YvcK of *Bacillus subtilis* is required for a normal cell shape and for growth on Krebs cycle intermediates and substrates of the pentose phosphate pathway

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The HPr-like protein Crh has so far been detected only in the bacillus group of bacteria. In *Bacillus subtilis*, its gene is part of an operon composed of six ORFs, three of which exhibit strong similarity to genes of unknown function present in many bacteria. The promoter of the operon was determined and found to be constitutively active. A deletion analysis revealed that gene *yvcK*, encoded by this operon, is essential for growth on Krebs cycle intermediates and on carbon sources metabolized via the pentose phosphate pathway. In addition, cells lacking YvcK acquired media-dependent filamentous or L-shape-like aberrant morphologies. The presence of high magnesium concentrations restored normal growth and cell morphology. Furthermore, suppressor mutants cured from these growth defects appeared spontaneously with a high frequency. Such suppressing mutations were identified in a transposon mutagenesis screen and found to reside in seven different loci. Two of them mapped in genes of central carbon metabolism, including *zwf*, which encodes glucose-6-phosphate dehydrogenase and *cggR*, the product of which regulates the synthesis of glyceraldehyde-3-phosphate dehydrogenase. All these results suggest that YvcK has an important role in carbon metabolism, probably in gluconeogenesis required for the synthesis of cell wall precursor molecules. Interestingly, the *Escherichia coli* homologous protein, YbhK, can substitute for YvcK in *B. subtilis*, suggesting that the two proteins have been functionally conserved in these different bacteria.

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INTRODUCTION

In the Gram-positive bacterium *Bacillus subtilis*, carbon catabolite repression (CCR) is mediated by phosphorylation of the phosphotransferase protein HPr and of its homologue Crh (Deutscher *et al.*, 2002). Whereas HPr is present in many bacteria, Crh proteins have been found exclusively in bacilli, i.e. *B. subtilis*, *Bacillus halodurans*, *Bacillus anthracis*, *Bacillus cereus*, *Bacillus licheniformis*, *Oceanobacillus iheyensis* and *Geobacillus stearothermophilus* (Rasko *et al.*, 2004; Veith *et al.*, 2004; for a review, see Warner & Lolkema, 2003). In *B. subtilis*, Crh is encoded within an operon composed of the six ORFs *yvcIJKL*, *crh* and *yvcN* (Galinier *et al.*, 1997). Three of these genes, *yvcI*, *yvcK* and *yvcL*, are clustered together with *crh* in all bacilli that possess a *crh* gene, whereas *yvcI* and *yvcN* are absent in some of these species. The gene

cluster *yvcJKL* is conserved in most Gram-positive bacteria. Homologues of genes *yvcJ* and *yvcK* are also present in several *Proteobacteria*, such as *Escherichia coli* (the designations of these genes in *E. coli* are *yhbJ* and *ybhK*, respectively), but they are located at different sites on the chromosome. In these bacteria, *yhbJ* is linked to genes related to the phosphoenolpyruvate:carbohydrate phosphotransferase system (PEP:PTS) (Boël *et al.*, 2003; Warner & Lolkema, 2003). The function of the genes *yvcJ* and *yvcK* and of their homologues in other bacteria remains unknown. Bioinformatic analyses propose that both proteins are cytoplasmic. YvcJ could be a nucleotide-binding protein containing a Walker A motif, whereas YvcK could belong to the subfamily of conserved hypothetical proteins that forms a sister group to the CofD family, involved in coenzyme F420 biosynthesis in archaea and high-G + C Gram-positive bacteria (Graupner *et al.*, 2002).

This paper describes a study of the *B. subtilis* *yvcI–N* operon. We characterized the promoter and showed that the gene *yvcK* encoded by this operon is essential for growth on intermediates of the Krebs cycle as well as on substrates of the pentose phosphate pathway (PPP), and in addition for a

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Abbreviations: EMP, Embden–Meyerhoff–Parnas; mTn10, mini-Tn10; PPP, pentose phosphate pathway; ST-PCR, semi-random, two-step PCR.

regular cell morphology. We propose that YvcK plays a role in carbon metabolism, presumably in gluconeogenesis.

METHODS

Plasmids, bacterial strains and general growth conditions.

The plasmids and *B. subtilis* strains used in this study are listed in Table 1. For cloning purposes, *E. coli* DH5 α (Woodcock *et al.*, 1989)

was used. DNA manipulations were performed following standard procedures (Sambrook & Russell, 2001). For the construction of a conditional *zwf* knockout mutation, the 5' part of *zwf* was amplified by PCR using the primers BG108 [*zwf* (−33 to −12)] and BG109 [*zwf* (+374 to +357)]. The PCR fragment was digested at the *Hind*III and *Eco*RI sites within the primers, and inserted between these sites in plasmid pMUTIN2 (Vagner *et al.*, 1998). The resulting plasmid pBGG9 was used to transform strain 168 by a single-cross-over event by selection for erythromycin resistance, resulting in

Table 1. *B. subtilis* strains used in this study

Strain	Relevant structure or genotype*	Source or reference
168	<i>trpC2</i>	Laboratory stock
GP 311	<i>trpC2</i> , Δ cggR <i>amyE</i> ::[cggR::lacZ <i>aphA3</i>]	Ludwig <i>et al.</i> (2001)
GM1501	<i>gapA</i> ::pMUTIN2	Fillinger <i>et al.</i> (2000)
SG1	<i>trpC2</i> , <i>amyE</i> ::[<i>yvcI</i> -5' (−419 to +105)::lacZ, <i>cat</i>]	pBGM9/ <i>ScaI</i> →168
SG2	<i>trpC2</i> , <i>amyE</i> ::[<i>yvcI</i> -5' (−311 to +105)::lacZ, <i>cat</i>]	pBGM10/ <i>ScaI</i> →168
SG3	<i>trpC2</i> , <i>amyE</i> ::[<i>yvcI</i> -5' (−205 to +105)::lacZ, <i>cat</i>]	pBGM11/ <i>ScaI</i> →168
SG5	<i>trpC2</i> , <i>amyE</i> ::[<i>yvcI</i> 5' (−20 to +105)::lacZ, <i>cat</i>]	pBGM13/ <i>ScaI</i> →168
SG6	<i>trpC2</i> , <i>amyE</i> ::[<i>yvcI</i> 5' (+1 to +105)::lacZ, <i>cat</i>]	pBGM14/ <i>ScaI</i> →168
SG29	<i>trpC2</i> , <i>amyE</i> ::[<i>yvcI</i> -5' (−176 to +105)::lacZ, <i>cat</i>]	pBGM21/ <i>ScaI</i> →168
SG30	<i>trpC2</i> , <i>amyE</i> ::[<i>yvcI</i> -5' (−153 to +105)::lacZ, <i>cat</i>]	pBGM22/ <i>ScaI</i> →168
SG31	<i>trpC2</i> , <i>amyE</i> ::[<i>yvcI</i> -5' (−141 to +105)::lacZ, <i>cat</i>]	pBGM23/ <i>ScaI</i> →168
SG32	<i>trpC2</i> , <i>amyE</i> ::[<i>yvcI</i> -5' (−80 to +105)::lacZ, <i>cat</i>]	pBGM25/ <i>ScaI</i> →168
SG33	<i>trpC2</i> , <i>amyE</i> ::[<i>yvcI</i> -5' (−61 to +105)::lacZ, <i>cat</i>]	pBGM26/ <i>ScaI</i> →168
SG34	<i>trpC2</i> , <i>amyE</i> ::[<i>yvcI</i> -5' (−40 to +105)::lacZ, <i>cat</i>]	pBGM27/ <i>ScaI</i> →168
SG56	<i>trpC2</i> , Δ (<i>yvcI</i> , <i>yvcK</i>)::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>)	pBGM48/ <i>ScaI</i> →168
SG59	<i>trpC2</i> , <i>amyE</i> ::[<i>yvcI</i> -5' (−141 to +19)::lacZ, <i>cat</i>]	pBGM52/ <i>ScaI</i> →168
SG61	<i>trpC2</i> , Δ (<i>yvcI</i> , <i>yvcK</i> , <i>yvcL</i>)::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>)	pBGM57/ <i>ScaI</i> →168
SG62	<i>trpC2</i> , Δ <i>yvcJ</i> ::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>)	pBGM59/ <i>ScaI</i> →168
SG63	<i>trpC2</i> , Δ <i>yvcK</i> ::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>)	pBGM61/ <i>ScaI</i> →168
SG64	<i>trpC2</i> , Δ <i>yvcL</i> ::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>)	pBGM67/ <i>ScaI</i> →168
SG65	<i>trpC2</i> , Δ (<i>crh</i> <i>yvcN</i>)::(<i>cat</i> , <i>rrnBt₁t₂λt₀</i>)	pBGM69/ <i>ScaI</i> →168
SG68	<i>trpC2</i> , <i>amyE</i> ::(<i>P_{spac}</i> ::lacZ)	Görke <i>et al.</i> (2004)
SG71	<i>trpC2</i> , <i>amyE</i> ::[<i>yvcI</i> -5' (−115 to +105)::lacZ, <i>cat</i>]	pBGM71/ <i>ScaI</i> →168
SG91	<i>trpC2</i> , Δ <i>yvcK</i> ::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>), Δ cggR <i>amyE</i> ::[cggR::lacZ <i>aphA3</i>]	SG63→GP311
SG92	<i>trpC2</i> , Δ <i>yvcK</i> ::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>), <i>gapA</i> ::pMUTIN2	GM1501→SG63
SG93	<i>trpC2</i> , <i>zwf</i> ::pMUTIN2	pBGG9→168
SG94	<i>trpC2</i> , Δ <i>yvcK</i> ::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>), <i>zwf</i> ::pMUTIN2	SG93→SG63
SG63-NB7 (8)	<i>trpC2</i> , Δ <i>yvcK</i> ::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>), cggR::Tn10 (position: +678; L→R)	mTn10 mutagenesis
SG63-NB6	<i>trpC2</i> , Δ <i>yvcK</i> ::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>), <i>yfnI</i> ::Tn10 (position: +234; R→L)	mTn10 mutagenesis
SG63-NB1 (2, 3, 5), -GN2	<i>trpC2</i> , Δ <i>yvcK</i> ::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>), <i>zwf</i> ::Tn10 (position: +1323; L→R)	mTn10 mutagenesis
SG63-GN1	<i>trpC2</i> , Δ <i>yvcK</i> ::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>), <i>yqfF</i> ::Tn10 (position: +1020; L→R)	mTn10 mutagenesis
SG63-GN5	As SG63-GN1, but Tn10 inserted in R→L direction	mTn10 mutagenesis
SG63-GN3	<i>trpC2</i> , Δ <i>yvcK</i> ::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>), <i>secDF</i> ::Tn10 (position: +12; L→R)	mTn10 mutagenesis
SG63-GN7	<i>trpC2</i> , Δ <i>yvcK</i> ::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>), <i>mfd</i> ::Tn10 (position: +2836; L→R)	mTn10 mutagenesis
SG63-GN9	<i>trpC2</i> , Δ <i>yvcK</i> ::(<i>lacZ</i> , <i>cat</i> , <i>rrnBt₁t₂λt₀</i>), <i>mrpB</i> ::Tn10 (position: +96; L→R)	mTn10 mutagenesis
SM-NB7	<i>trpC2</i> , cggR::Tn10 (position: +678; L→R)	SG63-NB7→168
SM-NB6	<i>trpC2</i> , <i>yfnI</i> ::Tn10 (position: +234; R→L)	SG63-NB6→168
SM-NB3	<i>trpC2</i> , <i>zwf</i> ::Tn10 (position: +1323; L→R)	SG63-NB3→168
SM-GN1	<i>trpC2</i> , <i>yqfF</i> ::Tn10 (position: +1020; L→R)	SG63-GN1→168
SM-GN3	<i>trpC2</i> , <i>secDF</i> ::Tn10 (position: +12; L→R)	SG63-GN3→168
SM-GN7	<i>trpC2</i> , <i>mfd</i> ::Tn10 (position: +2836; L→R)	SG63-GN7→168
SM-GN9	<i>trpC2</i> , <i>mrpB</i> ::Tn10 (position: +96; L→R)	SG63-GN9→168

*All positions are relative to the initiation codon of the respective gene.

strain SG93. Subsequently, the *zwf*::pMUTIN allele was crossed into strain SG63, resulting in strain SG94. Luria–Bertani (LB) broth was routinely used for bacterial growth. For propagation, the *yvcK* mutant strains were cultivated in LB supplemented with 1% (w/v) glucose. When necessary, the media were supplemented with the appropriate antibiotics (ampicillin at 100 µg ml⁻¹ for *E. coli*; chloramphenicol at 5 µg ml⁻¹, kanamycin at 5 µg ml⁻¹, erythromycin at 0.4 µg ml⁻¹ and spectinomycin at 100 µg ml⁻¹ for *B. subtilis*). *E. coli* and *B. subtilis* cells were transformed with DNA following standard procedures (Kunst & Rapoport, 1995; Sambrook & Russel, 2001). For the transfer of alleles such as the *zwf*::pMUTIN and the various mTn10 insertion mutations into other strains, chromosomal DNA of the respective mutant strain was prepared according to Cutting & Vander Horn (1990) and used for transformation.

Growth tests. Growth tests in liquid media were performed at 37 °C and with 160 r.p.m. agitation. Difco sporulation medium (DSM) was prepared according to Schaeffer *et al.* (1965). It contains 3 g Bacto beef extract l⁻¹, 5 g Bacto-peptone l⁻¹, 13.4 mM KCl, 1 mM MgSO₄, 10 µM MnCl₂, 0.5 mM CaCl₂ and 1 µM FeSO₄. CE-minimal medium contains 70 mM K₂HPO₄, 30 mM KH₂PO₄, 25 mM (NH₄)₂SO₄, 0.5 mM MgSO₄, 10 µM MnSO₄, 22 mg ferric ammonium citrate l⁻¹, 20 mg L-tryptophan l⁻¹ and 8 g potassium glutamate l⁻¹ (Martin-Verstraete *et al.*, 1990). The various carbon sources were added to a final concentration of 0.5% (w/v), with the exception of sodium succinate, which was used at a final concentration of 0.6% (w/v). An additional concentration of 1 mM MgCl₂ was added to the CE-citrate medium in order to improve the growth of *B. subtilis* on this carbon source (Warner & Lolkema, 2002). Note that the addition of glutamate is indispensable because the wild-type strain *B. subtilis* 168 is unable to utilize ammonium as sole nitrogen source when only poor carbon sources like succinate are available (Wacker *et al.*, 2003). Strain 168 is unable to grow on glutamate as a sole source of carbon, in other words it cannot grow in CE-medium unless a carbon source is added (data not shown).

For growth monitoring, pre-cultures were grown overnight in LB supplemented with 1% glucose. Thereafter, the cells were washed and inoculated to an OD₆₀₀ of 0.1 in the medium in which the growth test was subsequently performed. OD₆₀₀ readings were periodically taken during the incubation of the cultures. For growth monitoring on solid medium, the bacteria were streaked out from pre-cultures grown in LB-glucose and kept frozen at -70 °C.

Promoter deletion analysis. DNA fragments carrying sequences of the *trxB-yvcI* intergenic region were amplified by PCR using chromosomal DNA of strain 168 as template. The PCR reactions were carried out using the reverse primer BG17 [*yvcI* (+105 to +88); *Bam*HI site within the 5' end] in combination with one of various forward primers that carried an *Eco*RI or *Mun*I site at their 5' ends, respectively. The PCR fragments were digested by *Bam*HI and *Eco*RI or *Mun*I and inserted between the *Bam*HI and *Eco*RI sites on plasmid pAC6 (Stülke *et al.*, 1997). Plasmid pAC6 carries a promoterless *lacZ* reporter gene followed by *cat* downstream of the *Bam*HI/*Eco*RI cloning site encompassed by the *amyE*-5' and *amyE*-3' regions, respectively. The various upstream primers used, their annealing sites and the respective assigned plasmids were as follows: primer BG18 [*yvcI* (-419 to -403)] → plasmid pBGM9; primer BG19 [*yvcI* (-311 to -295)] → plasmid pBGM10; primer BG20 [*yvcI* (-205 to -189)] → plasmid pBGM11; primer BG36 [*yvcI* (-176 to -157)] → plasmid pBGM21; primer BG37 [*yvcI* (-153 to -135)] → plasmid pBGM22; primer BG57 [*yvcI* (-115 to -98)] → plasmid pBGM71; primer BG39 [*yvcI* (-80 to -62)] → plasmid pBGM25; primer BG40 [*yvcI* (-61 to -40)] → plasmid pBGM26; primer BG41 [*yvcI* (-40 to -22)] → plasmid pBGM27; primer BG1 [*yvcI* (-20 to -3)] → plasmid pBGM13; primer BG22 [*yvcI* (+1 to +17)] → plasmid pBGM14. For the construction of plasmid

pBGM23 carrying a fusion of *yvcI* (-141 to +105) to *lacZ*, we used the *Eco*RI site occurring naturally at position -140 to -135 in front of *yvcI*. The 1087 bp *Eco*RI-*Clal* fragment of plasmid pBGM10 was inserted between these sites on plasmid pAC6. The recombinant plasmids described above were linearized by digestion with *Sca*I and subsequently used to transform strain 168 by a double-crossover event with the *amyE* gene with selection for chloramphenicol resistance. The recombination events were verified by monitoring the absence of amylase activity, as described previously (Cutting & Vander Horn, 1990).

Construction of gene deletions within the *yvcI*-*N* operon.

For the construction of gene deletions, the generalized deletion vector pBGM35 was designed. To this end, the *Bam*HI-*Dra*I fragment of pMUTIN2 (Vagner *et al.*, 1998) encompassing the RBS_{spovG}-*lacZ* cassette was inserted between the *Bam*HI and *Sma*I sites of the previously described plasmid pBGM31 (Görke *et al.*, 2004). Plasmid pBGM35 therefore carries a *lacZ*-*cat*-*rrnBt1t2λt0* cassette encompassed upstream by *Sac*II/*Bam*HI and downstream by *Apa*I/*Kpn*I cloning sites. About 500 bp of the sequences flanking the gene(s) to be deleted was amplified by PCR and successively inserted between these sites. The resulting plasmids were linearized by *Sca*I digestion and used to transform *B. subtilis* 168 by a double-crossover event with selection for chloramphenicol resistance. The resulting gene deletions, and the designation of the respective strains and of the plasmids used to construct them, were as follows: Δ*yvcJ* (SG62; pBGM59), Δ*yvcK* (SG63; pBGM61), Δ*yvcJ*-*K* (SG56; pBGM48), Δ*yvcJ*-*K*-*L* (SG61; pBGM57), Δ*yvcL* (SG64; pBGM67), Δ[*crh*, *yvcN*] (SG65; pBGM69). Details of these constructions are available on request. In the resulting strains, the *lacZ*-*cat*-*rrnBt1t2λt0* cassette replaced the desired gene(s) and simultaneously a transcriptional fusion of *lacZ* to the gene(s) located upstream was created. The presence of the three transcriptional terminators prevented a transcriptional read-through into genes located downstream of the cassette.

Construction of expression plasmids for the complementation analysis.

The genes *yvcJ* and *yvcK* were amplified by PCR using chromosomal DNA of *B. subtilis* 168 as template and the primer pairs BG53 [*yvcJ* (+1 to +19)] with BG54 [*yvcJ* (+890 to 871)] and BG55 [*yvcK* (-42 to -24)] with BG56 [*yvcK* (+954 to 936)], respectively. Gene *ybhK* from *E. coli* was amplified by PCR using primers BG86 [*ybhK* (-1 to +18)] and BG87 [*ybhK* (+909 to +892)] and chromosomal DNA of *E. coli* W3110 (Bachmann, 1972) as template. The PCR fragments were digested at the *Stu*I sites introduced by the primers and inserted into the *Stu*I site of the *B. subtilis* replicative plasmid pDG148-*Stu* (Joseph *et al.*, 2001). The resulting plasmids pBGM53, pBGM54 and pBGM55 carry the genes *yvcJ*, *yvcK* and *ybhK*, respectively, under control of the IPTG-inducible *P*_{spac} promoter.

β-Galactosidase assays. Enzyme assays were according to Miller (1972), as previously described (Görke *et al.*, 2004). Samples were assayed in triplicate and each experiment was repeated at least twice using independent cultures. β-Galactosidase activities are expressed in Miller units. Standard deviations were below 15%.

RNA isolation and primer extension analysis. In order to determine the transcription start of the promoter present in front of *yvcI*, strain SG59 was used. This strain carries a transcriptional fusion of *yvcI* (-141 to +19) to *lacZ* integrated into the *amyE* locus. For its construction, a PCR fragment was amplified using primers BG20 [*yvcI* (-205 to -189)] and BG58 [*yvcI* (+19 to +1)], and digested with *Eco*RI (at position -140 to -135 in front of *yvcI*) and *Bam*HI (site located in primer BG58), and inserted between the corresponding sites of plasmid pAC6. The resulting plasmid pBGM52 was subsequently used to transform strain 168 by a double-crossover event with the *amyE* gene with selection for chloramphenicol resistance. For RNA isolation, strain SG59 was grown

overnight in CE-succinate, and 2 ml of the culture was harvested. Total RNA was isolated using the High Pure RNA isolation kit (Roche) according to the supplier's protocol, but DNase treatments and column purifications were repeated in order to eliminate contamination with genomic DNA. Primer extension reactions were carried out using 50 µg total RNA, 10 pmol end-labelled primer BG25 [primers in *lacZ* in reverse direction (positions +71 to +48)] and 200 units of SuperScript II RNaseH⁻-reverse transcriptase (Gibco-BRL) in 20 µl 1 × first-strand buffer (Gibco-BRL) containing 10 mM dithiothreitol and 0.5 mM each deoxyribonucleotide triphosphate (Amersham Pharmacia Biotech). The 5'-end labelling of the oligonucleotide was performed using T4 polynucleotide kinase (Amersham Pharmacia Biotech) and [γ -³²P]ATP (Amersham Pharmacia Biotech). Primer extension products were analysed by electrophoresis on a 6% polyacrylamide/6 M urea gel, alongside a sequencing ladder (lanes C, T, A and G) obtained with the same end-labelled primer and a PCR fragment encompassing the putative promoter [*yvcI* (-141 to +19)] as template. Sequencing reactions were carried out using Sequenase purchased from United States Biochemical.

Culture observation. Bacterial cells were observed by phase-contrast microscopy (Nikon Eclipse E800) and images were recorded using the Nikon Digital camera DXM 1200.

Construction of a chromosomal mini-Tn10 (mTn10) library and screening for suppression of the growth defect of strain SG63. For transposon mutagenesis, the mTn10 delivery vector pIC333 was introduced into strain SG63; transformants were selected at 28 °C on LB-glucose containing erythromycin. Ten independent colonies were inoculated into LB-glucose containing spectinomycin and grown overnight at 28 °C. These cultures were subsequently diluted 100-fold in 2 ml of the same medium and grown for 4 h at 28 °C. After shifting the temperature to 37 °C, the cultures were grown further for 5 h. Subsequently, the cells were washed with CE-medium lacking any carbon source and plated on DSM as well as on CE-gluconate containing spectinomycin and incubated at 37 °C. On both types of plates mutants appeared which resumed growth, and a representative number of clones were isolated. In order to verify that suppression of the growth defect was linked to the respective transposon insertion, chromosomal DNA was prepared from these suppressor mutants and retransformed into the original mutant strain SG63. Transformants were selected on LB-glucose containing spectinomycin and subsequently replica-plated onto the medium on which the respective transposon insertion was initially isolated. Those transformants for which 100% of the colonies were able to grow on both types of plate were kept for a further characterization. In addition, it was verified that the transformants were sensitive to erythromycin, in other words that the pIC333 vector had been lost.

Identification of mTn10 insertion sites by ST-PCR. The mTn10 insertion sites in the chromosomes of the various suppressing mutants were identified by the semi-random, two-step PCR (ST-PCR) protocol, which was originally developed for yeast (Chun *et al.*, 1997). The PCR conditions were as described by Chun *et al.* (1997), but the primer sequences were different. For the amplification of the sequences next to the left-hand end of the inserted mTn10, a first PCR was performed using the primers BG80 (5'-GGCCACGCGTCGACTAGTACNNNNNNNNNGATC) and BG84 [5'-AAGAGCGCCCAATACGCAAACCGCC; mTn10 (145–121)]. One microlitre of this PCR product was subsequently used as a template for a second PCR using the primer BG81 (5'-GCTCTAGAGGCCACGCGTCGACTAGTAC), which carries the sequence identical to the defined sequence preceding the ten random bases of primer BG80, and primer BG85, which anneals downstream of primer BG84 within the transposon [mTn10 (112–85)]. Similarly for amplification of the sequences next to the

right-hand end of the transposon, the first PCR reaction was performed by using the primers BG80 and BG82 [5'-TTTGCATGCTTCAAAGCCTGTCGGAATTGG; mTn10 (2189–2218)]. The second PCR reaction was carried out using the primers BG81 and BG83 [5'-GCTCTAGAATTCACGGTTACCCACTT-ATAAACAAAAGATCGG; mTn10 (2241–2275)], which anneals downstream of primer BG82. The various PCR products were subsequently sequenced by using the primers BG83 or BG85 for the sequences located to the right and the left-hand end of the transposon, respectively.

RESULTS AND DISCUSSION

Mapping of the promoter of the *yvcI*-*N* operon

In a former study (Galiniér *et al.*, 1997), it was shown by Northern blot analysis that gene *crh* is encoded within an operon composed of the six ORFs, *yvcIJKL*, *crh* and *yvcN*. In order to map its promoter, DNA fragments carrying gradually 5'-shortened versions of the *trxB*-*yvcI* intergenic region were transcriptionally fused to the *E. coli lacZ* reporter gene and integrated at the *amyE* locus of the *B. subtilis* chromosome. The recombinant strains were grown in CE-minimal medium supplemented with succinate as carbon source and the β -galactosidase activities were determined. As shown in Fig. 1(A), exclusively the strains that carried at least 61 bp of the sequence upstream of the putative translation initiation codon of *yvcI* expressed the *lacZ* gene. According to this result, sequence elements crucial for promoter activity must be located between positions -61 and -40 relative to the first codon of *yvcI*, and the sequence upstream of the promoter appears not to be important for its activity, suggesting the absence of regulatory sites in this region. A primer-extension experiment was performed to localize precisely the transcription start site in front of *yvcI* (see Methods). Two bands were revealed by the primer extension: one corresponds to a guanine located 23 bp upstream from the translation initiation codon of *yvcI*, and the other corresponds to the adenine just downstream (Fig. 1B). Most likely, this represents heterogeneity at the 5' terminus of the mRNAs resulting from multiple start sites of transcription (Sambrook & Russell, 2001). Upstream of this transcription start, sequences corresponding to a σ^A -dependent promoter (TTGTGG-17bp-TATAAT; Fig. 1B) can be found (Helmann & Moran, 2002).

Activity of the promoter of the *yvcI*-*N* operon in the presence of various carbon sources

Next, we determined whether the activity of the promoter in front of *yvcI* and thus the expression of the operon may be modulated by the carbon source. To this end, strain SG31 carrying a transcriptional fusion of this promoter to *lacZ* (cf. Fig. 1A) was grown in CE-minimal medium supplemented with different carbon sources. As shown in Table 2, the β -galactosidase activities varied only slightly with most carbon sources (8–11 units). However, the two Krebs cycle intermediates citrate and succinate caused about twofold higher activities (17 units) in comparison to the other

Fig. 1. Mapping of the promoter of the *yvcI*-N operon. (A) 5'-Deletion analysis of the *yvcI*-N promoter region. Gradually 5'-shortened versions of the putative promoter region as schematically depicted at the left of the figure were fused to the *lacZ* reporter gene and integrated into the chromosome. The designations of these strains are given on the right. The various strains were grown in CE-minimal medium supplemented with succinate, and the β -galactosidase activities they produced are indicated. Positions are indicated with respect to the *yvcI* start codon. (B) Mapping of the transcriptional start point in front of *yvcI*. Total RNA of strain SG59 was prepared and a reverse transcriptase reaction was performed using a primer specific for transcripts initiated at the *yvcI*-N promoter. The identical primer was used to obtain a sequencing ladder (lanes C, T, G and A) which was separated alongside the primer extension product on a 6% polyacrylamide/6 M urea gel. The mapped 5' end of the *yvcI*-N mRNA and the direction of transcription are indicated by the angled arrow above the sequence, which encompasses the region from -61 to +4 relative to the *yvcI* start codon. The -10 and -35 sequences of the corresponding promoter are in boxes and the Shine-Dalgarno site (SD) of *yvcI* is overlined. *B. s.*, *B. subtilis*.

substrates. Since this stimulatory effect was not seen with strain SG68 carrying a constitutively expressed *P_{spac}-lacZ* transcriptional fusion, it should be specific for the promoter in front of *yvcI*.

Deletion of *yvcK* results in a growth defect

The possible functions of the genes composing the *yvcI*-N operon were addressed. Therefore, a number of strains carrying deletions to different extents within the operon were constructed (Fig. 2A). All those strains that lacked *yvcK* (strains SG61, SG56, SG63), or were unable to express it (strain SG62), did not grow on sporulation (DSM) plates and exhibited slower growth on LB plates (data not shown). In contrast, deletion of *yvcL* (strain SG64), or *crh* and *yvcN* (strain SG65) simultaneously, did not yield a detectable phenotype. Moreover, strain SG63, which lacked *yvcK* but

still possessed *yvcI*, was also unable to grow on DSM plates, suggesting that deletion of *yvcK* is responsible for the growth impairment. In DSM liquid medium, all the strains lacking expression of *yvcK* (strains SG61, SG56, SG62, SG63) exhibited a decline of the OD₆₀₀ following a short period of normal initial growth (Fig. 2B).

Surprisingly, after about 9–10 h incubation, the cultures resumed growth. In principle, this could be ascribed either to an adaptation of the mutants to the growth conditions or to the appearance of compensatory mutations suppressing the growth defect. Since the phenotype of these bacteria was stable, i.e. the growth defect was not observable in subsequent growth tests (see below), it was concluded that suppressing mutations appeared and overcame the growth impairment. Growth of the other strains which still expressed *yvcK* (SG64, SG65) was not affected when

Table 2. Activity of the promoter of the *yvcI*–*N* operon in different carbon sources

Strain SG31 carrying a fusion of the *yvcI*–*N* promoter to *lacZ* was grown in DSM or in CE-minimal medium supplemented with the indicated carbon source to an OD₆₀₀ of 0.6–0.8 and the β-galactosidase activities were determined. Strain SG68 carrying a constitutively expressed *lacZ* gene served as control.

Carbon source or medium	β-Galactosidase activity (Miller units)	
	<i>yvcI</i> –5' (–141 to +105):: <i>lacZ</i> (SG31)	<i>amyE</i> :: <i>P</i> _{spac} :: <i>lacZ</i> (SG68)
Citrate	17	18
Succinate	17	18
Malate	9	26
Gluconate	10	18
Inositol	8	23
Glycerol	8	30
Glucitol	10	29
Mannitol	10	28
Fructose	11	25
Sucrose	10	27
Glucose	11	23
DSM	9	24

compared to the wild-type strain 168 (Fig. 2B). In LB liquid medium, all the strains grew, but growth of the *yvcK* mutants was significantly slower when compared to the wild-type (data not shown).

YvcK is required for growth on Krebs cycle intermediates and on PPP substrates

LB and DSM broth are both complex media based on enzymic digests of protein combined with yeast extract (LB) or beef extract (DSM). According to a typical analysis, LB broth contains 1.14 % (w/v) amino acids and 0.17 % carbohydrates, whereas DSM contains 0.48 % amino acids and only 0.03 % carbohydrates (Difco Manual, 11th edition, 1998, Difco Laboratories, Sparks, MD 21152, USA). Therefore DSM is a poor source of carbohydrates. When glucose was added to DSM or LB medium, the growth impairments of the strains lacking YvcK were completely cured (Fig. 2B; data not shown). These observations suggested that the *yvcK* mutants require the presence of certain carbohydrates, such as glucose, for growth. In order to determine these requirements in more detail, the various strains were examined in the chemically defined CE-minimal medium supplemented with different carbon sources, on plates as well as in liquid medium (Fig. 3). All the strains grew normally on PTS substrates (glucose, sucrose, salicin, maltose, fructose, mannitol) and on glucitol, glycerol and inositol (Fig. 3A; examples are shown for glucose and glucitol in Fig. 3B). All these substrates are catabolized via the Embden–Meyerhoff–Parnas (EMP) pathway. However, no growth of the strains lacking YvcK could be observed on plates containing

arabinose, ribose, gluconate, citrate or succinate, and growth was significantly impaired on fumarate and malate. In liquid medium supplemented with gluconate, fumarate (Fig. 3B) or malate (data not shown), the *yvcK* deletion strain SG63 started to grow and reached a certain cell density before a decline of the OD₆₀₀ could be observed. In the presence of citrate or succinate (Fig. 3B), strain SG63 grew slower than the wild-type and stopped growth when an OD₆₀₀ of about 0.5 was reached, whereas the wild-type strain grew to an OD₆₀₀ of 1.6 or 1.0, respectively. For the other strains lacking YvcK, comparable growth properties were observed (data not shown). Interestingly, growth of the *yvcK* mutants was impaired if gluconeogenesis was required, i.e. on carbon sources which are substrates of the Krebs cycle or of the pentose phosphate pathway (PPP).

The presence of the genes located downstream of *yvcK* appeared not to be required for its function, since the strains SG64 and SG65 deleted for these genes exhibited normal growth. However, it could not yet be ruled out that the presence of *yvcJ* was necessary for the function of YvcK. Therefore, a complementation analysis was carried out. Genes *yvcK* and *yvcJ* were cloned, respectively, under the control of an IPTG-inducible promoter on a plasmid able to replicate in *B. subtilis*, and subsequently used to transform strains SG63 (Δ*yvcK*) and SG56 [Δ(*yvcK*, *yvcJ*)]. Growth of these transformants was tested in DSM and in the various minimal media supplemented with different carbohydrates and with IPTG for the induction of expression of *yvcK* or *yvcJ*, respectively. The growth of the bacteria that harboured the *yvcK* expression plasmid was perfectly restored in DSM (crosses and diamonds in Fig. 4), whereas the transformants that carried the empty plasmid pDG148 (stars and short dashes in Fig. 4) or the *yvcJ* expression plasmid (circles and long dashes in Fig. 4) still exhibited the same growth defect as the untransformed mutant strains. Similarly, the *yvcK*-containing plasmid restored growth of these strains on all minimal media, as demonstrated for strain SG56 on plates (data not shown) and for SG63 in liquid minimal medium containing gluconate, fumarate or citrate as sole carbon source, respectively (circles in Fig. 3B). These results allow us to conclude that the genes *yvcJ*, *yvcL*, *crh* and *yvcN* are not required for the function of YvcK.

In principle, two general reasons could account for the observed growth defects: YvcK could be required either for the efficient uptake of the various ‘non-permissive’ carbon sources or for their subsequent metabolism in the cell. The transport proteins specific for the various substrates on which growth occurred or did not occur have no common characteristics. They belong to different superfamilies of transporters and use different forces to drive transport (Saier *et al.*, 2002). In addition, inositol and arabinose are both taken up by transporters of the major facilitator superfamily but they differed in allowing growth of the *yvcK* mutants. Therefore, it was concluded that the cellular metabolism rather than the transport of the various carbon sources was disturbed in the *yvcK* mutants.

Fig. 2. Gene *yvcK* is required for growth in sporulation medium and this requirement can be overcome by addition of glucose. (A) Construction of gene deletions within the *crh*-containing operon. The organization of the *yvcI-N* operon is drawn to scale at the top. The promoter is indicated by an arrow and the stem-loop structures correspond to transcriptional terminators. The horizontal bars below refer to the schematic drawing of the operon above and indicate the genes that were replaced by the *lacZ-cat-rrnBt₁t₂λt₀* cassette in each strain. The strain designations are given on the left. Strain 168 was the parent of the various deletion strains and is included as a control. (B) OD₆₀₀ monitoring of the six deletion strains and the wild-type strain in liquid DSM and DSM medium supplemented with 1 % (w/v) glucose. For the symbols of the seven curves, refer to the listing of the strains in (A). Note that in the right panel the growth curves corresponding to all the strains listed in (A) are present. Due to overlap the filled symbols are masked in this panel.

Deletion of *yvcK* leads to aberrant and strikingly different cell shapes in media in which growth is impaired

The courses of the growth curves of the *yvcK* mutants in the various 'non-permissive media' indicated that the cells were able to use the respective carbon sources at least for some generations before the cells stopped growth (Fig. 2). This suggested that either a toxic compound accumulated in the cells or that compound(s) or protein(s) required for growth could not be synthesized any more and were subsequently diluted by cell division. In CE minimal liquid media

containing gluconate, malate or fumarate as carbon source, the OD₆₀₀ of the YvcK-lacking strains declined after an initial phase of growth (Fig. 2), suggesting that the bacteria lysed. To check this observation, we grew the *yvcK* deletion strain SG63 and the wild-type strain 168 as a reference in various media. Samples were taken during the incubation and the bacteria were examined by microscopy. When grown in minimal medium containing glucose, strain SG63 exhibited a normal cell morphology, similar to that of the wild-type strain 168 (data not shown). In gluconate-containing minimal medium, the cells of strain SG63 adopted 'bubble'-like shapes, starting soon after inoculation

Fig. 3. Growth of strains carrying deletions within the *yvcI–N* operon on various carbon sources. (A) Schematic representation of the growth properties on CE-minimal medium plates supplemented with the indicated carbon sources. Strain 4 is a transformant of strain SG56 containing the replicative plasmid pBGM54 that carries gene *yvcK* under the control of the *P_{spac}* promoter. For induction of expression of *yvcK*, 1 mM IPTG was added to the medium. +, Growth; –, no growth. (B) Growth properties of strain SG63 ($\Delta yvcK$; triangles) and SG63 transformed with the *yvcK* expression plasmid pBGM54 (circles) in comparison to strain 168 (wild-type; squares) in liquid CE-minimal medium supplemented with different carbon sources, as indicated by the filled and open symbols.

of the medium, and resembled the L-form of *B. subtilis*, which has been shown to lack a cell wall (Gilpin *et al.*, 1973). This abnormality exhibited its most drastic extent 4–6 h after inoculation, shortly before the OD₆₀₀ declined (Fig. 5A). At that time, cell debris became visible, confirming that the bacteria had lysed. When the OD₆₀₀ of the culture rose again, bacteria exhibiting normal cell shapes started to appear, indicating that the compensatory mutation(s), which suppress the growth defect, also suppress the abnormal morphology.

In minimal medium containing malate, the *yvcK* mutants also swelled, but the effect was less drastic than that of gluconate (data not shown). Surprisingly, in DSM, strain SG63 exhibited a totally different phenotype. Very long filaments, indicative of a severe defect in septum formation and/or cell division, could be observed some hours after inoculation of the medium, and these filaments were longest when the lowest OD₆₀₀ value was reached during incubation in DSM (Fig. 5B). Thereafter, when the culture resumed growth, normally

shaped bacteria became visible, which again indicated that the suppressing mutation(s) restore both growth and normal cell morphology. Transformation of strain SG63 with plasmid pBGM54 carrying gene *yvcK* completely restored the normal morphological phenotypes during growth in DSM liquid medium as well as in CE-gluconate minimal liquid medium, rendering the cells indistinguishable from the wild-type strain 168 (data not shown).

High concentrations of Mg²⁺ restore growth and wild-type cell morphology of the *yvcK* mutant

Cell wall deficiency can be the result of degradation by cellular enzymes, a process known as autolysis, or due to a failure in biosynthesis or of the biosynthesis of precursors. Autolysis occurs in *B. subtilis* under any condition that causes the dissipation of protonic potential (Jolliffe *et al.*, 1981; Kemper *et al.*, 1993). An inhibited cell envelope synthesis has been observed in *B. subtilis* mutants defective in the pathway that converts glucose 6-phosphate into teichoic acids, which

Fig. 4. Plasmid-driven expression of *yvcK* and of *ybhK* from *E. coli* restores growth of the *yvcK* deletion strains. Strain SG56 lacking genes *yvcJ* and *yvcK*, as well as strain SG63 lacking *yvcK* only, were transformed with replicative plasmids carrying gene *yvcJ* (plasmid pBGM53), *yvcK* (plasmid pBGM54), *ybhK* from *E. coli* (plasmid pBGG1) or no gene (empty expression vector; pDG148-Stu) under the control of the IPTG-inducible P_{Spac} promoter. The OD₆₀₀ of these transformants as well as of the untransformed strains was monitored during growth in DSM supplemented with 1 mM IPTG. The graph symbols refer to the listing of the transformants given at the top of the figure.

are major constituents of the cell wall (Prasad & Freese, 1974; Lazarevic *et al.*, 2005). A *gtaC* mutant deficient in the α -phosphoglucosyltransferase catalysing the first step of this pathway, i.e. the interconversion of glucose 6-phosphate to α -glucose 1-phosphate, is morphologically similar to a *yvcK* mutant grown on gluconate (Fig. 5A; Lazarevic *et al.*, 2005). It has been reported that abnormal cell morphologies due to cell wall deficiency caused by mutations in the pathway leading to teichoic acids or due to a *mreB* null mutation can be cured by the addition of magnesium (Lazarevic *et al.*, 2005; Formstone & Errington, 2005). To check the idea that cell wall synthesis might be affected in the *yvcK* mutants when cultivated on the 'non-permissive media', the *yvcK* deletion strain SG63 and, as a reference, the wild-type strain 168 were grown in CE-gluconate minimal liquid medium in the presence or absence of an additional concentration of

25 mM MgCl₂. The addition of magnesium ions perfectly restored normal growth rates (Fig. 6A) and the wild-type cell morphology of strain SG63 (Fig. 6B). This result suggested that cell wall biosynthesis is affected in the *yvcK* mutant.

Suppressor mutations appear at high rates and cure the growth defects caused by deletion of *yvcK*

Liquid cultures of the *yvcK* deletion strains in DSM resumed growth after about 9–10 h shaking (Fig. 2B). Similarly, cultures of these mutants in minimal medium supplemented with one of the various non-permissive carbon sources were fully grown after an additional overnight incubation subsequent to the time range presented in Fig. 3B. The bacteria from the latter cultures exhibited normal cell shapes, suggesting that suppressor mutants had grown. Likewise, such suppressor mutants could be isolated as single colonies, when the strains were streaked on plates containing these media (data not shown). By a comparison of plating efficiencies on DSM and LB-glucose, we estimate that the suppressing mutants appear at the high rate of about 10^{-4} . When chromosomal DNA prepared from suppressor mutants isolated on DSM plates was back-crossed by transformation of the wild-type strain 168, chloramphenicol-resistant transformants were again unable to grow on DSM (data not shown). This demonstrated that the suppressing mutations can be separated from the *cat-lacZ* deletion cassette and must therefore be located *in trans* elsewhere on the chromosome. When growth of such spontaneous suppressor mutants was retested in the medium from which they were originally isolated, the bacteria grew normally like the wild-type strain 168. However, when suppressor mutants were tested for growth in the other media, the mutants split into several classes: some were able to grow on all media, whereas others grew only on a subset of the substrates on which growth of the original $\Delta yvcK$ mutant was impaired, and a third group acquired new growth defects, for example on CE-glucose (data not shown). This suggested that mutations in several different genetic loci are capable of suppressing the various growth defects, and consequently that YvcK may have a pleiotropic role. This also explained the high rate of spontaneously appearing suppressor mutants.

Identification of suppressor mutations by transposon mutagenesis

In order to gain insight into the reason for the growth impairments and for the cell wall deficiency exhibited by the *yvcK* mutant, we aimed to identify mutations able to suppress the growth defects. Therefore a transposon mutagenesis of the *yvcK* deletion strain SG63 was carried out using a mTn10 delivery system harboured on a temperature-sensitive plasmid. Briefly, several independent libraries of strain SG63 carrying random mTn10 insertions in the genome were generated and screened in parallel on DSM and on CE-gluconate plates for a resumption of growth. Several independent transposon mutants were isolated from

Fig. 5. Time-course phase-contrast images of cells of strain 168 (wild-type) and SG63 ($\Delta yvcK$) during growth in CE-gluconate minimal medium (A) and DSM (B). Samples of the cultures were taken at different times, as indicated by asterisks in the growth curves at the top of the figure, and examined microscopically. Scale bar, 5 μ m.

both types of plate. Control experiments verified that suppression of the growth defect was linked to the respective transposon insertion and was not due to a second mutation which could have occurred spontaneously during the procedure (see Methods). The sites of the mTn10 insertions in the chromosome were identified by ST-PCR. Finally, the mTn10 insertion sites of 13 mutants could be identified, of which seven were isolated on DSM plates and six on CE-gluconate plates (Fig. 7A).

On DSM, three types of insertions were found. These were insertions in genes *cggR*, *yfnI* and *zwf* (see Table 1 for the positions of the mTn10 insertions). In the case of *yfnI* and *zwf*, transcriptional terminators are present immediately downstream of these genes and according to database annotations they are supposedly transcribed monocistronically (<http://genolist.pasteur.fr/SubtiList/>; <http://bacillus.genome.jp/>). The function of *yfnI* is unknown; it encodes a membrane protein liberated into the medium following

Fig. 6. Mg^{2+} requirement for the $\Delta yvcK$ mutant. (A) OD_{600} monitoring of strain SG63 ($\Delta yvcK$, triangles) compared to strain 168 (wild type, squares) in CE-gluconate medium in the absence (filled symbols) or presence (open symbols) of an additional concentration of 25 mM MgCl_2 . (B) Phase-contrast microscopy images of cells of the cultures from (A) recorded 5 h after inoculation, as indicated by an asterisk in the corresponding growth curves.

membrane insertion in a SecA-dependent manner (Antelmann *et al.*, 2001; Hirose *et al.*, 2000). Gene *cggR* encodes a repressor protein that controls transcription of the bicistronic *cggR-gapA* messenger, and thus the expression of *gapA*, encoding glyceraldehyde-3-phosphate dehydrogenase A. GapA is a key enzyme of the EMP pathway and catalyses the conversion of glyceraldehyde 3-phosphate into 1,3-diphosphoglycerate, thereby connecting the upper and lower parts of this pathway (Fillinger *et al.*, 2000; Ludwig *et al.*, 2001). Gene *zwf* encodes the glucose-6-phosphate dehydrogenase, which catalyses the first reaction connecting the EMP pathway with the PPP, i.e. the conversion of glucose 6-phosphate into gluconate 6-phosphate (Zamboni *et al.*, 2004).

In the group of suppressor mutants isolated from the CE-gluconate plates, five different types of insertion were identified. One insertion was also found in *zwf* in the same position as in the *zwf* mutants isolated on DSM (see above). This was the only mutation that was found on both types of media used in the screen. The other insertions were located in genes *yqfF*, *secDF*, *mfd* and *mrpB*. The function of *yqfF* is unknown, and the transposon insertion might also affect the expression of genes located downstream. The gene *secDF* encodes a membrane protein required for the efficient secretion of proteins via the Sec-dependent pathway in *B. subtilis* (Bolhuis *et al.*, 1998). A transcriptional terminator is present downstream of *secDF*. Interruption of *secDF* in *B. subtilis* impairs the high-level production of secretory proteins (Bolhuis *et al.*, 1998), and might also affect the secretion of YfnI. In *E. coli*, SecDF couples protein translocation to the proton-motive force (pmf) (Duong & Wickner, 1997). The gene *mfd* encodes the transcription repair coupling factor, which displaces RNA polymerase (RNAP) stalled on the DNA by nucleotide lesions in the DNA template or by proteins that block elongation

downstream of a promoter (Ayora *et al.*, 1996; Roberts & Park, 2004). Finally, one insertion mapped in the gene *mrpB*, which is the second of seven genes within the *mrp* operon, whose products are all required for the activity of the Mrp antiporter that extrudes Na^+ or K^+ in exchange for protons (Krulwich *et al.*, 2001). The Mrp antiporter has been shown to play a central role in pH homeostasis and is required for cytoplasmic pH regulation in the presence of NaCl concentrations similar to those found in LB (Ito *et al.*, 1999, 2001). Accordingly, the *mrpB::mTn10* mutant was not viable on LB (Fig. 7; Kobayashi *et al.*, 2003).

It is noteworthy that not all these *mTn10* insertion mutations restored growth of the $\Delta yvcK$ mutant simultaneously in DSM and in gluconate minimal medium (Fig. 7). Indeed, the insertions in *yfnI*, *zwf*, *yqfF* and *mfd* restored growth in both media, whereas the remaining insertions suppressed the growth defect either exclusively in DSM (insertion in *cggR*) or in CE-gluconate (insertions in *secDF* and *mrpB*).

Effects of defined mutations in *zwf*, *gapA* and *cggR* on growth of the *yvcK* mutant

Most of the transposon insertions obtained in the screen were not very informative, since they resided in genes of unknown function (*yfnI* and *yqfF*) or presumably yielded pleiotropic effects (insertions in *secDF*, *mfd* and *mrpB*). However, the insertions in *zwf* and *cggR* affected central enzymes of carbon metabolism and caused growth impairments on substrates of the EMP pathway, such as glucose or glycerol (Fig. 7B). In order to explore in more detail the effects of these two transposon insertions, defined knock-out mutations were introduced into the $\Delta yvcK$ deletion strain SG63.

First we constructed a conditional *zwf* knock-out mutation in which expression of *zwf* is inducible by IPTG. The resulting

Fig. 7. Mutagenesis screen for mTn10 insertions that suppress the growth impairment caused by deletion of *yvcK*. (A) Schematic representation of the mTn10 insertion mutants obtained in the screen. Strain SG63 was subjected to mTn10 mutagenesis and screened for restoration of growth on DSM and CE-gluconate plates. The mTn10 insertion sites in the obtained mutants were determined by ST-PCR. The approximate location and the orientation of the transposon is indicated by a flag and by the highlighting of the ORF names in bold type (see Table 1 for details). The number of isolates of each mutant is given on the left and the number of independent mTn10 libraries that were the source for these mutants is given in parentheses. ORFs are shown as horizontal arrows in the direction of their transcription. Rho-independent transcriptional terminators are depicted as lollipops according to the annotations on the database <http://genolist.pasteur.fr/SubtilList/>. Promoters are represented as small horizontal arrows according to the following references: 1, Ludwig *et al.* (2001); 2, Northern analysis at database <http://bacillus.genome.jp/>; 3, predicted by http://cmgm.stanford.edu/~merino/Bacillus_subtilis/index_alpha.html; 4, Song & Neuhaud (1989); 5, Popham & Stragier (1991); 6, Hilden *et al.* (1995); 7, Zalieckas *et al.* (1998); 8, Bagyan *et al.* (1996); 9, Asai *et al.* (2000); 10, Tanaka *et al.* (2003); 11, Ito *et al.* (1999). (B) Schematic representation of the growth properties of the mTn10 insertion mutants on agar plates. The various mTn10/ $\Delta yvcK$ double mutant strains obtained in the screen were streaked out on plates containing DSM or CE-minimal medium supplemented with different carbon sources, and growth was monitored at 37 °C (upper line). In addition, the mTn10 insertions were examined in the wild-type background (bottom line). +, Growth; -, no growth; ±, delayed growth; ~, bacteria start to grow and lyse thereafter.

double-mutant strain $\Delta yvcK$, *zwf-5'* :: pMUTIN (SG94) was able to grow in both media, CE-gluconate as well as DSM, but exclusively in the absence of IPTG. Induction of *zwf* expression by the addition of IPTG resulted in the same growth defects observed for the $\Delta yvcK$ single mutant (data not shown). This result clearly establishes that the mTn10

insertion eliminated *Zwf* activity in the cell and that this abolition leads to suppression of the $\Delta yvcK$ phenotype.

To clarify the effect of the transposon insertion in *cggR*, we tested the suppressing effect of *gapA* and *cggR* mutations. A conditional *gapA* knockout mutation in which *gapA* is

expressed exclusively in the presence of IPTG (Fillinger *et al.*, 2000) was introduced into the *yvcK* mutant SG63. In the absence of IPTG, the resulting double-mutant strain $\Delta yvcK$, *gapA*-5'::pMUTIN2 (SG92) was unable to grow in DSM, in CE-gluconate as well as in CE-glucose (data not shown). This indicated that a switch-off of *gapA* expression does not lead to suppression of the growth defects. Next, we crossed into strain SG63 ($\Delta yvcK$) a *cggR*-frameshift mutation which leads to constitutive expression of *gapA* at a high level (Ludwig *et al.*, 2001). The resulting double mutant $\Delta yvcK$, $\Delta cggR$ (strain SG91) grew in all of the above-mentioned media, demonstrating that the $\Delta cggR$ allele completely suppressed the growth impairments of the *yvcK* mutant (data not shown). Thus, neither the *gapA*-knockout mutation nor the $\Delta cggR$ mutation resulted in the same growth properties caused by the *cggR*::mTn10 insertion (Fig. 7B). How can these differences be explained? It has been shown that a $\Delta cggR$ mutation also results in a higher expression level of the genes *pgk*, *tpiA*, *pgm* and *eno*, which are encoded downstream of *gapA*. Although these genes are transcribed from their own promoter located in the *gapA*-*pgk* intergenic region (see Fig. 7A), they can additionally be co-transcribed together with *gapA* from the promoter located in front of *cggR* (Ludwig *et al.*, 2001). The *cggR*::mTn10 mutant isolated here was unable to grow on glucose (Fig. 7B), which is indicative of a *gapA*-knockout mutation (Fillinger *et al.*, 2000; and see above). Thus, it may be concluded that the *cggR*::mTn10 mutation abolished *gapA* expression, perhaps by interference with the mRNA-processing event taking place at the 3' end of the *cggR* message (Meinken *et al.*, 2003), but led at the same time to an elevated expression of the genes downstream of *gapA*. This would mean that a higher expression of the genes downstream of *gapA* rather than of *gapA* itself might be responsible for suppression of the growth impairments. These genes encode enzymes of the lower part of the EMP pathway and are required for both glycolysis and gluconeogenesis.

The *B. subtilis* YvcK protein can be functionally replaced with its *E. coli* homologue YbhK

Homologues of YvcK are present in many Gram-positive as well as Gram-negative bacteria, suggesting that they accomplish an important function for the bacterial cell. The corresponding homologous protein YbhK from *E. coli* exhibits 34 % identity and 52 % similarity to YvcK at the amino acid sequence level. In order to test whether or not YbhK could substitute for YvcK in *B. subtilis*, *ybhK* was cloned under the control of an IPTG-inducible promoter on a plasmid able to replicate in *B. subtilis*. The resulting plasmid pBGG1 was used to transform the *yvcK* deletion strain SG63, and growth of the resulting transformant was tested in DSM in the presence of IPTG for the induction of *ybhK* expression. The presence of *ybhK* restored the growth of the $\Delta yvcK$ mutant, although not to the level that was observed when the *yvcK* expression plasmid pBGM54 was present in this strain (Fig. 4, compare filled triangles and

open diamonds). This result indicates that YbhK from *E. coli* can at least partially substitute for YvcK in *B. subtilis* and suggests that these proteins share some similar functions and interaction partners.

A recent global study of the protein-interaction network in *E. coli* proposes that several proteins interact with YbhK (Butland *et al.*, 2005). This is in agreement with our data indicating that YvcK may have several roles in the cell, and that its inactivation may yield pleiotropic effects. Among the putative interaction partners of YbhK, Butland *et al.* (2005) identified SecA, belonging to the Sec protein secretion machinery, and the essential cell division protein FtsK, involved in septum formation. This supports our finding that a *secDF* mutation partly suppresses the growth defect of the *yvcK* mutant (Fig. 7) and that the *yvcK* mutants exhibit a filamentous cell shape under certain growth conditions (Fig. 5). Furthermore, AceE and LpdA, two subunits of the pyruvate-dehydrogenase complex, were identified as YbhK interaction partners. The pyruvate-dehydrogenase complex converts pyruvate into acetyl-CoA, a key reaction in the lower part of the EMP pathway. This observation, together with our finding that mutations in *zwf* and *cggR* suppress the growth defect of the *yvcK* mutants, is intriguing.

In conclusion, we assume that an enzyme involved in the conversion of D-glyceraldehyde 3-phosphate into acetyl-CoA within the lower part of the EMP pathway, e.g. the pyruvate-dehydrogenase complex, may exhibit an abnormally high activity in the *yvcK* mutant and may redirect the carbon flux almost completely towards the production of acetyl-CoA. Alternatively, the activity of an enzyme required for gluconeogenesis, i.e. the conversion of oxaloacetate into glucose 6-phosphate, could be lowered or abolished in the *yvcK* mutant. This would slow down the synthesis of cell wall precursor molecules from carbon sources that do not directly enter the EMP pathway. It would explain the growth impairment and abnormal cell shape of the *yvcK* mutants on substrates of the PPP and of the Krebs cycle. This hypothesis is currently under investigation.

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