S-layer protein production by Corynebacterium strains is dependent on the carbon source

Emmanuelle Soual-Hoebek, Célia de Sousa-D’Auria, Mohamed Chami, Maire-France Baucher, Armel Guyonvarch, Nicolas Bayan, Karima Salim and Gérard Leblon

Three strains of Corynebacterium producing various amounts of PS2 S-layer protein were studied. For all strains, more PS2 was produced if the bacteria were grown in minimal medium supplemented with lactate than if they were grown in minimal medium supplemented with glucose. The consumption of substrate and PS2 production was studied in cultures with mixed carbon sources. It was found that the inhibitory effect of glucose consumption was stronger than the stimulatory effect of lactate in one strain, but not in the other two strains. The regulation of gene expression involved in S-layer formation may involve metabolic pathways, which probably differ between strains. S-layer organization was also studied by freeze-fracture electron microscopy. It was found that low levels of PS2 production correlated with the partial covering of the cell surface by a crystalline array. Finally, it was found that PS2 production was mainly regulated by changes in gene expression and that secretion was probably not a limiting step in PS2 accumulation.

Keywords: S-layer, freeze fracture, carbon regulation

INTRODUCTION

The ‘surface layer’ (S-layer) of many bacteria consists of a single (glyco)protein species that is assembled into a two-dimensional crystalline array enveloping the cell (Sleytr et al., 1996). Large amounts of the protein are produced (10–15% of total cell protein) and in most cases the protein is the major protein species produced by the cell (Messner & Sleytr, 1992). Genetic analyses have been used to investigate the mechanism and regulation of biosynthesis of the protein. The corresponding genes have been identified in many species (Boot & Pouwels, 1996). It has been calculated that approximately 5 x 10^9 monomers are required to cover a rod-shaped bacterium of average size (Sleytr & Messner, 1989). Thus, for a generation time of 20 min in a particular medium, a single gene must produce at least 500 protein molecules per second to cover the cell completely with the S-layer (Sleytr & Messner, 1989). In some cases, an excess of protein is even produced (Sleytr & Glauert, 1976; Lewis et al., 1987; Breitwieser et al., 1992). The high rate of S-layer protein synthesis is due to a high level of gene transcription and/or mRNA stability.

Very little is known about the regulation of S-layer protein synthesis. Multiple transcription initiation sites have been identified in the operon encoding cell-wall proteins in Bacillus brevis 47 (Adachi et al., 1989) and regulation of S-layer protein gene expression has also been observed (Adachi et al., 1991). The half-life of mRNA has been found to be long: 10–15 min in Caulobacter crescentus (Fisher et al., 1988), 22 min in Aeromonas salmonicida (Chu et al., 1993), 15 min in Lactobacillus acidophilus (Boot et al., 1996a) and 14 min in Lactococcus lactis (Kahala et al., 1997). Changes in the S-layer protein have been described in Campylobacter fetus (Garcia et al., 1995; Dwokin & Blaser 1996), Lactobacillus acidophilus ATCC 4356 (Boot et al., 1996b), Bacillus stearothermophilus (Särä & Sleytr, 1994; Särä et al., 1996) and Thermus thermophilus HB8 (Olabarria et al., 1996). In T. thermophilus HB8, the C-terminal fragment of the S-layer SlaA protein binds to the 5’ untranslated leader region of the slaA mRNA, providing evidence for translational auto-regulation in S-layer gene expression (Fernández-Herrero et al., 1997).

In the amino-acid-producing bacterium Corynebacterium glutamicum, two major proteins, PS1 and PS2, with apparent molecular masses of 67 and 63 kDa (Joliff et al., 1992) have been identified in the cell wall. Corynebacterium belongs to the actinomycete sub-

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division of the Gram-positive bacteria and has a high G+C content (Liebl & Sinskey, 1988). The gene encoding the PS2 protein (cspB) has been characterized in C. glutamicum (Peyret et al., 1993). Chami et al. (1995) showed that if C. glutamicum was grown on solid medium, the surface of cells was totally covered with a highly ordered, hexagonal surface layer, whereas if it was grown in liquid medium, the cell and fracture surfaces were only partially covered by ordered arrays. This partial covering was correlated with there being less PS2 associated with the cell wall. Typically, cells grown on solid medium contained 34 mg PS2 protein (g bacterial dry wt)\(^{-1}\), whereas cells grown in liquid medium to stationary phase contained 16 mg (g dry wt)\(^{-1}\). This suggests that PS2 production depends on the physiological and metabolic state of the cell.

Here we report that the amount of S-layer PS2 protein present depends on the carbon source available in the growth medium. We also show that a low level of PS2 production is associated with partial covering of the cell surface by a crystalline array.

**METHODS**

**Bacterial strains, plasmids and culture conditions.** The following *Escherichia coli* strains were used: JM110 [F' traD36 lacI\(^{q}\) Δ(lacZ)M15 proA\(^{B}\)/proAB/tpSl. (Stu') thr leu thi lacY galK galT ara fhuA dam cdm supE44 Δ(lac–proAB)] (Yanisch-Perron et al., 1985), DH5\(^{a}\) [F endA ΔbsdR17rrm\(_{e}\) supE44 thi-1 recA1 gyrA96 relA1 (Nal\(^{r}\) rpsL154) lacYΔ(−114::Tn5 prr-2) (Biolabs) and EB 101 [icd-1 dadR1 trpA62 trpE61 thr-1 lacI\(^{q}\)] (Apostolakos et al., 1982). The *Corynebacterium melassecola* ATCC 17965 and *Corynebacterium* sp. 2262 strains were supplied by Orsan and are described by Mondain-Monval (1988) and by Debay (1997), respectively. As stated by Peyret et al. (1993), *C. melassecola* ATCC 17965 is also known as *C. glutamicum* strain C. *melassecola*. *Corynebacterium* sp. 2262 is permissive for transformation with plasmid DNA extracted from *E. coli* DH5\(^{a}\) and *Corynebacterium glutamicum* ATCC 14752 is permissive for transformation with plasmid DNA from the *dam dcm* *E. coli* JM110 strain. *C. glutamicum* ATCC 14752 was obtained from the American Type Culture Collection, Manassas, VA, USA.

pMF2 was derived from pMC1403 (Casadaban et al., 1980) as follows. The *aphIII* gene from *Streptococcus faecalis* (Trieu-Cuot & Courvalin, 1983), isolated from pCGL243 (Reyes et al., 1991) as a XhoI/NotI cassette, and a NotI/XhoI fragment of the *icd* locus of *C. melassecola* ATCC 17965 were inserted together into the SalI site of pMC1403 to give pMF2. The *icd* gene encoding the isocitrate dehydrogenase of *C. melassecola* was cloned by heterologous complementation of the *C. melassecola* mutant EB 106 as described by Eikmanns et al. (1995). The chromosomal DNA library used for the isolation of the *icd* gene of *C. melassecola* was described by Reyes et al. (1991). The *E. coli–Corynebacterium* shuttle vectors pCGL482 and pCGL815 were described by Peyret et al. (1993).

Bacterial cells were cultured in MCGC minimal medium as described by Von der Osten et al. (1989), except that citrate (used as a chelating agent) was replaced by deferoxamine. This medium contained (l\(^{−}\)): 3 g Na\(_{2}\)HPO\(_{4}\), 6 g KH\(_{2}\)PO\(_{4}\), 2 g NaCl, 8 g (NH\(_{4}\))\(_{2}\)SO\(_{4}\), 0.4 g MgSO\(_{4}\), 7 H\(_{2}\)O, 40 mg FeSO\(_{4}\), 7 H\(_{2}\)O, 3.9 mg FeCl\(_{3}\), 0.9 mg ZnSO\(_{4}\), 7 H\(_{2}\)O, 0.3 mg CuCl\(_{2}\), 2 H\(_{2}\)O, 3.9 mg MnSO\(_{4}\), 2 H\(_{2}\)O, 0.1 mg (NH\(_{4}\))\(_{2}\)Mo\(_{7}\)O\(_{24}\), 4 H\(_{2}\)O, 0.3 mg Na\(_{2}\)B\(_{4}\)O\(_{7}\), 10 H\(_{2}\)O, 84 mg CaCl\(_{2}\), 4 mg biotin, 20 mg thiamin and 3 mg deferoxamine. The carbon sources were added to a final concentration of 180 mM (30 g l\(^{−}\) for glucose and 20 g l\(^{−}\) for lactate) or 90 mM for single or mixed carbon source experiments, respectively. *Corynebacterium* cells were grown aerobically at 34 °C with shaking (250 r.p.m.). Chloramphenicol (Cm; 15 µg ml\(^{−}\)) or kanamycin (Km; 25 µg ml\(^{−}\)) were added as required.

**Construction of the PcsPB–lacz fusion.** The *cspB* promoter (PcsPB) described by Peyret et al. (1993) was isolated as a 554 bp EcoRI/EcoNI fragment and ligated to an EcoNI/BamHI adaptor constructed with the two synthetic oligonucleotides 5′-TCAAGGAGCCTTCCGCTATATG-3′ and 5′-GATCCATAGAGGCGACCTGG-3′. The resulting EcoRI/BamHI fragment was inserted between the EcoRI and BamHI sites of pMF2 to give pCGL1502 (Fig. 1a), in

![Fig. 1. Restriction maps of pCGL1502 and pCGL1504. The positions of the promoter region of the cspB gene and the orientations of transcription of lacz, lacA, lacY, icd, aphIII, bla and cat are indicated. The positions of the origins of DNA replication (oriPMB1, oriM13/oriACY, repBL1) are also indicated.](image-url)
which the cspB and lacZ genes are fused at the BamHI site. Thus, pCGl1502 carries an in-phase fusion between the first codon of cspB and lacZ. A 7·92 kb PsI fragment from pCGl1502 containing the PCspB–lacZ fusion was inserted into the PsI site of the E. coli–Corynebacterium shuttle vector pCGl482 to give pCGl1504 (Fig. 1b).

**DNA manipulations.** Plasmid DNA was isolated from *Corynebacterium* strains by alkaline lysis (Birnboim & Doly, 1979). Genomic DNA was extracted as described by Ausubel et al. (1987). Integration into the *Corynebacterium* chromosome was checked by Southern blotting with non-radioactively labelled DNA probes as described by Labarre et al. (1993). Restriction endonucleases and DNA-modifying enzymes were purchased from Promega. *Corynebacterium* strains were transformed by electroporation as described previously (Bonamy et al., 1990), with 100–200 ng plasmid DNA bearing either kanamycin (pCGl1502) or chloramphenicol (pCGl1504) resistance genes.

**Extraction of PS2 protein and quantification of PS2 production.** Proteins were extracted from the cell walls of *Corynebacterium* cultures with 2% (w/v) SDS, as described by Peyret et al. (1993). A bacterial pellet was obtained from 2 ml cell culture and suspended in 200 µl Tris/HCl buffer (50 mM, pH 6·8) containing 2% SDS. The suspension was heated to 100 °C for 5 min and centrifuged at 16000 g for 3 min. The supernatant containing the proteins associated with the cell wall (mainly PS2) was collected. This treatment does not solubilize the cytoplasmic membrane or result in cell lysis (Bayan et al., 1993). SDS-PAGE was carried out as described by Laemmli (1970), with a 4% acrylamide stacking gel and a 10% acrylamide separating gel. The volume of protein extract used was calculated so as to give samples with equivalent optical density at 570 nm. The samples were denatured by heating at 100 °C for 5 min in the presence of 2% SDS and 1·25% (w/v) β-mercaptoethanol in 50 mM Tris/HCl pH 6·8. The samples were then subjected to electrophoresis; under these conditions, PS2 protein had an apparent molecular mass of 63 kDa (Joliff et al., 1992). After electrophoresis, the gels were stained with Coomassie brilliant blue R-250. PS2 protein was determined by densitometry (ImagQuant) and 1 µg β-galactosidase was loaded on the gel as a control for quantity. The amount of PS2 protein is expressed in mg protein (g bacterial dry wt)−1. Biomass (Bm) was determined as a function of optical density at 570 nm using a z correlation factor for each *Corynebacterium* strain; Bm = OD570/z. To determine the z factor, 200 ml bacterial cells were harvested from a culture in stationary phase with a known OD570 value by centrifugation for 20 min at 3500 g. The pellet was dried at 60 °C for 15 h and its weight was determined. This procedure was carried out for three independent cultures. The z factor was calculated for all carbon sources tested.

**Freeze-fracture electron microscopy.** A bacterial suspension was placed between a thin copper holder and a thin copper plate and quenched in liquid propane, as described by Gulik-Krzywicki & Costello (1978) and Aggerbeck & Gulik-Krzywicki (1986). The frozen sample was fractured at −125 °C in vacuum of about 1·333 x 10⁻⁵ Pa by removing the upper plate with a liquid-nitrogen-cooled knife in a Balzers 301 freeze-etching unit.

The fractured sample was etched at −105 °C for 3–5 min and a replica was produced with platinum-carbon or tungsten-carbon (1·0–1·5 nm of metal deposited), backed with about 20 nm of carbon. The replica was cleaned by incubation overnight with chromic acid, washed with distilled water and observed in a Philips 410 electron microscope.

**RESULTS**

**PS2 production as a function of carbon source**

We assessed the PS2 protein production of three strains of *Corynebacterium* grown in liquid minimal medium (MCG) supplemented with either glucose or lactate. PS2 production was assessed by densitometry (Fig. 2). The three strains produced different amounts of PS2.

**Glucose and lactate assays.** For glucose and lactate determinations in culture media, bacterial cells (2 ml) were centrifuged and the supernatant filtered through 0·45 µm filters (Millipore). Samples were stored at −20 °C until use. Extracellular glucose and lactate concentrations were determined with a sensitive colorimetric enzyme assay (Sigma Diagnostics glucose procedure no. 315 and Sigma Diagnostics lactate procedure no. 735, respectively). The kits for glucose and lactate assays were purchased from Sigma and used as recommended by the manufacturer.

**β-Galactosidase activity in cytoplasmic extracts.** Bacterial cells (20 ml of stationary phase culture) were collected by centrifugation and resuspended in 2 ml buffer A (100 mM Tris/HCl pH 8, 500 mM KCl, 1 mM MgSO4, 7H2O, 0·4 mM MnSO4, 4 mM DTT). The cell suspension was mixed with 1 g glass beads (106 µm diameter; Sigma), and was shaken for two periods of 5 min, each at 1800 vibrations min⁻¹ (Retsch MM 2000). The suspension was centrifuged at 20800 g and the supernatant was used as a cytoplasmic extract. β-Galactosidase activity was measured as follows. Cytoplasmic extract (270 µl) was mixed with 180 µl of a solution of 4 g ONPG l⁻¹. The absorbance of the mixture at 420 nm (A420) was monitored for 10 min at room temperature. The protein concentration of the extract was also determined by the Lowry method (DC protein assay, Bio-Rad). Specific β-galactosidase activity is expressed as [A420 min⁻¹ (µg protein)⁻¹] x 1000.
The largest amount of PS2 as a proportion of bacterial dry weight was detected if cells were grown in the presence of lactate: 30 mg PS2 protein (g bacterial dry wt)$^{-1}$ for *C. glutamicum* ATCC 14752, 98 mg (g dry wt)$^{-1}$ for *C. melassecola* ATCC 17965 and 15.8 mg (g dry wt)$^{-1}$ for *Corynebacterium* sp. 2262. The amount of PS2 produced in the presence of lactate was 4-fold higher than that in the presence of glucose for *C. glutamicum* ATCC 14752, 3-fold higher for *C. melassecola* ATCC 17965 and 13-fold higher for *Corynebacterium* sp. 2262. This suggests that lactate consumption increased PS2 production.

Fig. 3. Electron micrograph of a freeze-fracture and deep-etched preparation of *C. glutamicum* ATCC 14752 cells. F1, convex fracture surface; F2, concave fracture surface. Arrows indicate S-layer. (a, b) Cells grown on minimal medium supplemented with glucose. (c, d) Cells grown on minimal medium supplemented with lactate. Bar, 0.5 µm.
S-layer formation as a function of growth on glucose or lactate

As PS2 production was highly dependent upon the carbon source, we investigated whether carbon source also affected S-layer formation. We carried out freeze-fracture electron microscopy with all three strains grown on either glucose or lactate. This technique is suitable for the observation of the S-layer in *Corynebacterium* because the main fracture plane is propagated between the S-layer and the cell wall and produces two fracture surfaces: F1, the convex fracture surface, and F2, the concave fracture surface. The S-layer can be observed on the concave fracture surface (F2) and its imprint can be...
seen on the convex fracture surface (F1) (Chami et al., 1995). *Corynebacterium* ATCC 14752 cells grown on glucose had only a partial S-layer (Fig. 3a, b), with only small patches detectable. These patches are shown by arrows on convex fracture surfaces F1 (Fig. 3a) and on concave fracture surfaces F2 (Fig. 3b). A similar discontinuous S-layer has been described for *Corynebacterium* B15 (Peyret et al., 1993; Chami et al., 1995). In contrast, if the bacteria were grown on lactate (Fig. 3c, d), most were completely covered by the S-layer. Thus, the overproduction of PS2 protein in cultures grown on lactate [30 mg (g dry wt)$^{-1}$] resulted in an increase in the area covered by the S-layer. Similar results were obtained with *Corynebacterium* sp. 2262 (data not shown). Very few S-layer patches were observed in the presence of glucose, and significantly more patches were detected in the presence of lactate. However, *Corynebacterium* sp. 2262, unlike *Corynebacterium* ATCC 14752, was not completely covered with ordered arrays in the presence of lactate. The lower extent of the S-layer correlated with the lower amount of PS2 protein associated with the cell wall [15.8 mg (g dry wt)$^{-1}$] in this strain.

On glucose, *C. melasseecola* ATCC 17965 produced 31.5 mg PS2 (g dry wt)$^{-1}$ (Fig. 2) and had a complete S-layer [Fig. 4a (convex fracture surface F1) and 4b (concave fracture surface F2)]. In the presence of lactate, PS2 production increased to 98 mg (g dry wt)$^{-1}$ (Fig. 2), and ordered arrays were still present on the cell surface (Fig. 4c). The imprint of the S-layer (see the convex fracture surface) is barely visible, but remarkably, the concave fracture surface F2, is densely covered with non-ordered particles (Fig. 4d). This may result from the accumulation of an excess of PS2 protein under the S-layer.

**Effect of alteration of carbon source on PS2 production**

*C. glutamicum* ATCC 14752 cells were cultured overnight in glucose-containing medium. They were then used to inoculate a medium containing glucose (Fig. 5a), lactate (Fig. 5b) or a mixture of lactate and glucose (Fig. 5c). There was a large increase in PS2 production during the exponential phase of growth in cultures containing lactate as the sole carbon source (Fig. 5b). This suggests that PS2 accumulates in the cell wall after stimulation of its synthesis by lactate consumption. In cultures con-

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**Fig. 5.** Relationship between the consumption of various carbon sources and PS2 protein production by *C. glutamicum* ATCC 14752. At various time points, carbon sources [glucose ( ), and/or lactate ( )] were assayed, and cell wall proteins were recovered from the pellet; the amount of PS2 was quantified ( ) as described in Methods. Biomass (as OD$_{570}$) is also shown. (a–c) Cells were cultured overnight in MCGC minimal medium supplemented with glucose, then used to inoculate, to an OD$_{570}$ of 0.7, minimal medium supplemented with glucose (a), lactate (b) or lactate plus glucose (c). For PS2 determination, the volume of protein extract used was calculated so as to give samples of equivalent optical density at 570 nm (OD$_{570}$ = 1.05). (d–f) Cells were cultured overnight in MCGC minimal medium supplemented with lactate, then to inoculate minimal medium supplemented with glucose (d), lactate (e) or lactate plus glucose (f). For PS2 determination, the volume of protein extract used was calculated so as to give samples with equivalent optical density at 570 nm (OD$_{570}$ = 1.25).
glucose consumption was predominant in this strain. These observations suggest that the inhibitory effect of cultures from lactate-grown inocula (results not shown). In cultures with mixed carbon sources, no C. glutamicum obtained (data not shown). The single carbon source those observed with C. glutamicum grown and lactate-grown inocula, and then decreased in bacterial dry weight steadily increased throughout the stimulation of PS2 production in cultures with lactate as lactate consumption (Fig. 5f). We observed strong produced the large amounts of PS2 associated with source (Fig. 5d). Cultures with mixed carbon sources growth in cultures containing glucose as the sole carbon (Fig. 5e) or a mixture of lactate and glucose (Fig. 5f). We then cultured C. glutamicum ATCC 17965 and ATCC 14752 were then carried out with C. glutamicum. Experiments similar to those performed with C. glutamicum ATCC 17965 and 14752(pCGL1504) and 2262(pCGL1504) respectively.

### Table 1. β-Galactosidase activity produced under the control of a cspB promoter in various strains of Corynebacterium grown in MCGC minimal medium

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<td>C. glutamicum ATCC 14752(pCGL1504)</td>
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<tr>
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<td>Glucose</td>
<td>0.215±0.025</td>
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ND, Not detected.

*Activity was determined using cells in stationary phase and is expressed as \([A_{420} \text{min}^{-1} (\mu\text{g protein})^{-1}] \times 1000\). Values are the means of at least six independent determinations ± se.

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containing both lactate and glucose (Fig. 5c), an increase in PS2 production was also observed, suggesting that the stimulatory effect of lactate consumption on PS2 synthesis was independent of glucose.

We then cultured C. glutamicum ATCC 14752 cells overnight in lactate-containing medium and used them to inoculate medium containing glucose (Fig. 5d), lactate (Fig. 5e) or a mixture of lactate and glucose (Fig. 5f). There was a large decrease in PS2 production during growth in cultures containing glucose as the sole carbon source (Fig. 5d). Cultures with mixed carbon sources produced the large amounts of PS2 associated with lactate consumption (Fig. 5f). We observed strong stimulation of PS2 production in cultures with lactate as the sole carbon source. The amount of PS2 per g bacterial dry weight steadily increased throughout the exponential phase in lactate cultures from both glucose-grown and lactate-grown inocula, and then decreased in the stationary phase (Fig. 5b and 5e).

Experiments similar to those performed with C. glutamicum ATCC 14752 were then carried out with C. melassecola ATCC 17965 and Corynebacterium sp. 2262. For C. melassecola ATCC 17965, results similar to those observed with C. glutamicum ATCC 14752 were obtained (data not shown). The single carbon source results obtained with Corynebacterium sp. 2262 were similar to those for C. glutamicum ATCC 14752 (data not shown). In cultures with mixed carbon sources, no PS2 was produced in cultures from glucose-grown inocula and there was a decrease in PS2 production in cultures from lactate-grown inocula (results not shown). These observations suggest that the inhibitory effect of glucose consumption was predominant in this strain.

### cspB expression level as a function of carbon source

We investigated the regulation of PS2 production by constructing an in-phase fusion between the PsplB promoter and the E. coli lacZ gene. First, pCGL1502 carrying an in-phase fusion between lacZ and the first codon of cspB was constructed in E. coli DH5αF’ (Fig. 1a). As pCGL1502 cannot replicate in Corynebacterium, its transfer by electroporation may lead to its integration into the chromosome at either the icd locus or the cspB locus. We transformed the Corynebacterium sp. 2262 strain with pCGL1502 and selected five kanamycin-resistant recombinant strains. The structure of the insertion in these recombinant strains was determined by Southern blotting and a single copy of pCGL1502 was found to have inserted by homologous recombination at the icd locus in each (data not shown). One recombinant strain, Corynebacterium sp. 2262::pCGL1502, was studied further.

Plasmid pCGL1504 was then constructed, containing the PsplB–lacZ fusion, and able to replicate in Corynebacterium. This plasmid was constructed in E. coli DH5αF’ and transferred into E. coli JM110 (Fig. 1b). pCGL1504 was introduced by electroporation into C. glutamicum ATCC 14752 and Corynebacterium sp. 2262, producing the recombinant strains ATCC 14752::pCGL1504 and 2262::pCGL1504 respectively.

β-Galactosidase activity was measured after culture on MCGC minimal medium containing either glucose or lactate (Table 1). Activity was 2.4-fold higher in ATCC 14752(pCGL1504), 3.5-fold higher in 2262(pCGL1504) and 7.1-fold higher in Corynebacterium sp. 2262::pCGL1502 grown in the presence of lactate. These ratios are similar to those observed for PS2 production in lactate and suggested that regulation occurs mainly at the level of protein synthesis.

### DISCUSSION

Using three strains of Corynebacterium, each with a different rate of PS2 production, we found that the amount of S-layer protein depended on the carbon source available in the growth medium. We also found that the area covered by the S-layer closely correlated with the amount of PS2 produced. In C. glutamicum ATCC 14752 and Corynebacterium sp. 2262, which produces only small amounts of PS2 when cultured on glucose [8 mg (g dry wt)⁻¹ and 1.2 mg (g dry wt)⁻¹, respectively], only small patches of S-layer were observed. The cell surface was not completely covered by the S-layer if less than 30 mg PS2 (g dry wt)⁻¹ was produced, consistent with the results of Chami et al.
The current lack of knowledge about the function of the S-layer under the S-layer makes it difficult to relate its regulation by a carbon source to a particular function. The natural carbon source used by Corynebacterium in its biotope is unknown. It is commonly found in the soil (Trautwetter & Blanco, 1988) and organic acids may be its principal substrates. The S-layer may therefore be associated with adhesion sites for exoenzymes, surface recognition and cell adhesion to substrates, as has been suggested for the S-layers of several other organisms (Beveridge et al., 1997).

We showed, using a lacZ fusion, that PS2 production was mainly regulated by changes in cspB gene expression and that secretion was probably not a limiting step in PS2 accumulation at the cell surface. A few examples of the regulation of S-layer formation have been described (Bahl et al., 1997), but regulation by carbon source has not previously been reported. In B. brevis 47, one of the five promoters of the operon encoding cell wall proteins is specifically active in the exponential phase of growth (Adachi et al., 1989). We observed that growth phase had an effect on PS2 production but the promoter sequences of cspB involved have not yet been determined. In T. thermophilus HB8 (Fernández-Herrero et al., 1997), there is evidence that slrA encodes a transcriptional repressor of the S-layer gene. It has been suggested that SlrA is also linked with other metabolic pathways, such as those involved in cell wall synthesis. We observed co-metabolism of both substrates in all three strains, extending the observation by Cocaing (1992) of simultaneous glucose and lactate consumption in C. melassecola ATCC 17965. The inhibitory effect of glucose consumption on PS2 production was much greater in Corynebacterium sp. 2262 than in the other strains. This suggests that the use of metabolic pathways differs between strains. Glucose catabolism by central pathways in C. melassecola ATCC 17965 has been examined using NMR (Rollin et al., 1995), enzymic (Cocaing-Bousquet et al., 1996) and mathematical modelling (Pons et al., 1996) approaches. These studies have shown that the pentose pathway is responsible for almost 50% of glucose catabolism in this strain. One of the key aspects of sugar catabolism in C. melassecola is the manner in which an adequate supply of NADPH is generated to meet anabolic requirements. The pentose phosphate pathway, which involves two NADP-dependent dehydrogenases, is the principal source of NADPH during growth on glucose. The differences in flux distribution for glucose-grown and lactate-grown cells shows clearly that different NADPH-generating reactions operate in lactate-grown cells. During growth on lactate, a modified tricarboxylic acid cycle involving malic enzyme and an unknown enzyme with pyruvate carboxylating activity is thought to operate, accounting for the apparent shortfall of NADPH for anabolic requirements (Cocaing-Bousquet & Lindley, 1995). Such studies performed to analyse carbon flux in Corynebacterium sp. 2262 support the idea that major differences exist in metabolism of glucose in these two Corynebacterium strains (N. D. Lindley, personal communication).

ACKNOWLEDGEMENTS

C. de Sousa-D’Auria and E. Soual-Hoebeke made equal contributions to this work. We would like to thank T. Gulik-Krzewicz, E. Shechter and B. Holland for helpful discussions. We are also grateful to Orsan SA for providing Corynebacterium strains. This work was supported by grants from the Centre National de la Recherche Scientifique (CNRS), Orsan SA and the Université Paris-Sud (France).

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