Genomics in Bacillus subtilis
Noback, Michiel Andries

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CHAPTER VI

Identification and characterisation of the *Bacillus subtilis* gtaC gene, encoding the phosphoglucomutase involved in glucosylation of teichoic acid and phage susceptibility

VI.1. Summary

In the framework of the European *Bacillus subtilis* genome sequencing project an ORF, *yhxB*, was identified that displayed high homology to phosphoglucomutases and phosphomannomutases from both bacteria and eukaryotes. Phosphoglucomutase converts glucose-6-phosphate to glucose-1-phosphate. To study the possible function of the *yhxB* gene product, a Campbell-type mutant of *yhxB* with the concomitant formation of a promoter $P_{yhxB}$-lacZ fusion was constructed. The corresponding strain was subsequently analysed for growth, expression, phage φ25 and φ29 susceptibility and cell wall glucose content. We conclude from these analyses that *yhxB* encodes a phosphoglucomutase, probably involved in the glucosylation of teichoic acid, and that this gene corresponds to the gtaC marker. The gtaC (glucosylation of teichoic acid) marker was previously supposed to be located at around 77° on the *B. subtilis* 168 chromosome and to be responsible for the glucosylation of teichoic acid. We also conclude that the *B. subtilis* 168 chromosome does not encode a functional paralogue of *yhxB*. Gene *yhxB* is important for growth in glucose-based minimal medium, but not in nutrient broth. It is not essential for viability under the conditions tested.

VI.2. Introduction

Teichoic acids belong to the anionic polymers, a group of negatively charged polymers in the cell wall. Anionic polymers are divided into two classes: teichoic acids, in which a negative charge is provided by phosphodiester groups in the repeating units, and teichuronic acids, in which the negative charge is provided by the carboxyl groups of uronic acid residues. Teichoic acids vary in chemical composition between bacterial species and even between strains. In *Bacillus subtilis* 168, teichoic acid is a poly-1-*sn*-glycerol-3-phosphate in which the hydroxyl group at C-2 of the glycerol moiety may bear an α-glucosyl or D-alanyl ester substituent (Fig.VI.1.). The terminal phosphate bears a poly-

![Fig.VI.1. Teichoic acid structure in *B. subtilis*. R = H, glucosyl or alanyl residues. From Archibald, 1993.](image-url)
N-acetylhexosamine, linking it to an N-acetylmuramyl residue of the peptidoglycan (Archibald et al., 1993). Besides peptidoglycan, the anionic polymers constitute a substantial proportion of the weight of walls of many gram-positive bacteria (Archibald et al., 1993). Although it is probably essential for viability (Mauël et al., 1989), the precise function of teichoic acid in the cell wall of Bacillus is as yet not entirely understood, and neither is the function of glucosylation of this compound. Available experimental data suggest that cells require anionic groups, rather than teichoic acid per se, for normal cell division and the development of the typical morphology of B. subtilis cells and colonies (Archibald et al., 1993). From the work of Young (1967) it was known that B. subtilis 168 possesses a gene, gtaC, encoding the enzyme phosphoglucomutase, which is involved in the glucosylation of wall teichoic acid. Pooley and co-workers (1987) have demonstrated that gtaC is located close to argC on the genetic map of the B. subtilis chromosome, at about 77°C (Anagnostopoulos et al., 1993). The gtaC gene is possibly accompanied by an additional, regulatory gene, gtaE, controlling the levels of phosphoglucomutase (PGM) and UDPglucose pyrophosphorylase (Pooley et al., 1987). This finding was based on differences between mutants from the same linkage group with respect to their phage resistance pattern and cell wall galactosamine content. In this paper, we describe the function of gene yhxB in B. subtilis with respect to susceptibility to phages φ25, φ29, and SP10, cell-wall glucose content, and growth in glucose-based minimal medium. We postulate that yhxB probably corresponds to the gtaC marker, and that the putative regulatory gene, gtaE, is not encoded by the genes that are in the vicinity of yhxB.

**VI.3. Methods**

**Cloning and sequencing**

The cloning and sequencing of yhxB has been described in a previous paper (Noback et al., 1998).

**Homology analysis and sequence alignments**

Homology comparisons were carried out using the FASTA program (Pearson & Lipman, 1988), and multiple sequence alignments with program ClustalW at the EBI services homepage at [http://www2.ebi.ac.uk/services.html](http://www2.ebi.ac.uk/services.html) (Higgins et al., 1994).

**Media and growth conditions**

Strains were cultured in rich medium (TY), nutrient broth (NB) or minimal medium (MM). TY consists of 10 g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl, and 0.1 mM MnCl₂ at pH 7.2. Nutrient Broth contains 8 g/l Difco Bacto nutrient broth, 0.25 g/l MgSO₄·7H₂O, 1 g/l KCl, 0.01 mM MnCl₂, 0.001 mM FeSO₄, and 10 mM CaCl₂, at pH 7.1. Minimal Medium consists of Spizizen’s minimal salts (Spizizen, 1958), supplemented with glucose (0.5%), casein hydrolysate (0.02%; Difco Laboratories, Detroit, USA), and L-tryptophane (20 µg/ml).
Transformation and competence

*B. subtilis* cells were made competent essentially as described by Bron and Venema (1972). *E. coli* cells were made competent and transformed by the method of Mandel and Higa (1970).

Isolation of DNA

*B. subtilis* chromosomal DNA was purified as described by Bron (1990). Plasmid DNA was isolated by the alkaline-lysis method of Ish-Horowicz and Burke (1981).

PCR

PCR reactions were performed using Expand polymerase (Boehringer, GmbH, Mannheim, Germany) using buffers supplied with the enzyme, and according to protocols supplied by the manufacturer.

Protein determination

Protein concentration was measured using the Bio-Rad protein assay (Bio-Rad Laboratories GmbH, München, Germany) and the protocols supplied by the manufacturer.

β-Galactosidase assay

Culture samples of 1 ml were taken at appropriate time points, and the cells were harvested by centrifugation for 2 min at 12000 × g and stored at -20°C until use. Cells were lysed by incubation for 20 min at 37°C in 500 μl Z buffer containing per liter 10.7 g Na₂HPO₄·2H₂O (0.06 M), 5.52 g NaH₂PO₄·H₂O (0.04 M), 0.75 g KCl, 0.246 g MgSO₄·7H₂O. Before use, DTT was added to a final concentration of 1 mM and 1/100 (vol/vol) of lysis solution (1 mg/ml DnaseI and 10 mg/ml lysozyme in water). The samples were then centrifuged for 2 min at 12,000 × g, and the supernatant was collected and stored on ice. Subsequently, 200 μl of the protein sample was mixed with 600 μl of Z-buffer, and 200 μl ONPG solution containing 0.1 M Na₂HPO₄, 0.1 M NaH₂PO₄ and 4 mg/ml ONPG was added. Samples were then incubated at 28°C until a yellow colour appeared. The reactions were stopped by adding 500 μl 1 M Na₂CO₃. Extinction of the samples was measured at 420 nm and activity in Miller Units (M.U.: nmol ONPG × min⁻¹ × mg⁻¹ protein) was calculated as follows:

\[(\text{OD}420 \times 1.5) / (\text{sample vol} \times T \times 0.00486 \times \text{mg/ml protein})\].

Preparation of cell walls

A single colony was used to inoculate 1 litre of 2 × YT and the cells were grown overnight at 37°C with shaking. The cells were pelleted by centrifugation, and the supernatant discarded. The cells were then resuspended in 200 ml of 1M MES pH 6.5, 0.4% (w/v) SDS. The cell suspension was subsequently boiled for 15 min, and then washed three times in 500
ml of 1 M MES pH 6.5. The cell walls were then resuspended in 10 ml of water, freeze-dried overnight, and stored at 4°C.

**Removal of glucose from cell wall preparations**

A defined amount of cell wall (~20 mg) was resuspended in 1.5 ml of 2M HCl and boiled for three hours. The sample was diluted to 10 ml with water and then freeze-dried overnight. The hydrolysed walls were washed three times by resuspension in 5 ml of water and freeze-dried overnight. Finally, the samples were resuspended in 1 ml of water and stored at 4°C. As a control, 1 mg of glucose was treated as a regular sample to calculate the fraction of glucose lost during the hydrolysis procedure.

**Assay for glucose content using the glucose oxidase reaction**

Glucose oxidase reagent was prepared by resuspending 1 PGO tablet (peroxidase and glucose oxidase from Sigma-Aldrich Chemie, Zwijndrecht, the Netherlands) in 100 ml of water to which ABTS (diammonium 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonate), Boehringer) was added to a final concentration of 1 mg/ml. Reactions were prepared by mixing 100 µl of sample with 5 ml of glucose oxidase reagent, and the reaction was allowed to continue for 45 minutes at room temperature in the dark, after which the absorbance of the samples was determined at 450 nm. Water was taken as the blank, and 100 µg of glucose resuspended in 100 µl water as standard. The concentration of glucose in the sample was calculated as follows: \( \frac{\text{Absorbance of sample at 450 nm}}{\text{Absorbance of standard at 450 nm}} \times \text{(concentration of standard)} \).

**Phage titre determination**

The phage titre was determined by mixing 0.1 ml of phage suspension with 0.2 ml of *B. subtilis* culture grown in nutrient broth and 2 ml of molten soft agar (1%) at 45°C. The mixture was then vortexed for 10 seconds, poured onto a nutrient agar plate and the plate was incubated overnight at 37°C.

**VI.4. Results and discussion**

**Similarity analysis and regulon identification**

The deduced protein sequence of the *yhxB* gene was compared to all sequences in public databases, as well as to all available *B. subtilis* protein sequences. Besides numerous orthologs, one putative *B. subtilis* paralog of YhxB was identified, YbbT. However, its sequence is not included in the multiple sequence alignment presented in Fig. VI.2 because, although its sequence is 25% identical to *yhxB*, it lacks 24 of the 59 conserved amino acids of the phosphogluco- and phosphomannomutases. In this figure, for clarity reasons, only orthologs with the highest similarity are included, since there are at least forty sequences with significant homology to the YhxB protein sequence. The catalytic site residue, the serine at position 146 (in YhxB), forms the phosphoserine intermediate.
Identification of \textit{gtaC} 89

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**Fig.VI.2.** Multiple sequence alignment of (putative) phosphomanno- and phosphoglucomutases. Completely conserved residues are indicated by an asterisk; highly conserved residues with a double dot, and weakly conserved ones with a single dot. Ml: \textit{Mycobacterium leprae} putative phosphomanno-mutase (PMM); Mt: \textit{Mycobacterium tuberculosis} putative PMM/PGM (phosphogluco-mutase); Bs: \textit{Bacillus subtilis} YhxB; Bb: \textit{Borrelia burgdorferi} PMM; MP: \textit{Mycoplasma pirum} PMM; Mg: \textit{Mycoplasma genitalium} PMM.
**YhxB** is probably a monocistronic gene, since it is preceded and followed by a putative rho-independent terminator. The calculated free energies of these terminators are (at 37°C) -15.1 kCal (upstream of **yhxB**), and -14.8 kCal (downstream of **yhxB**). The 5’ upstream region contains a putative σA-dependent promoter: GTGACA-15nt-TATAAA.

**Mutant construction**

A Campbell-type mutant with concomitant transcriptional fusion to a lacZ reporter gene was constructed using plasmid pMUTin2 (Vagner *et al.*, 1998), which is the standard vector used for mutant construction and analysis in the European *B. subtilis* functional analysis program (Fig.VI.3.) An internal fragment of the **yhxB** coding region was amplified by PCR using primers TCS7 (HindIII tag; 5’-GCCGAAGCTTGTACGCGTCTTGAA-3’) and TCS8 (BamHI tag; 5’-CGCGGATCCATTGACGCGG-3’), and this fragment was cloned in pMUTin2. The resulting construct was used to transform *B. subtilis* 168 and transformants (Emr colonies) were checked for correct integration of the pMUTin2 derivative in **yhxB** (C).

Fig.VI.3. Construction of a Campbell-type mutant yielding a **yhxB** knock-out and P~**yhxB**-lacZ fusion. Indicated are the map of the integrational plasmid pMUTin2 (A), the chromosomal organisation in the **yhxB** region with the fragment that was amplified by PCR and cloned into pMUTin2, and the resulting situation (strain TCS789) after integration of the pMUTin2 derivative in **yhxB** (C).

using primers TCS7 (HindIII tag; 5’-GCCGAAGCTTGTACGCGTCTTGAA-3’) and TCS8 (BamHI tag; 5’-CGCGGATCCATTGACGCGG-3’), and this fragment was cloned in pMUTin2. The resulting construct was used to transform *B. subtilis* 168 and transformants (Emr colonies) were checked for correct integration of the pMUTin2 vector by PCR analysis and Southern hybridisation (data not shown). The resulting correct strain, *B. subtilis* TCS789, was used for all further analyses.

**Growth, expression, and standard functional analysis**

Since the **yhxB** mutant strain was viable, we concluded that gene **yhxB** is not essential. Growth and β-galactosidase activity were determined for the wild-type strain, *B. subtilis* 168, and the **yhxB** mutant, *B. subtilis* TCS789. The assays were carried out in minimal medium and nutrient broth and the data are summarised in Fig.VI.4. The results show that growth was slightly impaired when the **yhxB** mutant was grown in minimal medium. In this medium, the
mutant grew with a doubling time of 34.4 min as compared to 28.8 min for the wild-type, it reached a lower final cell density, and started to lyse already in early stationary phase. These differences were not observed in nutrient broth. Although yhxB was expressed during growth, its expression was highest around the switchpoint between logarithmic and stationary growth.

The yhxB mutant strain was also subjected to several tests that are employed in the European Bacillus subtilis functional analysis program, the aim of which is to analyse (and categorise) a large number of B. subtilis ORFs with unknown function with respect to processes such as protein secretion, sporulation, competence, recombination (measured as mitomycin C sensitivity), and stress responses. None of these tests revealed differences between the wild-type and yhxB mutant. Only during growth at increased (51°C) temperature, a difference was observed: although the yhxB mutant formed colonies of similar size as the wild-type strain, the centre of these colonies rapidly lysed after overnight incubation. No differences were observed during colony growth at 37°C or 15°C.

**Phage susceptibility and cell wall glucose content**

In order to demonstrate that yhxB corresponds to the gtaC marker, the yhxB mutant was analysed for cell wall glucose content and phage Φ25 and Φ29 susceptibility. In strain
TCS789, cell wall glucose content was reduced almost tenfold as compared to the wild-type strain, and the mutation in \( yhb \) rendered the cells fully resistant to both phages, \( \Phi25 \) and \( \Phi29 \) (Table VI.1.).

**Table VI.1.** Cell wall glucose content and phage \( \Phi25/\Phi29 \) susceptibility

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cell wall glucose (( \mu )g/mg)</th>
<th>Phage ( \Phi25/\Phi29 ) susceptibility $^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>( B. subtilis ) 168</td>
<td>163</td>
<td>normal; ( 10^9 ) p.f.u./ml</td>
</tr>
<tr>
<td>( B. subtilis ) TCS789</td>
<td>18</td>
<td>zero</td>
</tr>
</tbody>
</table>

$^5$: Determined by phage adsorption to the cells in liquid rich medium and subsequent plating on rich medium as an overlay in top agar. The titre after overnight incubation at 37°C was used as a measure for phage susceptibility.

In this paper, we have shown that a knock-out mutation could be constructed in ORF \( yhb \), indicating that this gene is not essential. ORF \( yhb \) probably encodes the enzyme phosphoglucomutase. In the \( yhb \) mutant strain, the cell wall glucose content and phage \( \Phi25/\Phi29 \) susceptibility were phenotypically similar to the corresponding properties of a phosphoglucomutase-deficient strain that was previously described by Young (1967). Although \( gtaC \) was previously mapped at 77°, and \( yhb \) is located at 86° on the \( B. subtilis \) chromosome (Anagnostopoulos *et al.*, 1993), the difference between these positions is in all probability only seeming, as the physical/genetic map of this region of the \( B. subtilis \) chromosome has recently been thoroughly revised (see chapter II of this thesis). Therefore, we conclude that ORF \( yhb \) most likely corresponds to the \( gtaC \) marker, responsible for glucosylation of teichoic acid.

We have also investigated the possibility that one of the genes in the vicinity of \( yhb \) could encode the other teichoic acid marker, \( gtaE \), the presence of which was postulated by Pooley and coworkers (1987). Upstream of \( yhb \), the \( glpPFKD \) operon is located, which is involved in uptake and catabolism of glycerol. These are not likely candidates, since \( gtaE \) was postulated to be a regulator of phosphoglucomutase and UDP-glucose pyrophosphorylase. Downstream of \( yhb \), we identified by similarity analysis more likely candidates: a putative two-component system of unknown function encoded by \( yhcY \) (sensory histidine kinase) and \( yhcZ \) (\( degU \)-like regulator). This two-component system is organised in an operon structure with a third ORF of unknown function which has one ortholog in *Escherichia coli*, \( yieF \), also of unknown function. Insertional mutants of these three genes were constructed in essentially the same manner as was done for \( yhb \), and these were analysed for phage \( \Phi25 \) and \( \Phi29 \) susceptibility. The three mutant strains all displayed wild-type sensitivity to both bacteriophages. We conclude from these experiments that neither the two-component system encoded by \( yhcYZ \), nor \( yhdA \) correspond to the \( gtaE \) marker.
References


