

The surface (S)-layer gene *cspB* of *Corynebacterium glutamicum* is transcriptionally activated by a LuxR-type regulator and located on a 6 kb genomic island absent from the type strain ATCC 13032

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The surface (S)-layer gene region of the Gram-positive bacterium *Corynebacterium glutamicum* ATCC 14067 was identified on fosmid clones, sequenced and compared with the genome sequence of *C. glutamicum* ATCC 13032, whose cell surface is devoid of an ordered S-layer lattice. A 5.97 kb DNA region that is absent from the *C. glutamicum* ATCC 13032 chromosome was identified. This region includes *cspB*, the structural gene encoding the S-layer protomer PS2, and six additional coding sequences. PCR experiments demonstrated that the respective DNA region is conserved in different *C. glutamicum* wild-type strains capable of S-layer formation. The DNA region is flanked by a 7 bp direct repeat, suggesting that illegitimate recombination might be responsible for gene loss in *C. glutamicum* ATCC 13032. Transfer of the cloned *cspB* gene restored the PS2⁻ phenotype of *C. glutamicum* ATCC 13032, as confirmed by visualization of the PS2 proteins by SDS-PAGE and imaging of ordered hexagonal S-layer lattices on living *C. glutamicum* cells by atomic force microscopy. Furthermore, the promoter of the *cspB* gene was mapped by 5' rapid amplification of cDNA ends PCR and the corresponding DNA fragment was used in DNA affinity purification assays. A 30 kDa protein specifically binding to the promoter region of the *cspB* gene was purified. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry and peptide mass fingerprinting of the purified protein led to the identification of the putative transcriptional regulator Cg2831, belonging to the LuxR regulatory protein family. Disruption of the *cg2831* gene in *C. glutamicum* resulted in an almost complete loss of PS2 synthesis. These results suggested that Cg2831 is a transcriptional activator of *cspB* gene expression in *C. glutamicum*.

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INTRODUCTION

The surface (S)-layer of many bacteria consists of single (glyco)proteins that are assembled in two-dimensional paracrystalline arrays. The assembly process of S-layer

protomers is entropy-driven, and gives rise to networks of highly ordered ultrastructures that can completely cover the surface of a bacterial cell (Sara & Sleytr, 2000). Due to their location, S-layers are generally involved in the interaction between a bacterium and its environment. The S-layers of several pathogenic bacteria have been reported to act as virulence factors by mediating resistance to bactericidal activities (Blaser *et al.*, 1987; Merino *et al.*, 1994) and adhesion to extracellular matrix proteins of the host (Schneitz *et al.*, 1993). Furthermore, S-layers can serve as molecular sieves and act as additional stability factors for the

Abbreviations: AFM, atomic force microscopy; MALDI-TOF MS, matrix-assisted laser desorption ionization time-of-flight mass spectrometry; RACE-PCR, rapid amplification of cDNA ends PCR.

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bacterial cell envelope, in both pathogenic and non-pathogenic bacteria (Sara & Sleytr, 2000).

It has been calculated that a rod-shaped bacterium of average size with a generation time of 20 min has to synthesize around 500 S-layer subunits per second to cover the cell surface completely (Sleytr & Messner, 1983). Accordingly, S-layer genes are expressed at extremely high levels, and S-layer proteins constitute 10 to 15% of the total protein content of the cell (Messner & Sleytr, 1992). The high-level expression of S-layer genes is based not only on strong promoters and efficient transcription but also on highly stable mRNA molecules (Boot & Pouwels, 1996; Fisher *et al.*, 1988; Kahala *et al.*, 1997). However, S-layer proteins are seldom found in culture supernatants, suggesting that their synthesis is tightly regulated.

A limited number of studies have determined that the biosynthesis of S-layer proteins is controlled at the transcriptional and post-transcriptional level, involving DNA-binding transcription factors and feedback control mechanisms by the S-layer protein. The S-layer mRNAs of *Bacillus anthracis*, *Lactobacillus brevis* and *Thermus thermophilus* contain 5' untranslated regions of up to 300 bp in length that might be involved in stabilization of the transcript (Mignot *et al.*, 2002; Vidgren *et al.*, 1992). For instance, the C-terminal part of the S-layer protein SlpA of *T. thermophilus* binds to the 5' leader region of the *slpA* mRNA, providing a clue to the mechanism of post-transcriptional autoregulation during the expression of *slpA* (Fernandez-Herrero *et al.*, 1997). The highly stable S-layer mRNAs of *Caulobacter crescentus* and *L. brevis* possess long half-lives of 10–15 min and 32 min, respectively (Fisher *et al.*, 1988; Kahala *et al.*, 1997). On the other hand, S-layer proteins may act as transcription factors, regulating the expression of their own genes. For instance, the S-layer protein AbcA of *Aeromonas salmonicida* activates *in trans* the expression of the S-layer gene when analysed in the heterologous host *Escherichia coli* (Chu & Trust, 1993; Noonan & Trust, 1995). S-layer gene expression of *B. anthracis* is moreover controlled by alternative sigma factors and the transcriptional master regulator AtxA (Mignot *et al.*, 2004).

The outermost surface of *Corynebacterium glutamicum*, a Gram-positive bacterium of great biotechnological importance (Hermann, 2003), also consists of a paracrystalline S-layer whose protomers are anchored in an outer-membrane-like structure (Chami *et al.*, 1997). The S-layer of *C. glutamicum* possesses a hexagonal lattice symmetry (Chami *et al.*, 1995), and has been classified into the M₆C₃-layer type by atomic force microscopy (Scheuring *et al.*, 2002). The S-layer of *C. glutamicum* is formed by the so-called PS2 protein, which is encoded by the *csfB* gene (Peyret *et al.*, 1993). Recently, a set of *C. glutamicum* isolates has been analysed systematically with respect to the presence of an S-layer that is found to be absent only in the completely sequenced type strain *C. glutamicum* ATCC 13032 (Hansmeier *et al.*, 2004). Sequence-based analyses of 28 different PS2 proteins have revealed a direct coherence between the primary sequence of

S-layer proteins and the corresponding morphology of S-layers (Hansmeier *et al.*, 2004).

First hints regarding the regulation of S-layer gene expression in *C. glutamicum* were published by Chami *et al.* (1995), who showed that *C. glutamicum* cells grown on solid medium are completely covered with an S-layer, whereas cells cultivated in liquid medium are only partially covered by an ordered lattice. Subsequent studies have demonstrated that the amount of S-layer protein synthesized by *C. glutamicum* is dependent on the carbon source of the growth medium (Soual-Hoebeke *et al.*, 1999). In particular, the addition of lactate to the growth medium has a stimulatory effect on PS2 production as well as on S-layer formation, whereas an inhibitory effect on PS2 synthesis is observed when glucose is used as carbon source. On the basis of these observations, a relationship between S-layer formation, carbohydrate metabolism and the physiological status of the cell in general has been suggested (Soual-Hoebeke *et al.*, 1999).

In the present study, the S-layer gene region of *C. glutamicum* ATCC 14067 was identified, sequenced and subsequently compared with the genome sequence of *C. glutamicum* ATCC 13032 to get clues on why this strain is devoid of an ordered S-layer. Furthermore, we examined the transcriptional regulation of the *C. glutamicum* S-layer gene and identified a putative activator of the *csfB* gene.

METHODS

Bacterial strains and growth conditions. *C. glutamicum* strains used in this study were obtained from the American Type Culture Collection. *E. coli* DH5 α MCR (Grant *et al.*, 1990) was used as host for recombinant plasmids. *E. coli* strains were routinely grown at 37 °C on solid Luria broth complex medium (Invitrogen). Selection for the presence of plasmids in *E. coli* was performed by addition of 50 μ g kanamycin ml⁻¹ or 100 μ g ampicillin ml⁻¹ to the growth medium. *C. glutamicum* strains were cultivated on solid Luria broth complex medium or in liquid minimal medium MM1 [MMYE without yeast extract (Katsumata *et al.*, 1984)]. Antibiotics for the selection of plasmids in *C. glutamicum* were kanamycin (25 μ g ml⁻¹) and chloramphenicol (5 μ g ml⁻¹).

DNA preparation, manipulation and transformation. Genomic DNA of *C. glutamicum* was isolated with the GenElute Bacterial Genomic DNA kit (Sigma-Aldrich) according to the protocol for Gram-positive bacteria. Plasmid and fosmid DNA was prepared from *E. coli* by the alkaline lysis technique using the QIAprep Spin Miniprep kit (Qiagen). Alkaline lysis of *C. glutamicum* cells was modified by using 20 mg lysozyme ml⁻¹ in resuspension buffer P1 at 37 °C for 2 h. All DNA manipulations were carried out as described by Sambrook *et al.* (1989). DNA transfer into competent *C. glutamicum* cells was performed by electroporation (Bonamy *et al.*, 1990).

PCR techniques. PCR experiments were performed with a PTC-100 thermocycler (MJ Research). Amplification of DNA was carried out with *Pfx* DNA polymerase according to the manufacturer's protocol (Invitrogen). Screening of the fosmid library by PCR was performed with the Qiagen *Taq* DNA polymerase. Oligonucleotides used in this study were purchased from Qiagen. PCR cycling times and temperatures were chosen according to the type of DNA polymerase, fragment length and oligonucleotide sequence. PCR products were purified by means of the QIAquick PCR Purification kit (Qiagen).

Construction of a *cg2831* mutant of *C. glutamicum*. To construct a *cg2831* insertion mutant of *C. glutamicum* ATCC 13058, a 469 bp DNA fragment was amplified by PCR using the primer pair Int1 (AAGCCAGCACGTCCAAC) and Int2 (TCCGGTTGGTGTGAGTGA). The PCR product was cloned into the *Sma*I site of pK18*mob* (Schäfer *et al.*, 1994), and the resulting plasmid pK18*mob*-Intcg2831 was transferred to *C. glutamicum* by electroporation. Gene disruption of *cg2831* was confirmed by Southern hybridization (Sambrook *et al.*, 1989).

Construction and screening of a *C. glutamicum* ATCC 14067 fosmid library. A genomic library of *C. glutamicum* ATCC 14067 was prepared in the pCC1FOS fosmid vector by IIT Biotech (Bielefeld). Briefly, genomic DNA of *C. glutamicum* was randomly sheared to give 40 kb fragments that were ligated into pCC1FOS. The ligated DNA was packaged with MaxPlax Lambda Packaging Extracts and transduced into *E. coli* EPI300 cells that were spread on Luria broth agar plates containing 12.5 µg chloramphenicol ml⁻¹. The resulting fosmid library was screened by applying PCR experiments with the *cskB*-specific primers *cskB_S1* (TGCTGGTCAATCGCAAT) and *cskB_S2* (AGAATGCTCGTCCGAACA). To facilitate DNA sequencing of the *cskB* gene region, fosmid clone pFOS-D1 was restricted either with *Eco*RI or with *Hind*III, and the resulting DNA fragments were subcloned in pUC19 (Yanisch-Perron *et al.*, 1985). Subclones pUC19*Eco*RI-01 and pUC19*Hind*III-12 were selected for DNA sequencing by primer walking (IIT Biotech). The nucleotide sequence of the *cskB* gene region was assembled with the STADEN software package (Staden, 1996). Annotation of the sequence was performed with the help of BLAST algorithms and Conserved Domain Database searches (Altschul *et al.*, 1997; Marchler-Bauer *et al.*, 2003).

Separation and identification of *C. glutamicum* proteins by SDS-PAGE and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS). S-layer proteins were extracted from the cell surface of *C. glutamicum* as described previously (Hansmeier *et al.*, 2004). The proteins were separated by one-dimensional 12.5% SDS-PAGE using the technique of Laemmli (1970). Gels were stained using Coomassie brilliant blue R-250 and G-250 (Sambrook *et al.*, 1989), and were briefly destained with 7% acetic acid to visualize protein bands. Molecular masses were determined by using the Broad Range Precision Protein Standard (Bio-Rad Laboratories) or the Prestained Precision Protein Standard (MBI-Fermentas).

For the identification of proteins, bands were excised from Coomassie-stained SDS-PAGE gels. Then, tryptic digestions and MALDI-TOF MS analysis were applied to generate peptide mass fingerprints (Hansmeier *et al.*, 2004). The Bruker Ultraflex MALDI-TOF mass spectrometer was used to generate mass spectra. Peptide mass fingerprints were compared with *in silico*-generated tryptic fingerprints by using the MASCOT software (Perkins *et al.*, 1999).

DNA affinity purification assay. To isolate proteins that bind to the putative promoter region of the *C. glutamicum cskB* gene, a DNA affinity purification method was used (Rey *et al.*, 2003). DNA fragments pF1-2 and pF3-4 were generated by PCR using the biotin-labelled oligonucleotides pF1 (GTAGTCCGAGGTTAAGTG), pF2 (GCAGCCTGTGTTGAGAA-BioTAG), pF3 (CAACGACAG-GCTGCTAAG) and pF4 (CAGTGC GGATACGATTGT-BioTAG). Unincorporated oligonucleotides and dNTPs were removed by means of the PCR Purification Spin kit (Qiagen). About 600 pmol of the biotin-labelled PCR products was immobilized to 200 µl streptavidin-coated magnetic particles (KMF Laborchemie). The magnetic particles were then equilibrated with protein binding buffer (20 mM Tris, 1 mM EDTA, 10% (v/v) glycerol, 0.05% (v/v) Triton X-100, 1 mM DTT, 100 mM NaCl, pH 8.0) and incubated with *C. glutamicum* crude cell extracts prepared from exponentially growing cultures. Unbound proteins were removed by using magnetic

separation with a magnetic particle concentrator and by two washing steps with protein binding buffer. DNA-bound proteins were finally eluted with 20 µl protein binding buffer containing 1 M NaCl. Eluted protein fractions were collected and analysed by SDS-PAGE and MALDI-TOF MS.

Atomic force microscopy (AFM) of *C. glutamicum* cells. *C. glutamicum* cells were cultivated on solid Luria broth medium for 2 days. The cells were washed twice in washing buffer (20 mM Tris/HCl, pH 7.5) and incubated on glass plates overnight. AFM images were recorded in tapping mode with silicon cantilevers (BS-Tap300AL, Budget Sensors) under ambient conditions by using a Nanoscope IIIa AFM system equipped with a Bioscope head (Veeco). AFM topographs and phase images were recorded simultaneously.

RNA isolation, rapid amplification of cDNA ends (RACE)-PCR and real-time RT-PCR. To isolate total RNA from *C. glutamicum* cells, cultures were grown in Luria broth medium. Approximately 1 × 10⁹ cells were harvested and total RNA was purified by using the RNeasy kit (Qiagen). To identify the transcription start site of the *C. glutamicum cskB* gene, 5' RACE-PCR experiments (Roche Diagnostics) were carried out with the specific oligonucleotides *cskB_sp1a* (CAACTGGCTGGATGGTGGAT), *cskB_sp1b* (GAAGC-CGTTGGTGATGTTGA) and *cskB_sp1c* (GTTGGTCTCCTGAGC-GAATG). Real-time RT-PCR was performed with the QuantiTect SYBR Green PCR kit (Qiagen) and the LightCycler system (Roche). Cycling times and temperatures were chosen according to the Light-Cycler manufacturer's protocols in dependence on the oligonucleotide sequences (*cskB*1, TGGTAAGCAACGGACTC; *cskB*2, GGC-TTCAACGATGCTGAT; *cg2831*1, AAGCCAGCACGTCCAAC; *cg2831*2, TCCGGTTGGTGTGAGTGA). Determination of crossing points was done by using the second derivative maximum data analysis method. The crossing point of the wild-type was used for determining relative gene expression (Pfaffl, 2001). All real-time RT-PCR experiments were carried out in duplicate with three biological replicates.

RESULTS

The *C. glutamicum* S-layer gene *cskB* is located on a 5.97 kb DNA fragment flanked by a 7 bp direct repeat and is absent in the *C. glutamicum* ATCC 13032 genome

In order to identify components necessary for S-layer gene expression and formation, we cloned and sequenced the *cskB* gene region of the wild-type strain *C. glutamicum* ATCC 14067 (formerly named *Brevibacterium flavum*), which is capable of synthesizing the S-layer protomer PS2. A genomic library of randomly sheared chromosomal DNA fragments of *C. glutamicum* ATCC 14067 was constructed in fosmid vector pCC1FOS. About 1400 individual clones, representing an estimated 16-fold genome coverage based on the complete sequence of *C. glutamicum* ATCC 13032, were transferred into *E. coli* EPI300 and used for further experiments. The fosmid library was screened for the presence of the S-layer gene by PCR techniques, using the *cskB*-specific primer pair *cskB_S1* and *cskB_S2* and a defined combination of DNA pools of clones from microtitre plates (Capela *et al.*, 1999). Despite the high genome coverage of the fosmid library, only four fosmids revealed a PCR fragment of the expected size of about 1 kb, representing an internal fragment of the *cskB* gene (data not shown). Fosmid

pFOS-D1 was selected for further cloning, since it covered the entire *ospB* gene region. Fosmid DNA was digested with either *EcoRI* or *HindIII*, and restriction fragments were subcloned into pUC19. Two DNA fragments with sizes of approximately 5 and 6 kb overlapped the *ospB* gene region, and were finally chosen for nucleotide sequencing. A DNA region of 11 294 bp was determined on both strands by using a primer-walking strategy. The DNA sequence was analysed and annotated with the BLAST tools and by Conserved Domain Database searches. The resulting annotation and relevant characteristics of the predicted proteins are summarized in Table 1.

A total of 13 coding sequences were predicted on the sequenced *ospB* gene region. Comparison with the known genome sequence of *C. glutamicum* ATCC 13032 (Kalinowski *et al.*, 2003) showed that the deduced gene products of *bf2708* to *bf2713* are highly similar to proteins (Cg2708 to Cg2713) of the PS2⁻ strain *C. glutamicum* ATCC 13032 (Table 1). Most interestingly, alignments of *bf2714a* with the genome sequence of *C. glutamicum* ATCC 13032 showed 99% identity to the 5' region of *cg2714* (nucleotides 1–385), whereas part of *bf2714f* was nearly identical to the 3' region (nucleotides 378–879) of *cg2714*. Consequently, the 5' and 3' ends of *cg2714* are separated in *C. glutamicum* ATCC 14067 by an additional 5.97 kb DNA segment. This DNA segment is flanked by the 7 bp direct repeat CGCGATG, which represents the deduced border of the *ospB* gene region additionally present in the *C. glutamicum* ATCC 14067 chromosome. Accordingly, *cg2714* is most likely a chimeric coding sequence, resulting from a defined event of gene loss in the chromosome of *C. glutamicum* ATCC 13032.

In addition to the *ospB* gene, six coding sequences, designated *bf2714a* to *bf2714f*, were identified on the sequenced DNA fragment of *C. glutamicum* ATCC 14067. All coding sequences were preceded by a ribosome-binding site in front of the predicted translational start codon. Palindromic stem-loop structures resembling transcriptional terminators were predicted downstream of each coding sequence, with the exception of *bf2714e*. The deduced proteins for *bf2714a* to *bf2714f* revealed similarities to alcohol dehydrogenases, amidohydrolases, oxidoreductases and carboxylases of different organisms (Table 1). According to this bioinformatic prediction, no functional link of the six proteins to S-layer synthesis was apparent, suggesting that S-layer expression and formation might be possible in the PS2⁻ strain *C. glutamicum* ATCC 13032 solely by transferring the *ospB* structural gene.

The *ospB* gene region is conserved in several *C. glutamicum* wild-type strains

Earlier examination of 28 different *C. glutamicum* isolates revealed a general occurrence of the S-layer gene *ospB*, with the exception of the sequenced type strain *C. glutamicum* ATCC 13032 (Hansmeier *et al.*, 2004). To determine how conserved the genetic arrangement of the *ospB* gene region is in different *C. glutamicum* strains, a systematic PCR

mapping approach was applied (Fig. 1a). According to a previously published classification scheme based on sequence and structural similarities of the corynebacterial S-layer, *C. glutamicum* wild-type strains are divided into five classes (Hansmeier *et al.*, 2004). To gain a wide diversity of *C. glutamicum* isolates in the present study, one representative strain of each class was chosen for the PCR mapping approach, namely *C. glutamicum* ATCC 17965 (class 1), *C. glutamicum* 22243 (class 2), *C. glutamicum* ATCC 13058 (class 3), *C. glutamicum* ATCC 31808 (class 4) and *C. glutamicum* ATCC 14017 (class 5). The nucleotide sequence of the *ospB* gene region from *C. glutamicum* ATCC 14067 was used to design 13 primer pairs capable of amplifying a distinct set of DNA fragments that should completely cover structurally similar gene regions in other wild-type strains (Fig. 1a). Accordingly, chromosomal DNA of *C. glutamicum* ATCC 14067 (class 5) served as control in PCR experiments. PCR mapping of the hitherto unknown *ospB* gene region of *C. glutamicum* ATCC 13058, a member of S-layer class 3, is shown as an example in Fig. 1(b). The resulting PCR products were analysed by agarose gel electrophoresis, and all of the amplified DNA fragments revealed the expected sizes. Mapping analysis of representatives of other S-layer classes showed identical results (data not shown). Consequently, the PCR mapping approach demonstrated that the *ospB* gene region is conserved in different *C. glutamicum* wild-type strains capable of S-layer formation.

Transfer of the *ospB* gene to *C. glutamicum* ATCC 13032 is sufficient for S-layer formation

To restore the PS2⁻ phenotype of the wild-type strain *C. glutamicum* ATCC 13032, plasmid pCGL824 was transferred by electroporation. This plasmid is a low-copy-number vector carrying the entire *ospB* gene of *C. glutamicum* ATCC 17965 under the control of its own promoter (Houssin *et al.*, 2002; Peyret *et al.*, 1993). The resulting strain *C. glutamicum* ATCC 13032 (pCGL824), as well as the control strains *C. glutamicum* ATCC 13032 and *C. glutamicum* ATCC 14067, were then cultivated on solid Luria broth complex medium. Cell surface proteins were extracted by using chaotropic detergents (Hansmeier *et al.*, 2004), and were finally visualized by SDS-PAGE (Fig. 2). Comparison of the protein profile of *C. glutamicum* ATCC 13032 (pCGL824) with that of *C. glutamicum* ATCC 13032 showed an additional protein that was of a similar size to the most prominent protein of *C. glutamicum* ATCC 14067 (Fig. 2). Both proteins were subsequently identified as PS2 proteins by MALDI-TOF MS and peptide mass fingerprinting. These data demonstrated that the *ospB* gene is efficiently expressed in *C. glutamicum* ATCC 13032, resulting in a considerable amount of PS2 protein that can be extracted from the cell surface.

To assess whether, in addition, S-layer lattices were formed by *C. glutamicum* ATCC 13032 (pCGL824), images of the bacterial cell surface were taken by AFM. For this purpose, *C. glutamicum* ATCC 13032 (pCGL824) and the control strains *C. glutamicum* ATCC 13032 and *C. glutamicum* ATCC 14067 were cultivated on solid Luria broth medium

Table 1. Molecular features of the predicted proteins encoded by the *cspB* gene region of *C. glutamicum* ATCC 14067

Feature of <i>cspB</i> protein				Related protein					
Name	Length (aa)	Size (kDa)	TM‡	Name	Organism§	Accession no.	Length (aa)	Predicted function	Percentage identity (amino acids)
Bf2708*	129	13.7	–	MsiK1	<i>C. glutamicum</i> ATCC 13032	CAF21135	376	ATPase of sugar transport system (Cg2708)	99 (1–128)
Bf2709†	228	25.1	–	PpmC	<i>C. glutamicum</i> ATCC 13032	NP601663	228	Glycosyltransferase (Cg2709)	99 (1–228)
Bf2710	227	25.8	–	Int3	<i>C. glutamicum</i> ATCC 13032	CAF22220	227	Integrase (Cg2710)	99 (1–227)
Bf2711†	141	15.6	–	–	<i>C. glutamicum</i> ATCC 13032	CAF21128	230	Secreted protein (Cg2711)	97 (1–141)
Bf2712†	185	20.5	–	–	<i>C. glutamicum</i> ATCC 13032	CAF21129	187	AraC-type regulator (Cg2712)	96 (1–187)
Bf2713†	208	22.2	–	DhaS	<i>C. glutamicum</i> ATCC 13032	CAF21130	263	Aldehyde dehydrogenase (Cg2713)	96 (56–263)
Bf2714a	366	38.4	–	AdhD	<i>C. glutamicum</i> ATCC 13032	CAF21131	292	Alcohol dehydrogenase (Cg2714)	99 (1–107)
				AreB	<i>Acinetobacter</i> sp.	AAD34026	371	Benzyl alcohol dehydrogenase	48 (5–368)
CspB	491	53.5	1	PS2	<i>C. glutamicum</i> ATCC 14067	AY842007	491	Surface-layer protein	100 (1–491)
Bf2714b	60	16.0	2	–	<i>Bu. mallei</i>	YP102219	177	Hypothetical membrane protein	25 (34–128)
Bf2714c	208	22.1	–	–	<i>B. linens</i>	ZP00378379	212	Nicotinamidase-related amidohydrolase	56 (1–205)
Bf2714d†	266	29.0	–	–	<i>R. xylanophilus</i>	ZP00352018	339	Flavin-dependent oxidoreductase	32 (116–333)
Bf2714e	284	30.5	–	–	<i>C. efficiens</i> YS-314	NP738969	298	Hypothetical protein (Ce2359)	72 (15–298)
Bf2714f	185	18.4	2	AirC	<i>C. efficiens</i> YS-314	NP738970	223	Carboxylase (Ce2360)	78 (39–223)
				AdhD	<i>C. glutamicum</i> ATCC 13032	CAF21131	292	Alcohol dehydrogenase (Cg2714)	99 (113–292)

*Deduced from truncated coding sequence due to *EcoRI* cloning.

†Defective proteins due to 5' or 3' truncation of the corresponding coding region.

‡Number of transmembrane regions predicted by TMHMM (Krogh *et al.*, 2001).

§Genus abbreviations: *B.*, *Brevibacterium*; *Bu.*, *Burkholderia*; *C.*, *Corynebacterium*; *R.*, *Rubrobacter*.

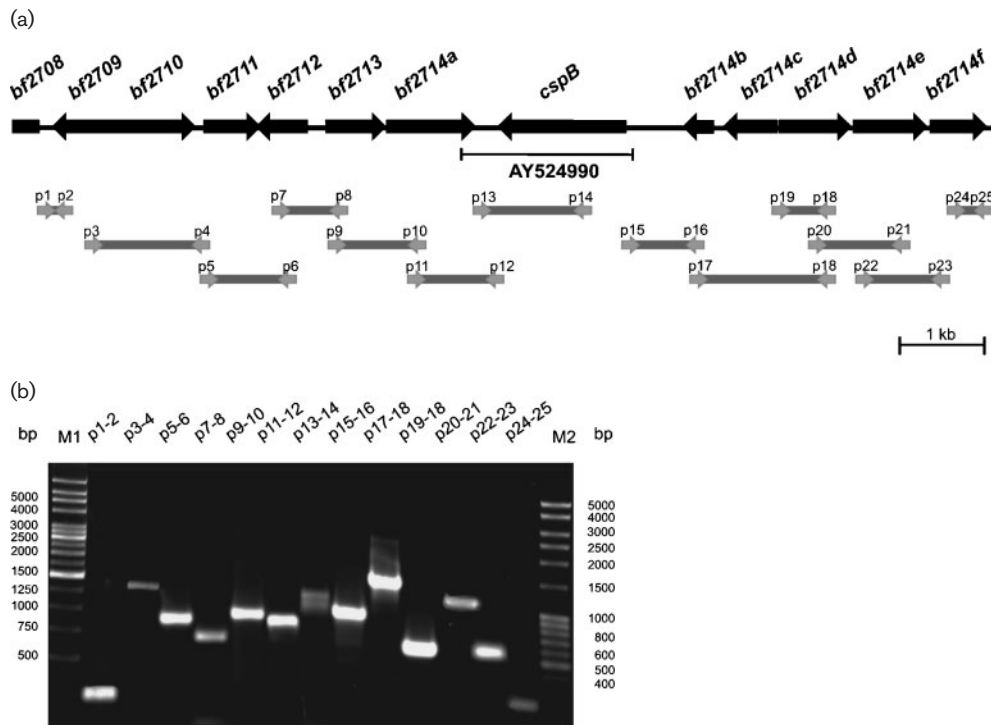


Fig. 1. Analysis of conservation of the *cspB* gene region in *C. glutamicum* ATCC 13058 by PCR experiments. (a) Schematic presentation of the *cspB* gene region as deduced from *C. glutamicum* ATCC 14067. Predicted coding sequences are marked by black arrows. Sequence AY524990 was known from a previous study (Hansmeier *et al.*, 2004). The approximate position and length of DNA fragments amplified by PCR with the primers P1 to P25 is shown. (b) Agarose gel of PCR products detected in *C. glutamicum* ATCC 13058. The specific PCR products varied in length between 283 and 1501 bp and covered the complete *cspB* gene region. Lane M1, MBL 250 bp DNA ladder; lane M2, MBL 100 bp DNA ladder. Primers used were: P1 (nucleotide position 369–386), P2 (635–652), P3 (850–867), P4 (2223–2240), P5 (2276–2293), P6 (3123–3140), P7 (3044–3061), P8 (3703–3720), P9 (3689–3706), P10 (4617–4634), P11 (4593–4610), P12 (5427–5444), P13 (5326–5343), P14 (6498–6515), P15 (7022–7039), P16 (7970–7987), P17 (7780–7797), P18 (9264–9281), P19 (8637–8654), P20 (8972–8989), P21 (10117–10134), P22 (9867–9884), P23 (10425–10442), P24 (10727–10744), P25 (11008–11025).

at 30 °C for 48 h. The cells were then washed twice with Tris/HCl buffer before they were adsorbed on glass plates for AFM imaging. Fig. 3(a) shows AFM phase images of the corynebacterial cell surfaces that were recorded in tapping mode. The cell surface of *C. glutamicum* ATCC 13032 (Fig. 3a, 1) is devoid of any ordered structure that resembles hexagonal S-layers. On the other hand, *C. glutamicum* ATCC 13032 carrying pCGL824 with the *cspB* gene (Fig. 3a, 2) and *C. glutamicum* ATCC 14067 (Fig. 3a, 3) showed ordered cell surface lattices. This structure represents the typical hexagonal S-layer of *C. glutamicum* cells (Chami *et al.*, 1997; Hansmeier *et al.*, 2004; Scheuring *et al.*, 2002). Morphological differences between the cell surfaces of *C. glutamicum* ATCC 13032 and the PS2⁺ strains *C. glutamicum* ATCC 13032 (pCGL824) and *C. glutamicum* ATCC 14067 were further emphasised by a more detailed view of selected areas of the images (Fig. 3b). Consequently, transfer of the *cspB* gene into *C. glutamicum* ATCC 13032 was sufficient for expression of the PS2 protein and formation of an ordered S-layer.

The promoter of the *cspB* gene was mapped by identifying the transcriptional start site

The DNA sequence determined on fosmid pFOS-D1 revealed that the intergenic region between *cspB* and *bf2717b* had a size of 637 nucleotides. To precisely map the promoter of the *cspB* gene within this DNA region, the 5' RACE-PCR method was applied. Total RNA was isolated from exponentially growing *C. glutamicum* cells and purified to amplify and sequence the 5' end of the *cspB* transcript. The resulting nucleotide sequence allowed us to determine the transcriptional start site of the *cspB* gene, which was located at an adenine residue 152 nucleotides upstream of the translational start codon of PS2 (Fig. 4a). Thus, the *cspB* mRNA of *C. glutamicum* contains a long 5' untranslated region, as reported previously for S-layer mRNAs of other bacteria (Castan *et al.*, 2001; Mignot *et al.*, 2002). The identified transcriptional start point was subsequently used to deduce the putative promoter sequence of the *cspB* gene according to the consensus sequence of *C. glutamicum* promoters

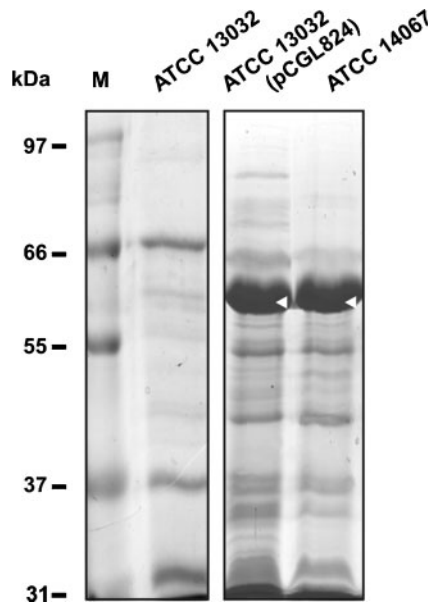


Fig. 2. SDS-PAGE of cell surface proteins of *C. glutamicum* ATCC 13032, *C. glutamicum* ATCC 13032 (pCGL824) and *C. glutamicum* ATCC 14067. The proteins were separated by 12.5% SDS-PAGE and visualized by staining with Coomassie blue. The white arrowheads indicate the S-layer protein PS2 that was identified by MALDI-TOF MS and peptide mass fingerprinting. The strains were grown on solid Luria broth medium. Lane M, Bio-Rad Broad Range Precision Protein Standard (kDa).

defined by Patek *et al.* (1996). The deduced -35 (TGGCAA) and -10 (TACAGT) boxes of the *cspB* promoter are separated by a spacer of 17 nucleotides (Fig. 4a). The -10 box of the *cspB* promoter is almost identical with the consensus sequence of σ^A -dependent promoters of *C. glutamicum* [TA(^{T/C})AAT], whereas the -35 hexamer shares only four out of the six bases with the consensus sequence TTGCCA (Patek *et al.*, 1996). These data suggested that the *cspB* gene of *C. glutamicum* is transcribed by the RNA polymerase- σ^A holoenzyme.

The LuxR-type transcriptional regulator Cg2831 was found to be involved in the activation of the *cspB* gene

The knowledge of the location of the *cspB* promoter enabled us to search for regulatory DNA-binding proteins interacting with this DNA region. For this purpose, DNA affinity purification assays with magnetic streptavidin beads were performed. The biotinylated DNA fragments pF1-2 and pF3-4, covering different portions of the *cspB* upstream region (Fig. 4a), were amplified and linked to streptavidin-coated magnetic beads. The immobilized DNA was then incubated with crude protein extracts from *C. glutamicum* cells grown in minimal medium MM1 with glucose as carbon source. Proteins were purified by binding to the DNA fragments, followed by elution with an appropriate

buffer and separation by SDS-PAGE (Fig. 4b). The resulting protein gel showed a number of proteins binding to both the *cspB* promoter region present on pF1-2 and the pF3-4 region that served as control. Of special interest was a single protein band that was exclusively detected with the pF1-2 promoter fragment of the *cspB* gene (Fig. 4b). This protein band was excised from the gel, and the protein was digested with trypsin and analysed by peptide mass fingerprinting. This procedure resulted in the identification of protein Cg2831, which has been annotated in the course of the *C. glutamicum* genome project as a putative regulatory protein of the LuxR family (Kalinowski *et al.*, 2003). An additional 14 protein bands were excised from each lane of the protein gel and analysed by MALDI-TOF MS and fingerprint analysis, but no further transcriptional regulator was detected. These other proteins were identified either as being subunits of RNA or DNA polymerase, or as having other DNA-binding activities, such as helicases, methyltransferases and single-strand binding protein (data not shown). In fact, the other proteins were purified by means of both the pF1-2 promoter region and the pF3-4 control fragment, apparently excluding a regulatory role for these proteins in *cspB* gene expression.

Inspection of the *C. glutamicum* genome sequence suggested that *cg2831* represents a separate transcription unit that is located downstream of a gene (*cg2830*) encoding a putative adenosylcobalamin-dependent diol dehydratase and upstream of *cg2833*, which encodes a cysteine synthase (Rey *et al.*, 2003). The *cg2831* coding region has a length of 846 nucleotides and is preceded by a ribosome-binding site (AGGAGG) eleven nucleotides in front of the predicted translational initiation codon. Downstream of the coding region, a rho-independent transcriptional terminator ($\Delta G = -12.8 \text{ kcal mol}^{-1}$, 53.6 kJ mol^{-1}) was predicted (Combet *et al.*, 2000). The deduced protein of *cg2831* consists of 281 amino acids with a molecular mass of 30.8 kDa that corresponds very well to the experimentally determined size of the protein when isolated by DNA affinity purification (Fig. 4b). A putative helix-turn-helix motif of the C-terminal-effector-domain type was identified at the C-terminal end of Cg2831 (amino acids 235–256), which might be indicative of a transcriptional activator function (Brune *et al.*, 2005). InterProScan of the Cg2831 amino acid sequence revealed the presence at the N-terminus (amino acids 6–109) of a putative GAF domain (encountered in cGMP-specific phosphodiesterases, adenylyl cyclases and formate hydrogen lyases), which is generally capable of binding second messenger molecules, such as cGMP, and might be required for triggering the regulatory activation of the protein (Ho *et al.*, 2000). Furthermore, weak similarity (32% identity within a stretch of 77 amino acids) was detected to the ATP-dependent transcriptional activator MalT of *E. coli* that binds to the asymmetrical operator sequence GGGGA(^{T/G})GAGG in front of several genes belonging to the maltose regulon (Vidal-Ingigliardi *et al.*, 1991). Although the similarity of Cg2831 from *C. glutamicum* to MalT from *E. coli* is low, it is nevertheless worth mentioning in view of earlier observations that the

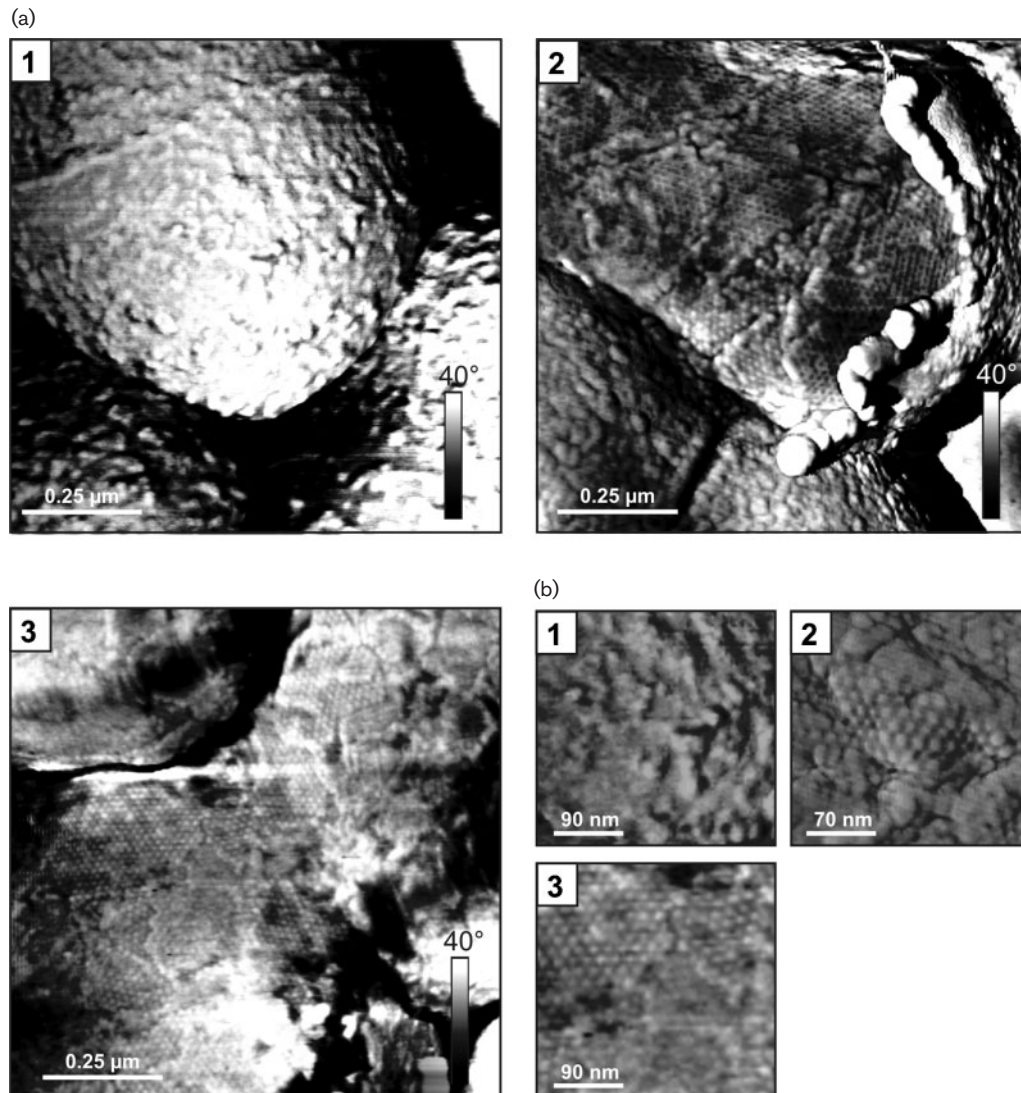


Fig. 3. AFM of *C. glutamicum* cells. (a) AFM phase images of cells from (1) *C. glutamicum* ATCC 13032, (2) *C. glutamicum* ATCC 13032 (pCGL824) and (3) *C. glutamicum* ATCC 14067 were recorded in tapping mode to show the cell surface morphology. The strains were grown on solid Luria broth medium. *C. glutamicum* ATCC 13032 (pCGL824) and *C. glutamicum* ATCC 14067 show large patches of the hexagonal S-layer lattice, whereas *C. glutamicum* ATCC 13032 is apparently devoid of an ordered surface structure. (b) Magnification of specific areas of the AFM deflection images of (1) *C. glutamicum* ATCC 13032, (2) *C. glutamicum* ATCC 13032 (pCGL824) and (3) *C. glutamicum* ATCC 14067.

expression of the S-layer is dependent on the carbon source of the growth medium (Soual-Hoebek *et al.*, 1999). Additionally, the sequence motif GGGGATGGGT, showing striking similarity to the MalT operator, was identified upstream of the *cspB* promoter region (Fig. 4a), which is consistent with the putative function of Cg2831 as transcriptional activator.

Inactivation of *cg2831* in *C. glutamicum* ATCC 13058 abolished S-layer formation

To analyse the putative regulatory role of Cg2831 in S-layer formation, the *cg2831* coding region was disrupted in *C.*

glutamicum ATCC 13058. This strain was chosen because it produces an ordered S-layer (Hansmeier *et al.*, 2004), and is easily accessible to molecular genetic engineering. An internal fragment of the *cg2831* gene was amplified by PCR and cloned into the pK18*mob* vector, which allowed gene disruption to be performed in *C. glutamicum* (Schäfer *et al.*, 1994). Disruption of the *cg2831* gene in the chromosome was confirmed by Southern hybridization (data not shown). Subsequently, expression of the *cspB* gene was analysed on the mRNA and protein level in the resulting mutant, designated *C. glutamicum* Intc_{g2831}, and the corresponding wild-type strain.

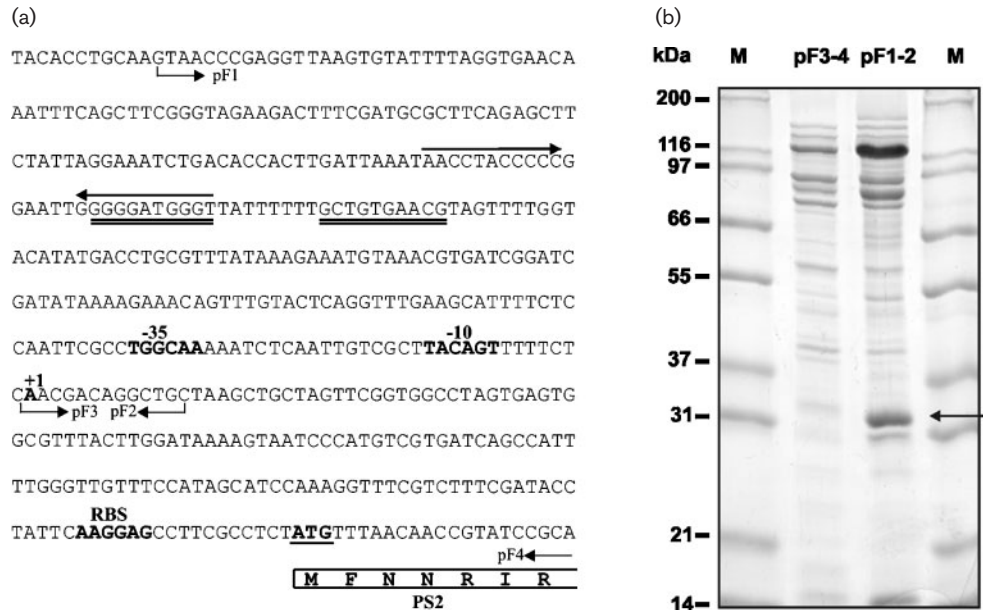


Fig. 4. Genetic features of the *cspB* promoter region of *C. glutamicum* ATCC 14067 and visualization by SDS-PAGE of proteins binding in front of the *cspB* gene. (a) The nucleotide sequence of the *cspB* promoter region is shown. The transcription start site (+1), the deduced -35 and -10 promoter sequences and the putative ribosome-binding site (RBS) are marked. The translational start codon is shown in bold, underlined type. Arrows above the nucleotides indicate a palindromic sequence previously described by Peyret *et al.* (1993). Oligonucleotides used for amplification of DNA fragments pF1-2 and pF3-4 are depicted. Putative regulatory binding sites with similarity to the MalT operator (Boos & Shuman, 1998; Vidal-Ingigliardi *et al.*, 1991) are double underlined. (b) SDS-PAGE of proteins binding to DNA fragments pF1-2 and pF3-4. The proteins were isolated by DNA affinity purification, separated by 12.5% SDS-PAGE and visualized by staining with Coomassie blue. The arrow indicates a protein (Cg2831) specifically binding to the promoter region of the *cspB* gene. Lane M, Bio-Rad Broad Range Precision Protein Standard (kDa).

The effect of *cg2831* disruption on *cspB* expression was first measured on the mRNA level. Both *C. glutamicum* ATCC 13058 and *C. glutamicum* *Intcg2831* were therefore cultivated in complex Luria broth medium, and aliquots of the cells were harvested during exponential growth and stationary phase of the cultures. Subsequently, total RNA was isolated, purified, and applied in real-time RT-PCR experiments, using the *cspB*-specific primers *cspBLC1* and *cspBLC2*. These assays were repeated three times with independently grown cultures. It was found that the relative expression of the *cspB* gene in *C. glutamicum* *Intcg2831* was drastically decreased, by a factor of approximately 10 000-fold, compared with the expression of the wild-type strain. Moreover, a dependence on the growth phase of *cspB* gene expression was detected in the wild-type strain, since *cspB* expression was reduced ~2 000-fold in the stationary phase compared with expression during exponential growth of the culture. A similar expression pattern was detected for *cg2831*, although transcription was reduced only fivefold when the culture entered stationary phase. On the other hand, expression of the *cspB* gene in *C. glutamicum* *Intcg2831* remained unaffected by changes of the growth phase. These observations might indicate that the *cspB* gene is mainly expressed during exponential growth of the culture.

To determine the amount of PS2 protein synthesized by *C. glutamicum* *Intcg2831* and the wild-type, cell surface proteins were extracted from both strains and analysed by SDS-PAGE. Equal amounts of bacterial cells were harvested, and cell surface proteins were separated according to the protocol of Hansmeier *et al.* (2004). The proteins were further characterized by SDS-PAGE combined with MALDI-TOF MS and peptide mass fingerprinting (Fig. 5a). The disruption of *cg2831* resulted in an almost complete loss of the PS2 protein band. However, small amounts of PS2 were detected by MALDI-TOF MS in the mutant strain *C. glutamicum* *Intcg2831* (Fig. 5a).

To assess whether S-layer lattices were formed on the cell surface of *C. glutamicum* *Intcg2831*, AFM images of the respective cells were recorded in tapping mode by phase imaging contrast, as described above. The cell surface of *C. glutamicum* *Intcg2831* displayed only very small patches of the hexagonal S-layer that were primarily located at the bacterial cell poles (Fig. 5b). These patches covered only about 1% of the cell surface of *C. glutamicum* *Intcg2831*. Consequently, the Cg2831 protein plays an important role as transcriptional activator of *cspB* gene expression, resulting in an enhanced transcription of the *cspB* gene and the

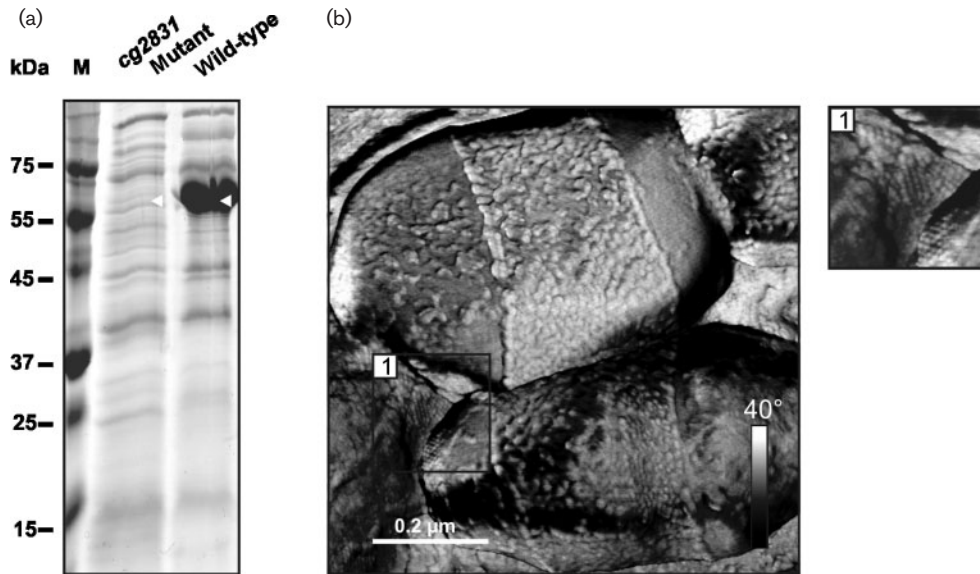


Fig. 5. SDS-PAGE of cell surface proteins prepared from *C. glutamicum* Intcg2831 and AFM phase-contrast image of *C. glutamicum* Intcg2831 cells. (a) Proteins were extracted from the cell surface by treatment with 4% SDS. The PS2 protein expressed by the wild-type *C. glutamicum* ATCC 13058 and the mutant strain *C. glutamicum* Intcg2831 is marked by white arrowheads. Lane M, MBI-Fermentas Prestained Precision Protein Standard (kDa). (b) AFM phase-contrast image of cells from *C. glutamicum* Intcg2831 recorded in tapping mode. The cells were cultivated on solid Luria broth medium. *C. glutamicum* Intcg2831 cells showed only marginal S-layer patches at the bacterial cell poles, as visualized by magnification of area 1 of the image.

formation of an ordered S-layer lattice on the surface of *C. glutamicum* cells.

DISCUSSION

The PS2⁻ phenotype of *C. glutamicum* ATCC 13032 is apparently caused by the loss of a 5.97 kb chromosomal DNA fragment

Recently, we investigated the occurrence of the S-layer gene *cspB* in 28 *C. glutamicum* wild-type strains (Hansmeier *et al.*, 2004). Only the sequenced type strain *C. glutamicum* ATCC 13032 was devoid of the *cspB* gene along with a downstream ORF encoding a putative Zn-dependent alcohol dehydrogenase. It is a well-known phenomenon that bacteria can lose the ability for S-layer formation under specific culture conditions (Blaser *et al.*, 1987; Fujimoto *et al.*, 1989; Sleytr, 1997). In the present study, we demonstrated that *C. glutamicum* ATCC 13032 has apparently lost the ability for S-layer formation due to the chromosomal deletion of a 5.97 kb DNA region. The deleted DNA region is flanked by a 7 bp direct repeat, suggesting that illegitimate recombination was responsible for gene loss. Illegitimate recombination processes using very short stretches of homologous DNA have been observed before in *C. glutamicum*. Mateos *et al.* (1996) have shown that random integration of cloning vectors into the chromosome of *C. glutamicum* is dependent on the presence of homologous nucleotide sequences of only

8–12 bp in length. This type of illegitimate recombination is detectable with very low frequencies in *C. glutamicum*, and it is postulated that this process occurs in a RecA-independent way by specific enzymes that recognize very short regions of DNA homology. Similar mechanisms of illegitimate recombination have been described in several micro-organisms (Farabaugh *et al.*, 1978; Kumagai & Ikeda, 1991; Lopez *et al.*, 1984; Yi *et al.*, 1988).

Loss of S-layer gene expression has also been analysed in more detail in *Campylobacter fetus* strains. In both *Camp. fetus* TK and *Camp. fetus* 23B, the promoter region of the S-layer gene *sapA* was found to be truncated (Fujita *et al.*, 1997; Tummuru & Blaser, 1992). A Chi-like sequence located upstream of the S-layer gene, and most likely recognized by the RecBCD system of the cell, might be responsible for inactivation of the S-layer gene (Tummuru & Blaser, 1993). Loss of S-layer gene expression results in variation of antigenicity of *Camp. fetus*, and thus contributes to the virulence of this pathogenic bacterium (Garcia *et al.*, 1995). The current lack of knowledge about the function of the *C. glutamicum* S-layer makes it difficult to relate the loss of the S-layer locus to the fitness of this bacterium. *C. glutamicum* is commonly found in soil, and the S-layer might be associated with adhesion of exoenzymes and substrates or with other surface recognition processes (Beveridge *et al.*, 1997). However, the loss of the S-layer locus might simply be the result of prolonged cultivation of the type-strain on

synthetic medium since its discovery in 1957 and of its extensive use in the fermentation industry (Hermann, 2003; Sleytr & Sara, 1997). Thus, loss of the *cspB* gene region under favourable culture conditions might reflect a mechanism of deactivation of a non-required surface structure that is, moreover, synthesized in considerable amounts (Sleytr, 1997). Accordingly, it would be interesting to analyse original *C. glutamicum* ATCC 13032 isolates from the late 1950s for the presence of the *cspB* gene region.

Cg2831, a member of the LuxR regulator family, is an activating element for the transcription of the S-layer gene *cspB*

To gain insight into transcriptional regulation of S-layer formation in *C. glutamicum*, we mapped the *cspB* promoter region and identified a DNA-binding transcriptional regulator. The *cspB* promoter shared nine out of 12 nucleotides with the promoters of the *hom* and *gltA* genes that are of medium strength in *C. glutamicum* and allow only weak gene expression in the heterologous host *E. coli* (Eikmanns *et al.*, 1994; Mateos *et al.*, 1994). Only a very low basal level of *cspB* expression was detected in *C. glutamicum* Intcg2831, indicating that the S-layer gene is expressed by a rather weak promoter. On the other hand, electron microscopic images of *C. glutamicum* show that the complete cell surface can be covered by an ordered hexagonal S-layer lattice (Peyret *et al.*, 1993). To cope with the high amount of S-layer proteins necessary to cover the complete bacterial cell surface, high expression of the S-layer gene is mandatory. Generally, a high expression level of the S-layer gene is achieved by using very stable mRNAs and additional regulatory factors, such as DNA-binding transcriptional regulators (Mignot *et al.*, 2002, 2004; Vidgren *et al.*, 1992). In *C. glutamicum*, a high expression level of the *cspB* gene is apparently obtained by the LuxR-type transcriptional regulator Cg2831 that binds to the *cspB* promoter region. LuxR-type regulators act as transcriptional activators and control a wide variety of functions in various biological processes, for instance in biofilm and spore formation, cell division, plasmid transfer and bacterial virulence (Cui *et al.*, 2005; Fuqua *et al.*, 1994; Guvener & McCarter, 2003; Schweizer, 1991). Database searches revealed significant amino acid similarities of Cg2831 to putative LuxR-type regulatory proteins of other species, but none of the regulators has hitherto been functionally analysed. A weak similarity of Cg2831 was also observed to the LuxR-type activator MalT of in *E. coli* (Boos & Shuman, 1998). Expression of the *malT* gene is subject to catabolite repression, and therefore dependent on the intracellular cAMP level. Likewise, Cg2831 contains an N-terminal GAF domain that might be necessary for activation of the regulatory protein by second messenger molecules. Additionally, a DNA motif with similarity to the MalT operator consensus sequence was identified upstream of the *cspB* promoter. A binding site upstream of a promoter is consistent with an activating function of the respective regulatory protein (Madan Babu & Teichmann, 2003).

The role of Cg2831 as transcriptional activator of S-layer gene expression was deduced from the characterization of the mutant strain *C. glutamicum* Intcg2831 by real-time RT-PCR, SDS-PAGE and AFM: (i) relative expression of the *cspB* gene was drastically decreased in *C. glutamicum* Intcg2831 compared with expression in the wild-type strain; (ii) SDS-PAGE showed a nearly complete loss of the PS2 protomer in the mutant strain *C. glutamicum* Intcg2831 in comparison to the protein profile of the *C. glutamicum* wild-type; (iii) AFM images of *C. glutamicum* Intcg2831 showed that only the bacterial cell poles exhibited small S-layer patches, covering in total approximately 1% of the cell surface. Furthermore, real-time RT-PCR revealed that *cg2831* and the *cspB* gene were expressed at higher rates during exponential growth than during stationary phase. In earlier studies, Soual-Hoebeke *et al.* (1999) have demonstrated that the amount of S-layer protein is dependent on the growth phase of *C. glutamicum* and is related to the environmental stimulus of the carbon source available within the growth medium. For instance, the use of lactate as carbon source results in an enhanced formation of the S-layer, and, as a consequence thereof, *C. glutamicum* cells are densely covered by an ordered S-layer lattice. On the other hand, the use of glucose as carbon source shows the contrary effect on S-layer formation, and the cells are only partially covered by a hexagonal S-layer. Since LuxR regulators are known to act in dependence on distinct environmental stimuli, such as growth condition, cell density and stress (Boos & Shuman, 1998; Suzuki *et al.*, 2002), it is likely that Cg2831 is the regulatory element responsible for the observed differences in S-layer gene expression and formation.

From these results, it is apparent that the Cg2831 protein plays an important role as transcriptional activator of *cspB* gene expression, resulting in an enhanced transcription of the *cspB* gene and formation of an ordered S-layer lattice on the surface of *C. glutamicum* cells. Since genes orthologous with *cg2831* are present in other corynebacterial genome sequences that do not encode S-layer protomers, a more global role of Cg2831 in regulation of gene expression in corynebacteria is likely (Brune *et al.*, 2005). The exact physiological function of the transcriptional regulator Cg2831 and the regulatory link with the carbohydrate metabolism of *C. glutamicum* remain to be elucidated.

NOTE ADDED IN PROOF

While this manuscript was going to press, Cramer *et al.*, (2006) provided further information on the LuxR-type regulator Cg2831 that activates transcription of the *cspB* gene. These authors named this regulator RamA (regulator of acetate metabolism A) and showed convincingly that it activates transcription of a genetic network involved in several aspects of acetate metabolism in *C. glutamicum*. In addition, they identified a consensus binding DNA motif that resembles very much the palindromic sequences double-underlined in Fig. 4.

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