation of this operon is therefore likely to occur at

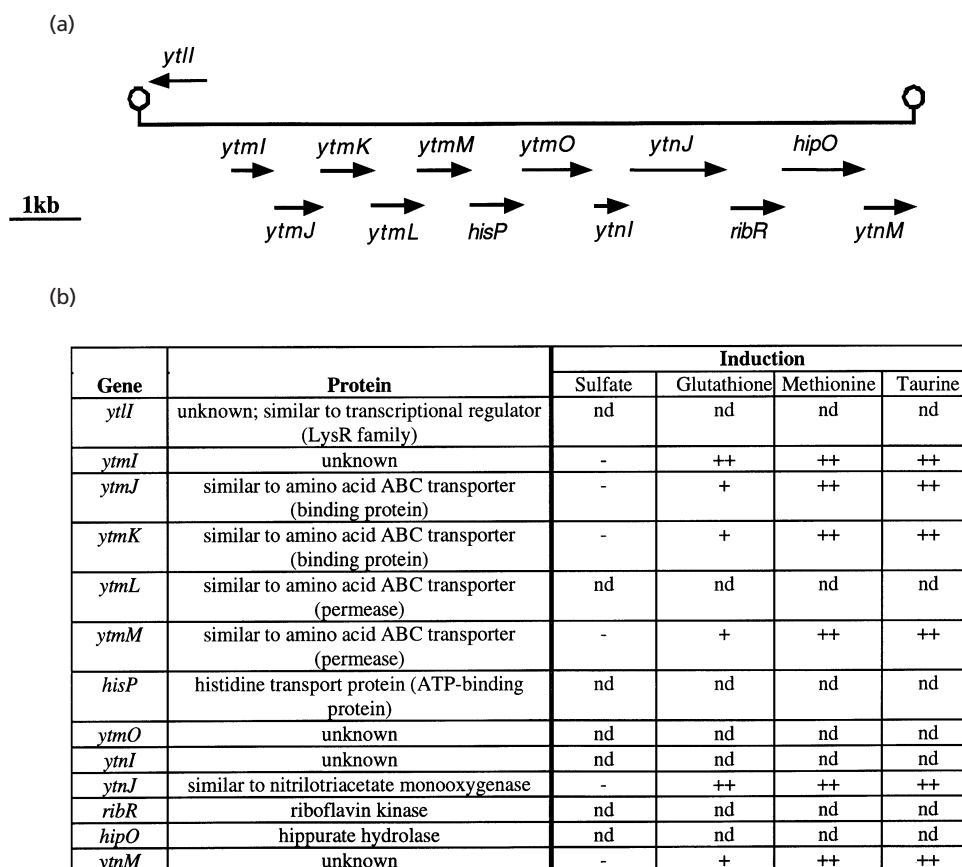
the transcriptional level, as previously observed (van der Ploeg *et al.*, 1998).

DppE, the dipeptide-binding protein of the high-affinity ABC permease of dipeptides (Mathiopoulos *et al.*, 1991), was also more abundant in cells grown on glutathione than in those grown on sulfate. Slot-blot analysis performed with a probe covering *dppE* revealed a slight induction of this gene: its expression was increased only 1.5-fold with glutathione as compared to its expression with sulfate (Fig. 2).

Two proteins of unknown function, YtmI and YurL, were detected on 2D gels only in extracts obtained after growth with glutathione (Fig. 1). YurL shares sequence similarities with the PfkB family of carbohydrate kinases (Wu *et al.*, 1991). Using a strain carrying a *yurL-lacZ* fusion the expression of this gene was shown to be induced 40-fold on glutathione [430 U (mg protein)<sup>-1</sup>] as compared to its expression on sulfate [10 U (mg protein)<sup>-1</sup>]. Expression of *yurL* is thus strongly repressed at the transcriptional level during sulfur limitation.

#### Coordinated regulation of the *ytmI* locus

The synthesis of the YtmI protein is strongly increased in the presence of glutathione. The regulation of expression of the corresponding gene and the genetic organization of the *ytmI* region were therefore studied in more detail. In order to investigate the regulation of the *ytmI* gene in response to sulfur availability, a fusion between the *ytmI* and *lacZ* genes was constructed. Strain BFS71 contains a *ytmI-lacZ* transcriptional fusion and a *ytmI* gene disruption (Table 1). The effect of several sulfur sources on the expression of this fusion was tested (Table 3). The  $\beta$ -galactosidase activity of strain BFS71 after growth on glutathione was 350-fold higher than that after growth on sulfate. This result confirms the data obtained by 2D gel analysis and indicates that expression of the *ytmI* gene is regulated at the transcriptional level. The expression of this gene was also



**Fig. 3.** Analysis of the chromosomal region containing *ytmI*. (a) Organization of the *ytmI* locus. For each gene, the direction of transcription is indicated by an arrow whose size is proportional to the length of the corresponding gene. Putative transcription terminators are represented by loops. (b) Sulfur regulation of the genes located at the *ytmI* locus. For each gene product, similarities with proteins of known function are indicated according to SubtiList (<http://genolist.pasteur.fr/SubtiList>). When *lacZ* fusions were available, the expression of these genes was analysed on solid minimal medium supplemented with sulfate, glutathione, methionine or taurine as sole sulfur source (-, no expression; +, weak expression; ++, high expression; nd, not determined).

strongly increased in the presence of taurine, isethionate, methanesulfonate and methionine (Table 3 and data not shown). In contrast, the transcription of *ytmI* appeared to be very weak in the presence of sulfate, thiosulfate and cysteine (Table 3).

The *ytmI* gene belongs to a locus of 12 genes which are transcribed in the same direction (Fig. 3a). No terminator could be identified within this locus, suggesting that it forms an operon (d'Aubenton Carafa *et al.*, 1990). Transcriptional fusions of *lacZ* and *ytmJ*, *ytmK*, *ytmM*, *ytnJ* or *ytnM* were constructed as described for *ytmI* (Table 1). These fusions were tested on plates containing minimal medium supplemented with sulfate, glutathione, methionine or taurine as sole sulfur source. For all the fusions tested, we observed a minimal expression level in the presence of sulfate and a clear increase in expression with taurine, glutathione or methionine (Fig. 3b). These results indicate a co-regulation of all these genes. In contrast, the *ytmI-lacZ* fusion (*ytmI* is downstream of *ytmM*) did not respond to any regulation by the sulfur source. These results strongly suggest the

existence of a large operon (from *ytmI* to *ytmM*) regulated in response to sulfur availability. The role of these genes in sulfur assimilation in *B. subtilis* is not yet known. However, a significant alteration of the growth on taurine of the strain carrying a *ytmI* gene disruption (BFS71) was observed, while its growth with sulfate, methionine, cysteine and thiosulfate was unaffected (Table 3). This result indicates that at least one of the genes of the *ytmI* locus is directly or indirectly involved in the assimilation of taurine. This growth defect on taurine could be due to the involvement of *ytmI* in taurine utilization but also to polar effects on genes located downstream of *ytmI*.

The YtmJ, YtmK, YtmL, YtmM and HisP polypeptides share similarities with amino acid ABC transport systems (<http://www.biology.ucsd.edu/~ipaulsen/transport>). We therefore tested the possible role of this ABC permease in the uptake of sulfur-containing amino acids. The *ytmM* mutant was able to grow with methionine and cysteine as sulfur sources (data not shown). This indicates either that this ABC permease is

probably not involved in the uptake of these two amino acids or that there exist several uptake systems for these amino acids, as observed for methionine in *E. coli* (Greene, 1996).

### YtlI positively regulates the expression of the *ytml* gene in response to sulfur availability

The *ytll* gene is located upstream of *ytml* and is transcribed divergently. The product of the *ytll* gene displays significant similarities with the LysR family of transcriptional regulators. The YtlI protein might therefore encode the transcriptional activator of the *ytml* gene. To test this possibility, a kanamycin-resistance cassette was inserted into the *ytll* gene. Chromosomal DNA from strain BSIP1214 (*ytll::aphA3*) was used to transform strain BFS71 containing a *ytml-lacZ* fusion, resulting in strain BSIP1223. The  $\beta$ -galactosidase activity was measured after growth of the wild-type strain and the *ytll* mutant in the presence of various sulfur sources (Table 3). Disruption of the *ytll* gene led to the complete loss of *ytml* expression in the presence of methionine, taurine or glutathione as sulfur source. This indicates that *ytll* positively regulates the expression of *ytml* in response to the availability of sulfur sources. The *ytll* gene probably controls the expression of the entire *ytml* locus. Indeed, when *ytll* is disrupted, we also observed lack of expression of the *ytNJ* and *ytmO* genes in the presence of either taurine, glutathione or methionine (data not shown). The involvement of YtlI in the regulation of the expression of the *serA*, *glyA*, *ssuA*, *cysK* and *yurL* genes was also investigated. The inactivation of *ytll* did not modify the expression pattern of *serA-lacZ*, *ssuA-lacZ*, *yurL-lacZ* and *cysK-lacZ* fusions (data not shown). Slot-blot analyses revealed that YtlI does not regulate the expression of *glyA*. Indeed, this expression remained 3.5-fold higher on sulfate than on glutathione if *ytll* was inactivated (data not shown). One may therefore conclude that YtlI is probably not involved in the global regulation of transcription of these genes in response to sulfur availability.

## DISCUSSION

The metabolism of sulfur in Gram-positive bacteria remains poorly understood despite its importance for the synthesis of many cofactors and amino acids. To get insight into the sulfur metabolism of *B. subtilis*, we have compared the proteome of a wild-type strain grown with either sulfate or glutathione as sole sulfur source. Glutathione occurs in animal tissues, plants and many micro-organisms in significant concentrations (Meister, 1988). In higher eukaryotes, reduced glutathione is metabolized by peptide cleavage, which yields glycine and cysteine. In *B. subtilis*, the transport and metabolic pathways of glutathione are not clear (Sekowska *et al.*, 2000). Glutathione is probably slowly degraded into cysteine, so that growth with this sulfur source induces the expression of sulfur-scavenging proteins in *E. coli* and *B. subtilis* (van der Ploeg *et al.*, 1997, 1998).

Using MALDI TOF spectrometry, 15 proteins whose synthesis is up- or down-regulated by sulfate or glutathione have been identified. Nine other sulfur-limitation-regulated (Slr) proteins remained unidentified. For seven of them, this could be attributed to their low molecular mass (25 kDa). In the presence of sulfate, the amount of proteins involved in the metabolism of C<sub>1</sub> units (SerA, GlyA, FodD), in the biosynthesis of nucleotides (PurQ, Upp, PyrAA, PyrF, Xpt) and in the synthesis of chorismate (AroF) is increased. However, the increase of expression of the corresponding genes is weak (Fig. 2). At this time, it is difficult to discriminate between a modification of expression due to differences in the growth rate, a general derepression during nutrient starvation or a specific regulation during sulfur limitation.

Analysis of the proteome of the wild-type strain grown in the presence of glutathione revealed that the synthesis of at least six proteins is significantly enhanced. Five of them are related to ABC transport and/or desulfonation systems: SsuA, SsuD, DppE, YurL and YtmI. ABC permeases which actively transport substrates (e.g. sugars, amino acids, peptides) across biological membranes (Quentin *et al.*, 1999) probably play an essential role in the adaptation of *B. subtilis* to its environment.

The *yurL* gene is located in the *yurONML* region. These genes encode proteins sharing similarities with sugar ABC permeases and sugar kinases. Surprisingly, YurL contains six cysteine residues while all the other proteins induced by glutathione contain no or one cysteine residue, like the majority of the *B. subtilis* proteins (Danchin *et al.*, 2000). A similar specific induction of proteins containing very few cysteine residues under sulfur limitation conditions had been previously described for *E. coli* (van der Ploeg *et al.*, 1996) and *Calothrix* (Mazel & Marlière, 1989). The up-regulation of a cysteine-rich protein such as YurL under sulfur-limiting conditions might not be specific for sulfur deprivation but reflect a more general induction due to nutrient limitation.

The *dppE* gene belongs to an operon encoding an ABC permease for dipeptides (Mathiopoulos *et al.*, 1991). This operon, which is upregulated rapidly after induction of sporulation, may facilitate adaptation to nutrient limitation instead of favouring sporulation. The regulation of *dppE* expression might be a way for the cell to scavenge sulfur-containing peptides in response to sulfur limitation.

Proteins directly related to sulfur metabolism are also more abundant in the presence of glutathione: CysK, SsuA and SsuD. CysK, which catalyses the biosynthesis of cysteine from acetylserine and sulfide, is involved in the sulfate-starvation response of *E. coli*. In this bacterium, reduced-sulfur limitation is known to derepress the genes of the cysteine biosynthetic pathway (Kredich, 1996). Based on sequence similarity with the SsuD protein from *E. coli* (63% identity), SsuD of *B. subtilis* is probably a monooxygenase which catalyses the oxygenolytic conversion of sulfonates to sulfite and the

corresponding aldehydes (Eichhorn *et al.*, 1999). SsuA displays 32% identity with the aliphatic sulfonate binding protein of *E. coli* (van der Ploeg *et al.*, 1999). In *B. subtilis*, the expression of the *ssu* operon is repressed by sulfate and cysteine, while it is strongly induced by glutathione, taurine and methionine, and to a lesser extent by sulfonates (this work; van der Ploeg *et al.*, 1998). In *E. coli*, the *ssuEADCB* operon (utilization of a broad range of sulfonates) and the *tauABCD* operon (utilization of taurine) are also upregulated in the absence of sulfate and cysteine (van der Ploeg *et al.*, 1996, 1999). When sulfate and sulfonates are present, *E. coli* preferentially uses the inorganic sulfur source and represses the enzymes involved in the degradation of organosulfur compounds. Full expression of the genes involved in sulfonate utilization requires two LysR-type activators, CysB and Cbl (Kredich, 1996; Iwanicka-Nowicka & Hryniewicz, 1995).

The *ytml* gene is located in a locus containing 12 genes which seem to form an operon. The *ribR* gene encodes a riboflavin kinase (Solovieva *et al.*, 1999). Based on sequence similarities a potential function has been assigned to seven of these genes (Fig. 3). Five of them (*ytmJ*, *ytmK*, *ytmL*, *ytmM*, *hisP*) are part of ABC transport systems. Interestingly, two different binding proteins, YtmJ and YtmK, are present, suggesting that this transport system is involved in the uptake of two different substrates. The *ytmJ* gene product shares similarities with monooxygenases (30–40% identity) and that of *hipO* with amino acid amidohydrolases (35–40% identity). The functions of YtmL, YtmO and YtmM are unknown, while YtmI shares weak similarities with a putative glutaredoxin from *Thermotoga maritima*. The significant alteration in growth on taurine of a *ytml* mutant suggests that at least one of these 12 genes plays a role in taurine assimilation. These genes are probably involved in the uptake and oxidation of alternative sulfur sources. The elucidation of their role in sulfur assimilation in *B. subtilis* deserves further investigation.

The *ytml* gene is weakly expressed during growth with sulfate, cysteine and thiosulfate while its expression is strongly increased in the presence of glutathione, taurine and methionine. The *ytmJ*, *ytmK*, *ytmM*, *ytmI* and *ytmN* genes are regulated in the same way. We therefore propose that either some intermediary of the cysteine biosynthetic pathway mediates repression, or the expression of this operon is induced during sulfur limitation (glutathione and taurine) and in the presence of methionine.

Interestingly, YtlI, a LysR-type regulator, is the transcriptional activator of the *ytml* operon. YtlI is the first regulator involved in the control of expression in response to sulfur availability to be identified in *B. subtilis*. In *E. coli*, three LysR-type proteins play a role in the regulation of sulfur metabolism: Cbl, CysB and MetR (Iwanicka-Nowicka & Hryniewicz, 1995; Kredich, 1996; Greene, 1996). However, YtlI seems not to be involved in the global regulation of the sulfur

limitation response in *B. subtilis* as it is not responsible for the regulation of *serA*, *glyA*, *ssuA*, *cysK* and *yurL*.

Several mechanisms for regulation of sulfur metabolism exist in *B. subtilis*. The S-box sequence involved in premature termination of transcription is found upstream of several genes involved in the methionine biosynthetic pathway (Grundy & Henkin, 1998). In the present work, we have identified genes regulated in response to sulfur availability and the YtlI protein, a positive regulator of expression of the *ytml* operon. The *cysK*, *yurL*, *glyA* and *serA* genes and the *ssu* operon are regulated by other mechanisms. The identification of other regulators is a challenging question for the future.

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