Effects of α -phosphoglucomutase deficiency on cell wall properties and fitness in *Streptococcus gordonii*

Alain Bizzini, Paul Majcherczyk, Siham Beggah-Möller, Blazenka Soldo, José M. Entenza, Muriel Gaillard, Philippe Moreillon and Vladimir Lazarevic

Département de Microbiologie Fondamentale, Bâtiment Biophore, Université de Lausanne, Quartier UNIL-Sorge, CH-1015 Lausanne, Switzerland

Correspondence

Vladimir Lazarevic

vladimir.lazarevic@unil.ch

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Streptococcus gordonii α -phosphoglucomutase, which converts glucose 6-phosphate to glucose 1-phosphate, is encoded by *pgm*. The *pgm* transcript is monocistronic and is initiated from a σ^A -like promoter. Mutants with a gene disruption in *pgm* exhibited an altered cell wall muropeptide pattern and a lower teichoic acid content, and had reduced fitness both *in vitro* and *in vivo*. *In vitro*, the reduced fitness included reduced growth, reduced viability in the stationary phase and increased autolytic activity. *In vivo*, the *pgm*-deficient strain had a lower virulence in a rat model of experimental endocarditis.

INTRODUCTION

α -Phosphoglucomutase (PGM) converts glucose 6-phosphate to glucose 1-phosphate, which is the precursor of UDP-glucose. UDP-glucose serves as a glucosyl donor in several metabolic pathways. In Gram-positive bacteria, UDP-glucose may be used as the precursor of wall teichoic acids (WTAs), lipoteichoic acids, membrane glycolipids, capsules and exopolysaccharides (Boels *et al.*, 2001; Lazarevic *et al.*, 2005).

PGM deficiency has pleiotropic effects, the most common being morphological changes and slower growth (Hardy *et al.*, 2000; Buchanan *et al.*, 2005; Lazarevic *et al.*, 2005). In the soil bacterium *Bacillus subtilis*, altered cell morphology of PGM-deficient mutants correlates with a deficiency in membrane glycolipids and is reversible by addition of Mg^{2+} (Lazarevic *et al.*, 2005). Moreover, these mutants have a reduced ability to form biofilms and this may be attributed to their inability to synthesize a hypothetical glucose-containing exopolysaccharide. Finally, changes in WTA content and structure, resulting from an inability to synthesize UDP-glucose, confer resistance to some phages.

In *Streptococcus pneumoniae*, an important cause of respiratory infections, PGM loss is associated with a reduced virulence, possibly due to a reduction in capsule production (Hardy *et al.*, 2000, 2001). Similarly, in *Streptococcus iniae*, a fish pathogen that can cause invasive infection in humans,

PGM deficiency correlates with diminished capsule production and reduced virulence, which might be due to an increased susceptibility to phagocytic clearance (Buchanan *et al.*, 2005).

Streptococcus gordonii is a primary colonizer of the tooth surface, where it contributes to the formation of dental plaque. The bacterium may enter the bloodstream and, in susceptible individuals, cause infective endocarditis (Douglas *et al.*, 1993). However, the use of *S. gordonii* strains harbouring foreign immunogenic peptides on their surface has been considered as an immunization technique (Pozzi *et al.*, 1992). In the present work, we assessed the physiological implications of PGM in *S. gordonii* by studying the effects of PGM deficiency on *in vitro* and *in vivo* fitness, as well as on cell wall properties.

METHODS

Strains and growth conditions. *S. gordonii* Challis was routinely grown at 37 °C without aeration, either in brain heart infusion (BHI) broth (Oxoid) or on BHI agar plates. *Escherichia coli* DH5 α , used as a host for plasmid clones, was grown at 37 °C, either in Luria–Bertani (LB) broth (Difco) with aeration or on LB agar plates. Growth of the cultures was followed by monitoring OD₆₀₀ with a Novaspec II spectrophotometer (Pharmacia LKB). When appropriate, erythromycin was added to the media at a final concentration of 5 μ g ml⁻¹ for *S. gordonii*. For *E. coli* transformants, ampicillin was used at a concentration of 50 μ g ml⁻¹. Bacterial stocks were stored at -80 °C in broth supplemented with 10 % (v/v) glycerol.

Construction of a *pgm* knockout mutant. The protein sequence deduced from the *B. subtilis* *pgcA* gene encoding PGM was used to search for homology against the unfinished *S. gordonii* Challis genomic sequence available at The Institute for Genomic Research

Abbreviations: PGM, α -phosphoglucomutase; PSD, post-source decay; WTA, wall teichoic acid.

The GenBank/EMBL/DDBJ accession number for the nucleotide sequence of *Streptococcus gordonii* *pgm* reported in this paper is DQ234767.

website (<http://www.tigr.org>). The 1977 bp region comprising the *S. gordonii* orthologue of *B. subtilis* *pgcA* was designated *pgm*, and it was PCR-amplified and sequenced; no discrepancies were found.

A 1038 bp *pgm* segment was PCR-amplified using oligonucleotides PGM_5' and PGM_3'_B, and cloned into pBAD-TOPO vector (Invitrogen) to obtain plasmid pBAD-TOPO_PGM. The 29 bp internal part of the cloned *pgm* segment (residues 584–612 with respect to the putative *pgm* translational start) was excised by *HindIII*/*Sall* and replaced by the 1083 bp erythromycin-resistance cassette, which includes its own promoter. The latter was obtained by the *HindIII*/*Sall* digestion of the PCR product generated with oligonucleotides VL602 and Ery_3'_Sall using pJDC9 DNA (Chen & Morrison, 1988). The resulting plasmid pBAD-TOPO_ΔPGM was digested with *MluI* and used to transform *S. gordonii* competent cells. Correct *pgm* knockout of Δ*pgm*::*erm*, a randomly chosen erythromycin-resistant transformant, was verified by PCR (data not shown).

Transformation. *S. gordonii* competent cells were prepared as described by Pozzi *et al.* (1990). Transformants were selected on Columbia agar (Difco) plates supplemented with 3% human blood and 5 µg erythromycin ml⁻¹.

Oligonucleotides. The following oligonucleotides were used in this study:

Ery_3'_Sall, 5'-GTCGACCTTGGGAAGCTGTCAGTAGTATACCTA-3'; PGM_3'_B, 5'-AAAACGTGAACATGGTTGC-3'; PGM_5', 5'-AAAGGAAGATGCCTTTTATACCAA-3'; VL602, 5'-AAAGCTTAACGAAATGATACACCAATCAGTGCA-3'; VL837, 5'-TTGAGGTGGCGAACGGCGAATGAAAGTTCA-3'; VL838, 5'-TCGATAGCAGGGATGTAAGTTGTCA-3'; VL840, 5'-CACTTCCACACCAACACGGTCAGCA-3'; VL851, 5'-GCCAAACCTTCAGTCGCTTGACGGACAACA-3'; VL852, 5'-CTGGAAGATCAGCGAAATCGA-3'; VL1008, 5'-GCATCAATATCTATGTTGTCCGTCA-3'. Underlined portions of the oligonucleotides represent engineered restriction sites: G/TCGAC, *Sall*; A/AGCTT, *HindIII*.

RNA extraction. Total RNA was extracted by using the RNeasy Protect Bacteria Mini Kit (Qiagen). Briefly, 4 ml RNeasy Protect Bacteria Reagent (Qiagen) was added to a 2 ml aliquot of *S. gordonii* culture in BHI broth and vortexed for 5 s. After 5 min incubation at room temperature, the suspension was centrifuged at 3200 g for 10 min. The supernatant was discarded, and the pellet was resuspended in 100 µl TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0) containing 15 mg lysozyme ml⁻¹ and incubated at room temperature for 15 min. The manufacturer's protocol was followed from this point.

Mapping of transcriptional start site. The 5' end of the *pgm* transcript was mapped with the BD SMART RACE cDNA Amplification Kit (BD Biosciences), according to the manufacturer's protocol. Briefly, gene-specific first-strand cDNA, synthesized from 560 ng total RNA extracted at an OD₆₀₀ of 0.6 with *pgm*-specific primers VL838 and VL840, was tailed with the BD SMART II A oligonucleotide. The products were then amplified with a nested gene-specific primer VL837 and the Universal Primer A Mix in a PCR involving 30 cycles of denaturation at 95 °C for 30 s, annealing at 54 °C for 30 s and extension at 72 °C for 3 min. The PCR product was purified with the GFX PCR DNA and Gel Band Purification Kit (Amersham Biosciences), and a nested PCR was carried out under the conditions described above using Nested Universal Primer A and oligonucleotide VL851. The nested PCR product was sequenced using primer VL852 (Synergene Biotech).

Northern blot. Total RNA was extracted from cells at an OD₆₀₀ of 0.2. After incubation in 20 µl (total volume) of denaturation buffer (1 M glyoxal, 25% DMSO, 10 mM sodium phosphate, pH 7.0) for 1 h at 50 °C, total RNA (10 µg) was separated by agarose

electrophoresis and transferred onto a Hybond-N+ nylon membrane (Amersham Biosciences). RNA was blotted in 10 × SSC with the VacuGene XL system (Amersham Biosciences) for 3.5 h under a vacuum of 50 mbar. After transfer, RNA was fixed to the membrane with a UV cross-linker (CX-2000, UVP) at a dose of 0.3 J cm⁻². A 367 bp *pgm* probe labelled with digoxigenin was synthesized with a PCR DIG Labelling Mix (Roche Diagnostics) using oligonucleotide primers VL838 and VL1008. Hybridization and immunological detection were performed according to the supplier's instructions (Roche Diagnostics), using alkaline-phosphatase-conjugated anti-digoxigenin Fab fragments to probe the digoxigenin-labelled DNA, and disodium 3-(4-methoxyphosphoryl)-1,2-dioxetane-3,2'-(5'-chloro)tricyclo[3.3.1.1^{3,7}]decan-4-yl)phenyl phosphate (CSPD) as the reagent for chemiluminescent detection. Light emission was detected by using Hyperfilm ECL (Amersham Biosciences).

Cell extract preparation. Bacteria were grown in 200 ml BHI medium at 37 °C. At different points during growth, cells were recovered by centrifugation at 7000 g and 4 °C. The pellets were rinsed with ice-cold distilled water and resuspended in 5 ml 100 mM triethanolamine, pH 7.4, containing 5 mM MgCl₂ and 2.2 mM EDTA. The cells were disrupted by two passes through a One Shot cell disruptor (Constant Systems) at a pressure of 2.5 kbar (2.5 MPa). The cell debris was removed by centrifugation at 15 000 g for 15 min and the supernatant was frozen at -80 °C. The protein concentration was determined with the BCA Protein Assay Reagent (Pierce).

PGM assay. PGM activity was assayed as previously described (Lazarevic *et al.*, 2005). The reaction mixture contained cell extract (20 µg protein), 0.6 mM β-NADP, 25 µM glucose 1,6-diphosphate and 1 unit glucose-6-phosphate dehydrogenase (Sigma), in 1 ml (total volume) of 100 mM triethanolamine, pH 7.4, containing 5 mM MgCl₂ and 2.2 mM EDTA. The reaction was started by adding 1.5 mM α-D-glucose 1-phosphate. Under the assay conditions used, one unit of enzyme activity corresponds to the conversion of 1 nmol substrate min⁻¹ (mg cell protein)⁻¹.

Phase-contrast microscopy. Samples were fixed by addition of 0.1 vol. 20% formaldehyde and applied to a slide. Phase-contrast images were captured with a ×100 Plan-Neofluar objective on a Axioskop 2 microscope (Carl Zeiss) equipped with MetaVue imaging software (version 6.1r5; Molecular Devices).

Viable cell estimation. The cell viability was estimated by using the LIVE/DEAD BacLight Bacterial Viability Kit for microscopy and quantitative assays (Molecular Probes). This kit differentially stains live or undamaged cells versus dead or damaged cells by using two nucleic acid stains. The Syto 9 green-fluorescent nucleic acid stain labels all cells whether living or dead, while the red-fluorescent nucleic acid stain propidium iodide enters only the cells with damaged membranes. The applicability of the kit to *S. gordonii* was verified with cells killed by incubation in 70% 2-propanol for 1 h. Briefly, cells from samples of between 0.5 and 2 ml were harvested by centrifugation for 10 min at 10 000 g, washed with 1 ml 0.9% NaCl and resuspended in 200 µl 0.9% NaCl. Syto 9 (component A) and propidium iodide (component B) were mixed at a 1:1 ratio and 0.6 µl aliquots were added to each sample of cells. The suspensions were incubated for 15 min at room temperature in the dark. Bacteria were visualized with a DM IRE2 inverted laser scanning confocal microscope (Leica Microsystems) equipped with an HCX PL APO CS ×100 1.4 oil objective and Leica Confocal Software version 2.5. Images were taken using 488 and 543 nm laser excitations and 503–523 nm and 595–631 nm band-pass filters, for the Syto 9 and propidium iodide stains, respectively. The cells were counted in at least eight random fields and the proportion of viable or undamaged (green-stained) cells was calculated as a percentage of all the cells.

Cell wall preparation. Cell walls were purified from bacteria grown to an OD₆₀₀ of 0.3 in BHI, as described by de Jonge *et al.* (1992).

Estimation of cell wall phosphate. Lyophilized cell walls were mineralized according to Ames (1966). A 50 µl sample of a 1 mg ml⁻¹ cell wall suspension was mixed with 50 µl 10% Mg(NO₃)₂ (w/v in 96% ethanol) in a borosilicate glass test tube and dried at 95 °C for 3–4 h. Samples were heated in a Bunsen flame for 2 min. After cooling, 300 µl 1 M HCl was added and tubes were heated for 15 min in a water bath at 100 °C. The phosphate concentration was determined according to Chen *et al.* (1956) against a standard curve generated using known concentrations of K₂HPO₄.

Muropeptide analysis. WTAs were removed from the cell walls by gently mixing the cell wall suspension in hydrofluoric acid (49%) for 48 h at 4 °C. The peptidoglycan was recovered, digested with mutanolysin (Sigma), and reduced and separated by HPLC, as described by de Jonge *et al.* (1992). The HPLC system (Hitachi) consisted of an L-7200 autosampler, an L-7100 gradient pump with low-pressure mixing and an L-7400 UV detector. Column temperature was maintained at 34 °C using the L-7360 column oven. Separation was performed by injection of a 100 µl sample, containing 100 µg of wall digest, onto a 250 × 4.6 mm reverse-phase column (ODS-Hypersil 3 µm; Keystone Scientific). The material was eluted at 0.5 ml min⁻¹ using a linear gradient of 5% (v/v) methanol in 100 mM sodium phosphate (pH 2.5) to 30% (v/v) methanol in 100 mM sodium phosphate (pH 2.8) over 160 min. Eluted material was detected by its absorbance at 210 nm. The results were analysed using the D-7000 HPLC System Manager program (Hitachi). Principal peaks were collected and desalted using C18 ZipTips (Millipore), according to the manufacturer's protocol. The mass of desalted peaks was determined by reflector positive-ion mode MALDI-TOF MS (Autoflex; Bruker Daltonics) using a 5-chloro-2-mercaptobenzothiazole matrix in water, tetrahydrofuran and ethanol (1:1:1). Pure peaks were sequenced by post-source decay (PSD) analysis (Xu *et al.*, 1997).

Autolysis of whole cells. Cells were grown in BHI medium at 37 °C. At an OD₆₀₀ of 0.2, cells from a 3.5 ml sample were recovered by centrifugation for 3 min at 10 000 g, washed with PBS buffer (0.68% NaCl, 0.148% Na₂HPO₄, 0.043% KH₂PO₄, pH 7.2) and suspended in 1 ml 30 mM potassium phosphate (pH 7.0). The suspension was incubated at 37 °C and the OD₆₀₀ was monitored. Results were expressed as percentages of the initial OD₆₀₀.

Experimental endocarditis. Sterile aortic vegetations were produced in rats, as described by Heraief *et al.* (1982). Groups of animals were inoculated by intravenous challenge with 10⁶ c.f.u. exponential-phase streptococci. For the parent strain, this inoculum size produced endocarditis in >90% of the rats, and thus it permitted a clear differentiation between virulent and putatively less virulent strains (Entenza *et al.*, 1997; Stutzmann Meier *et al.*, 2001). Rats were sacrificed 16 h after bacterial inoculation and viable colony counts in the vegetations and spleens were determined. The frequency of infection was compared by using Fisher's exact test. The median bacterial titres were compared by using a non-parametric Mann-Whitney unpaired test; *P* < 0.05 was considered significant.

RESULTS

Sequence analysis and expression of *pgm*

The 572-residue protein encoded by the *S. gordonii* *pgm* gene showed 96.7% identity to the PGM of *S. pneumoniae* (Hardy *et al.*, 2000) and 48.8% identity to the PGM of *B. subtilis* (Lazarevic *et al.*, 2005). Analysis of the nucleotide

sequence revealed that the 30 nt segment located 8 nt downstream of the *S. gordonii* *pgm* stop codon could form a stem-loop structure corresponding to a transcriptional terminator. The presence of a terminator-like sequence 193 nt upstream of the *pgm* start codon, followed by a functional promoter (Fig. 1a; see below), suggests that *pgm* forms a one-gene operon. The Northern hybridization experiments yielded a major product of the expected length of about 1.8 kb (Fig. 1b), confirming a monocistronic transcription of *pgm*. The minor band of about 1.5 kb was most probably an artefact caused by the partially degraded mRNA and the abundance of 16S rRNA.

To identify promoters of the *pgm* gene, the 5' end of the *pgm* transcript was determined by 5'-RACE PCR, using RNA from exponentially growing *S. gordonii* cells. A band corresponding to a PCR product with an estimated length of 0.5 kb was revealed by agarose gel electrophoresis and sequenced (Fig. 2). The transcriptional start located 75 nt upstream of the putative *pgm* start codon was preceded at a distance of 6 nt by the sequence ATATGCTACAAT (Fig. 1a). This sequence exhibited a strong (10/11) adherence to the so-called extended -10 consensus sequence (RTRTGNTATAAT) of *B. subtilis* and *S. pneumoniae* σ^A -type promoters (Helmann, 1995; Sabelnikov *et al.*, 1995). Only a poorly matching (3/6) -35 region was found at the usual distance of 11 nt upstream of the extended -10. Therefore, it seems likely that, as in *B. subtilis* (Helmann, 1995) and *S. pneumoniae* (Sabelnikov *et al.*, 1995), the extended -10 site may function without a recognizable -35 region. Moreover, in *S. gordonii*, transcription initiation from such promoters seems to be relatively frequent (M. Haenni and V. Lazarevic, unpublished).

pgm knockout

To confirm that *pgm* encodes PGM, we constructed a knockout mutant by inserting an erythromycin-resistance cassette into *pgm* and assayed cell extracts for PGM activity during the exponential phase of growth. In contrast to the parent strain (Fig. 3b), no detectable PGM activity was found in the Δ *pgm::erm* mutant, strongly suggesting that *pgm* encodes a unique PGM in *S. gordonii*. In the wild-type strain, after growth from an OD₆₀₀ of 0.03 to a final reading of 1.39, PGM activity increased by a factor of 2.3.

Cell morphology

On solid BHI and blood agar, the *pgm*-deficient mutant formed somewhat smaller colonies than the parent strain (not shown). In liquid BHI medium, the *pgm*-deficient mutant grew at a slightly slower rate (mean doubling time of 59 ± 3 min, versus 48 ± 1 min for the wild-type) and the OD₆₀₀ in the stationary phase reached only 39% of the wild-type level (Fig. 3a).

Microscopic examination of the Δ *pgm::erm* mutant grown in BHI liquid medium revealed an enlarged diameter

Fig. 1. Transcriptional unit of the *pgm* operon. (a) Gene organization of the chromosomal region containing *pgm* and the strategy for *pgm* inactivation. Arrows correspond to *pgm* and neighbouring genes. Stem-loop structures indicate possible transcriptional terminators. Insertion of the plasmid clone used for *pgm* inactivation is represented by bold horizontal lines. The -35 and extended -10 promoter regions and the ribosome-binding site (RBS) are labelled. Transcriptional and putative translational starts are indicated by white and black triangles, respectively. *erm*, erythromycin-resistance cassette. The DIG-labelled probe used for hybridization is indicated by a white bar. (b) Northern hybridization analysis of the *pgm* gene. The position of the 16S rRNA is shown on the left of the blot, and the sizes (nt) of the RNA molecular mass markers (Roche Diagnostics) are shown on the right.

compared with that of wild-type cells (Fig. 4). The cell size varied within the same chain. This aberrant cell morphology, which is in accordance with the observations recently made in *S. iniae* (Buchanan *et al.*, 2005), was not reversible by addition of Mg^{2+} , as shown for *B. subtilis* PGM-deficient mutants (Lazarevic *et al.*, 2005).

Cell viability in vitro

The viability of the $\Delta pgm::erm$ mutant was investigated using the LIVE/DEAD BacLight Bacterial Viability Kit, which includes stains to determine whether cells have an intact or damaged membrane. In the exponential phase, at an OD_{600} of 0.3, 99 and 93 % of the wild-type and *pgm*-deficient cells, respectively, were stained as viable (Table 1). In contrast, a striking difference in viability was observed after 24 h batch cultivation. Wild-type cells showed only a 2 % decline in the viable count, whereas the *pgm*-deficient mutant exhibited >30 % loss of viability under the same conditions.

In vivo virulence

The role of PGM was further evaluated in a rat model of experimental endocarditis. The results are presented in Table 2. Whereas the parent strain infected 100 % of both vegetations and spleens, the *pgm* deletion mutant infected only 54 % of these tissues ($P < 0.05$). Moreover, the rats inoculated with the parent strain had significantly higher vegetation and spleen bacterial densities than those inoculated with the $\Delta pgm::erm$ mutant ($P < 0.05$). Thus, in agreement with previous findings demonstrating that mutations in PGM had a significant effect on the virulence of *S. pneumoniae* and *S. iniae* in different animal models (Buchanan *et al.*, 2005; Hardy *et al.*, 2001), our results showed that the *S. gordonii* $\Delta pgm::erm$ mutant was also less virulent in experimental endocarditis.

Cell autolysis and cell wall analyses

The autolysis of whole cells was monitored in terms of the decrease in OD_{600} of exponentially grown bacteria

Fig. 2. Determination of the transcriptional start site by 5'-RACE PCR. (a) Agarose gel electrophoresis of PCR-amplified cDNA tailed with BD SMART II Oligonucleotide. DNA was stained with SYBR Safe and visualized under UV light. (b) A chromatogram from the sequencing of the 5'-RACE-PCR product. The nucleotide complementary to the start of the transcript is highlighted and it is followed by the BD SMART II Oligonucleotide tail (dashed arrow).

Fig. 3. Growth in BHI medium and change in PGM activity during growth. (a) Overnight cultures grown in BHI at 37 °C were diluted into fresh medium to an OD₆₀₀ of 0.01–0.013. Cultures were incubated at 37 °C and the OD₆₀₀ of the wild-type (○) and *pgm*-deficient strains (■) was monitored. (b) PGM activity [nmol min⁻¹ (mg cell protein)⁻¹] and error bars (SD) are indicated for the wild-type strain only, since activity was not detected in the $\Delta pgm::erm$ strain. Samples were taken from exponential (OD₆₀₀ 0.03, 0.2 and 1.0) and stationary growth phases (OD₆₀₀ 1.39).

resuspended in a hypotonic phosphate buffer. In these conditions, the *pgm*-deficient strain lysed at a faster rate than the wild-type (Fig. 5). The increased autolysis might be due to an altered cell wall structure that allows hydrolytic enzymes increased access to the peptidoglycan.

Fig. 4. Cell morphology of the wild-type (WT) and mutant *S. gordonii* strains. Cells were grown overnight in BHI medium at 37 °C. Bar, 10 µm.

In order to check whether the mutant had an altered cell wall structure, its cell wall mucopeptide pattern was compared with that of its parent. Cell wall mucopeptides were prepared from both the mutant and the parent on three different occasions and analysed separately. Both the parent and the mutant had complex mucopeptide patterns that did not vary between culture days (Fig. 6). The composition of the components present in individual peaks was determined (Table 3). Identical structures were present in both the parent and the mutant, but the relative amounts varied between strains (Fig. 6, Table 4). These differences were quantified by calculating the relative amounts of the different peaks labelled in Fig. 6. Peaks 1, 2, 5b and 7 were significantly increased in the $\Delta pgm::erm$ strain compared with the wild-type, whereas peaks 3, 4, 5a and 6 were significantly decreased. No significant difference was observed in the amounts of peak 5c found in either strain (Table 4). Peaks 1, 2, 3, 4, 6 and 7 were found to be pure by MALDI-TOF MS and were identified by PSD analysis (Table 3). Peaks 5a, 5b and 5c contained several components and were not further analysed in this study. The fact that peak 2 was more abundant in the mutant than in the parent, and that it was a simple monomer, suggests that the wall of the mutant might be simpler than that of the parent.

To test whether the *pgm* knockout affects the synthesis of a cell wall anionic polymer, we compared the cell wall

Table 1. Effect of the *pgm* knockout on viability and cell wall phosphate content

Strain	Viable cells (%) [*]		Cell wall phosphate [µmol (mg cell wall) ⁻¹] [†]
	Exponential phase	Stationary phase	
Wild-type	99.1 ± 0.3	96.8 ± 1.8	0.221 ± 0.018
$\Delta pgm::erm$	92.5 ± 0.1	60.8 ± 1.8	0.153 ± 0.013

^{*}Mean (±SD) of three independent experiments.

[†]Mean (±SD) based on three independent measurements obtained on two cell wall preparations.

Table 2. Infectivity rate and bacterial densities in vegetation and splenic tissues of rats 16 h after bacterial challenge with an inoculum of 10^6 c.f.u.

Strain	Vegetation tissue		Splenic tissue	
	No. infected rats/total (%)	Median (range) [$\log_{10}(\text{c.f.u. g}^{-1})$]	No. infected rats/total (%)	Median (range) [$\log_{10}(\text{c.f.u. g}^{-1})$]
Wild-type	10/10 (100 %)	6.67 (4.05–7.63)	10/10 (100 %)	3.12 (2.08–3.72)
$\Delta\text{pgm}::\text{erm}$	6/11 (54 %)*	4.11 (2.00–7.44)*	6/11 (54 %)*	1.66 (1.00–2.60)*

* $P < 0.05$ versus wild-type *S. gordonii*.

phosphate content of the $\Delta\text{pgm}::\text{erm}$ and the wild-type strains. The phosphate content of the wild-type (Table 1) was very close to $0.23 \mu\text{mol phosphate (mg cell wall)}^{-1}$, a value that has been reported for *S. gordonii* NCTC 7865 (Rose *et al.*, 1997). Phosphate measurements revealed about 30 % reduction in the cell wall of the PGM-deficient mutant (Table 1). This decreased value is to be expected for walls impoverished in sugar phosphate polymers.

DISCUSSION

S. gordonii deficient in PGM, the enzyme providing the link between glycolysis and biosynthetic processes requiring UDP-glucose, exhibited multiple phenotypic changes. These changes, especially the reduced survival of the organisms in stationary phase, increased autolytic activity and reduced virulence in infective endocarditis, point to a global loss of bacterial fitness.

Other studies have shown that at least part of the virulence attenuation observed in PGM-deficient *S. iniae* and *S.*

pneumoniae is likely to be due to a diminished capsule expression (Buchanan *et al.*, 2005; Hardy *et al.*, 2001). Interestingly, *S. gordonii* appears to be an unencapsulated bacterium. Therefore, the reduced *in vivo* and *in vitro* fitness in the PGM-deficient mutant may be caused by changes in other cell envelope components.

The cell wall of most Gram-positive bacteria is composed of peptidoglycan, which is a structurally defining cell wall polymer that contains covalently attached anionic polymers, such as WTA and teichuronic acid. The repeating units of most WTAs contain substituted polyol phosphate. WTAs contribute to the cell wall electrolyte properties, modify the activity of peptidoglycan hydrolases and maintain cation homeostasis (Neuhaus & Baddiley, 2003). In the rod-shaped

Fig. 5. Autolysis at pH 7.0 of whole cells from an exponential-phase culture of *S. gordonii*. The mean and SEM were calculated from values obtained in three experiments. ○, Wild-type; ■, $\Delta\text{pgm}::\text{erm}$ mutant. **Fig. 6.** Muropeptide fingerprints of the wild-type *S. gordonii* and its PGM-deficient mutant. Principal peaks numbered were analysed by MALDI-TOF MS. The mutant ($\Delta\text{pgm}::\text{erm}$) and parent (WT) contained the same peaks, but the proportion of certain peaks varied between the strains, notably the increase of peak 2 in the PGM-deficient mutant.

Table 3. Molecular mass and composition of pure mucopeptides from wild-type and *Δpgm::erm* peptidoglycan

Principal peaks were analysed by MALDI-TOF MS. Peaks 1, 2, 3, 4, 6 and 7 were pure and were identified by PSD analysis. Peaks 5a, 5b and 5c contained several components and were not further analysed in this study. Identical structures were present in the parent and mutant. [M+Na]⁺, mass of sodium adduct; G-M, *N*-acetylglucosamine-*N*-acetylmuramic acid; Ala, alanine; Glx, glutamine/glutamate; Lys, lysine; ND, not determined.

Peak	[M+Na] ⁺		Proposed structures
	Observed	Calculated	
1	948	ND	ND
2	991	991	G-M Ala Glx Lys-Ala Ala
3	1061	1062	G-M Ala Glx Lys-Ala Ala Ala
4	1133	1133	G-M Ala Glx Lys-Ala-Ala Ala Ala
6	2154	2154	G-M G-M Ala Ala Glx Glx Lys-Ala-Ala Lys-Ala-Ala-Ala Ala Ala
7	3176	3175	G-M G-M Ala G-M Ala Glx Ala Glx Lys-Ala-Ala Glx Lys-Ala-Ala-Ala Lys-Ala-Ala-Ala Ala Ala

bacterium *B. subtilis*, as well as in the spherical *Streptococcus oralis*, WTA deficiency correlates with abnormal shape and cell growth (Horne & Tomasz, 1993; Soldo *et al.*, 2002). Since most species of viridans streptococci do not contain polyol-WTA (Rose *et al.*, 1997), their sugar-phosphate polymers probably perform some of the roles carried out by WTA. Moreover, Hancock & Baddiley (1985) proposed that these so-called sugar 1-phosphate polymers should be recognized as WTAs. Although the structure of *S. gordonii* Challis sugar-phosphate polymer has not been determined, it may be similar to those found in the viridans group of

streptococcal strains whose repeated unit is a glucose- and phosphate-containing hexasaccharide or heptasaccharide (Cisar *et al.*, 1997). Therefore, the PGM deficiency that prevents the synthesis of UDP-glucose, which is an important glucosyl donor for the synthesis of a variety of cell wall polymers, may lead to the abnormality in sugar-phosphate polymer metabolism and, as a consequence, altered cell morphology.

Since PGM does not appear to be involved in the mucopeptide biosynthetic pathway, PGM deficiency does

Table 4. Muropeptide composition of the parent and PGM-deficient strains

Muropeptides were prepared from the wild-type and $\Delta pgm::erm$ strains on three separate occasions. Each batch of muropeptides was analysed in duplicate by HPLC. The values are percentages of the sum of all integrated peak areas presented in Table 3 and they are means $\pm$ SEM of the six analyses. The percentage peak areas were compared by Student's two-tailed distribution test.

Peak	Wild-type	$\Delta pgm::erm$	Variation factor*	Pvalue
1	1.63 $\pm$ 0.7	2.95 $\pm$ 0.47	1.81	0.001
2	3.90 $\pm$ 1.4	11.21 $\pm$ 0.96	2.87	0.000
3	8.54 $\pm$ 0.2	5.14 $\pm$ 0.36	0.60	0.000
4	17.83 $\pm$ 0.8	15.67 $\pm$ 0.65	0.88	0.002
5a	7.65 $\pm$ 0.5	7.04 $\pm$ 1.09	0.92	0.000
5b	5.64 $\pm$ 0.6	8.11 $\pm$ 0.20	1.44	0.001
5c	7.59 $\pm$ 0.6	7.34 $\pm$ 0.67	0.97	0.396
6	28.93 $\pm$ 2.1	21.25 $\pm$ 2.71	0.73	0.003
7	18.29 $\pm$ 1.5	21.29 $\pm$ 3.04	1.16	0.052

*Factor by which the composition of the muropeptide of $\Delta pgm::erm$ differs from the wild-type.

not provide a straightforward explanation for changes in the muropeptide pattern of the $\Delta pgm::erm$ strain. Moreover, we are not aware of any other study linking PGM deficiency to a change in the muropeptide pattern in other bacterial species. One possibility is that changes in WTA metabolism may influence the activity of peptidoglycan-hydrolytic or -biosynthetic enzymes, resulting in an altered muropeptide composition and an increased autolysis rate.

It has been hypothesized that the altered cell morphology in a PGM-deficient *B. subtilis* strain might be due to deficiencies in glucose 1-phosphate metabolites, monoglucosyldiacylglycerol and/or diglucosyldiacylglycerol (Lazarevic *et al.*, 2005). In *B. subtilis*, synthesis of these glycolipids requires YpP, a processive enzyme that catalyses successive addition of glucosyl residues from UDP-glucose to diacylglycerol (Jorasch *et al.*, 1998). Interestingly, sequence similarity searches failed to reveal orthologues of *B. subtilis* yppP in *S. gordonii* and other streptococci. Therefore, the synthesis of membrane glycolipids in streptococci seems to involve a family of enzymes that are evolutionarily distinct from those operating in bacilli and staphylococci. Nevertheless, a deficiency in membrane glycolipids may be due to an inability to synthesize the putative glycosyl donor UDP-glucose.

S. gordonii is a commensal oral bacterium that has been considered as a potential live vaccine vector in humans (Pozzi *et al.*, 1992; Kotloff *et al.*, 2005). It may be theoretically advantageous to use a *pgm*-deficient *S. gordonii* as a vector in cases where only transient antigen exposure is required. An attenuated strain would be naturally out-competed by the resident microflora, preventing long-term antigen stimulation. The use of such a strain may also reduce

the risk of infective endocarditis after accidental entry of the bacterium into the bloodstream. Whether or not the diminished virulence properties conferred by *pgm* deficiency may offer an advantage for the use of *S. gordonii* as a potential vaccine vector in humans warrants further investigation.

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REFERENCES

- Ames, B. N. (1966). Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol* **8**, 115–118.
- Boels, I. C., Ramos, A., Kleerebezem, M. & de Vos, W. M. (2001). Functional analysis of the *Lactococcus lactis* *galU* and *galE* genes and their impact on sugar nucleotide and exopolysaccharide biosynthesis. *Appl Environ Microbiol* **67**, 3033–3040.
- Buchanan, J. T., Stannard, J. A., Lauth, X., Ostland, V. E., Powell, H. C., Westerman, M. E. & Nizet, V. (2005). *Streptococcus iniae* phosphoglucomutase is a virulence factor and a target for vaccine development. *Infect Immun* **73**, 6935–6944.
- Chen, J. D. & Morrison, D. A. (1988). Construction and properties of a new insertion vector, pJDC9, that is protected by transcriptional terminators and useful for cloning of DNA from *Streptococcus pneumoniae*. *Gene* **64**, 155–164.
- Chen, P. S., Toribara, T. Y. & Warner, H. (1956). Microdetermination of phosphorus. *Anal Chem* **18**, 1756–1758.
- Cisar, J. O., Sandberg, A. L., Reddy, G. P., Abeygunawardana, C. & Bush, C. A. (1997). Structural and antigenic types of cell wall polysaccharides from viridans group streptococci with receptors for oral actinomyces and streptococcal lectins. *Infect Immun* **65**, 5035–5041.
- de Jonge, B. L., Chang, Y. S., Gage, D. & Tomasz, A. (1992). Peptidoglycan composition of a highly methicillin-resistant *Staphylococcus aureus* strain. The role of penicillin binding protein 2A. *J Biol Chem* **267**, 11248–11254.
- Douglas, C. W., Heath, J., Hampton, K. K. & Preston, F. E. (1993). Identity of viridans streptococci isolated from cases of infective endocarditis. *J Med Microbiol* **39**, 179–182.
- Entenza, J. M., Caldelari, I., Glauser, M. P., Francioli, P. & Moreillon, P. (1997). Importance of genotypic and phenotypic tolerance in the treatment of experimental endocarditis due to *Streptococcus gordonii*. *J Infect Dis* **175**, 70–76.
- Hancock, I. C. & Baddiley, J. (1985). Biosynthesis of the bacterial envelope polymers teichoic acid and teichuronic acid. In *The Enzymes of Biological Membranes*, pp. 279–307. Edited by A. N. Martonosi. New York: Plenum.
- Hardy, G. G., Caimano, M. J. & Yother, J. (2000). Capsule biosynthesis and basic metabolism in *Streptococcus pneumoniae* are linked through the cellular phosphoglucomutase. *J Bacteriol* **182**, 1854–1863.
- Hardy, G. G., Magee, A. D., Ventura, C. L., Caimano, M. J. & Yother, J. (2001). Essential role for cellular phosphoglucomutase in virulence of type 3 *Streptococcus pneumoniae*. *Infect Immun* **69**, 2309–2317.

- Helmann, J. D. (1995).** Compilation and analysis of *Bacillus subtilis* σ^A -dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. *Nucleic Acids Res* **23**, 2351–2360.
- Heraief, E., Glauser, M. P. & Freedman, L. R. (1982).** Natural history of aortic valve endocarditis in rats. *Infect Immun* **37**, 127–131.
- Horne, D. S. & Tomasz, A. (1993).** Possible role of a choline-containing teichoic acid in the maintenance of normal cell shape and physiology in *Streptococcus oralis*. *J Bacteriol* **175**, 1717–1722.
- Jorasch, P., Wolter, F. P., Zahringer, U. & Heinz, E. (1998).** A UDP glucosyltransferase from *Bacillus subtilis* successively transfers up to four glucose residues to 1,2-diacylglycerol: expression of *yppP* in *Escherichia coli* and structural analysis of its reaction products. *Mol Microbiol* **29**, 419–430.
- Kotloff, K. L., Wasserman, S. S., Jones, K. F., Livio, S., Hruby, D. E., Franke, C. A. & Fischetti, V. A. (2005).** Clinical and microbiological responses of volunteers to combined intranasal and oral inoculation with a *Streptococcus gordonii* carrier strain intended for future use as a group A streptococcus vaccine. *Infect Immun* **73**, 2360–2366.
- Lazarevic, V., Soldo, B., Medico, N., Pooley, H., Bron, S. & Karamata, D. (2005).** *Bacillus subtilis* α -phosphoglucosyltransferase is required for normal cell morphology and biofilm formation. *Appl Environ Microbiol* **71**, 39–45.
- Neuhaus, F. C. & Baddiley, J. (2003).** A continuum of anionic charge: structures and functions of D-alanyl-teichoic acids in Gram-positive bacteria. *Microbiol Mol Biol Rev* **67**, 686–723.
- Pozzi, G., Musmanno, R. A., Lievens, P. M., Oggioni, M. R., Plevani, P. & Manganelli, R. (1990).** Method and parameters for genetic transformation of *Streptococcus sanguis* Challis. *Res Microbiol* **141**, 659–670.
- Pozzi, G., Contorni, M., Oggioni, M. R., Manganelli, R., Tommasino, M., Cavalieri, F. & Fischetti, V. A. (1992).** Delivery and expression of a heterologous antigen on the surface of streptococci. *Infect Immun* **60**, 1902–1907.
- Rose, R. K., Matthews, S. P. & Hall, R. C. (1997).** Investigation of calcium-binding sites on the surfaces of selected Gram-positive oral organisms. *Arch Oral Biol* **42**, 595–599.
- Sabelnikov, A. G., Greenberg, B. & Lacks, S. A. (1995).** An extended –10 promoter alone directs transcription of the *DpnII* operon of *Streptococcus pneumoniae*. *J Mol Biol* **250**, 144–155.
- Soldo, B., Lazarevic, V., Pooley, H. M. & Karamata, D. (2002).** Characterization of a *Bacillus subtilis* thermosensitive teichoic acid-deficient mutant: gene *mnaA* (*yvyH*) encodes the UDP-N-acetylglucosamine 2-epimerase. *J Bacteriol* **184**, 4316–4320.
- Stutzmann Meier, P., Entenza, J. M., Vaudaux, P., Francioli, P., Glauser, M. P. & Moreillon, P. (2001).** Study of *Staphylococcus aureus* pathogenic genes by transfer and expression in the less virulent organism *Streptococcus gordonii*. *Infect Immun* **69**, 657–664.
- Xu, N., Huang, Z. H., de Jonge, B. L. & Gage, D. A. (1997).** Structural characterization of peptidoglycan mucopeptides by matrix-assisted laser desorption ionization mass spectrometry and postsource decay analysis. *Anal Biochem* **248**, 7–14.

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