

Genome Engineering Reveals Large Dispensable Regions in *Bacillus subtilis*

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Bacterial genomes contain 250 to 500 essential genes, as suggested by single gene disruptions and theoretical considerations. If this view is correct, the remaining nonessential genes of an organism, such as *Bacillus subtilis*, have been acquired during evolution in its perpetually changing ecological niches. Notably, ~47% of the ~4,100 genes of *B. subtilis* belong to paralogous gene families in which several members have overlapping functions. Thus, essential gene functions will outnumber essential genes. To answer the question to what extent the most recently acquired DNA contributes to the life of *B. subtilis* under standard laboratory growth conditions, we initiated a “reconstruction” of the *B. subtilis* genome by removing prophages and AT-rich islands. Stepwise deletion of two prophages (SP β , PBSX), three prophage-like regions, and the largest operon of *B. subtilis* (*pks*) resulted in a genome reduction of 7.7% and elimination of 332 genes. The resulting strain was phenotypically characterized by metabolic flux analysis, proteomics, and specific assays for protein secretion, competence development, sporulation, and cell motility. We show that genome engineering is a feasible strategy for functional analysis of large gene clusters, and that removal of dispensable genomic regions may pave the way toward an optimized *Bacillus* cell factory.

Introduction

Bacillus subtilis is a well-studied Gram-positive soil bacterium, which undergoes two interesting developmental processes: morphological differentiation into spores (Piggot and Losick 2001) and physiological differentiation into a state of competence for genetic transformation (Berka et al. 2002). Furthermore, *B. subtilis* and closely related *Bacillus* species are nonpathogenic, free of endotoxins, and well known with respect to fermentation technology, which makes them important cell factories for industrial enzymes, fine biochemicals, antibiotics, and insecticides (Bron et al. 1999; van Dijl et al. 2001).

Characterized genomes of eubacteria differ in size from 580 kb for *Mycoplasma genitalium* (Fraser et al. 1995) to 9,200 kb for *Myxococcus xanthus* (He et al. 1994). One of the key questions emerging from the still expanding data set of complete genome sequences is how many genes are essential for the life of an organism such as *B. subtilis*. Itaya (1995) has used a small set of randomly selected genetic loci in *B. subtilis* to determine the percentage of genes that could be disrupted without loss of viability. This led to the hypothesis that the minimal *B. subtilis* genome may comprise about 318–562 kb (Itaya 1995) which, given the average size of ~1 kb for a bacterial protein-encoding gene,

corresponds to 300–500 genes (Kunst et al. 1997). In a systematic approach of single gene disruptions covering the complete *Bacillus* genome, about 270 genes have thus far been found to be indispensable for growth of *B. subtilis* in a rich medium at 37°C (Kobayashi et al. 2003). This finding is in line with a global transposon mutagenesis study showing that 265–350 of the 517 genes of *M. genitalium* are essential for growth under laboratory conditions (Hutchison et al. 1999). Moreover, it suggests that the majority of the *B. subtilis* genome would be dispensable for growth under defined conditions. It is intriguing to consider whether such a minimal genome would encode a healthy cell. A first step toward an answer can come from the construction of a *Bacillus* cell that has been deprived of sequences encoding functions that are nonessential for propagation and fermentation. As a consequence of such a chromosome reduction, cellular metabolite and energy resources would not be expended to maintain and express the deleted genetic information. Thus, the consumption of substrates would be optimally directed toward the synthesis of both essential and desired gene products. Concomitantly, the metabolic waste might decrease, because fewer unwanted proteins are synthesized. In this respect, it is important to note that every dispensable protein produced by a cell factory can represent a potential contaminant in the purification of desired proteinaceous products (Kolisnychenko et al. 2002).

For *B. subtilis*, a large number of potentially dispensable chromosomal loci can be inferred from the complete nucleotide sequence of the chromosome (4,188-kb) (Kunst et al. 1997). On average, the G + C ratio of the

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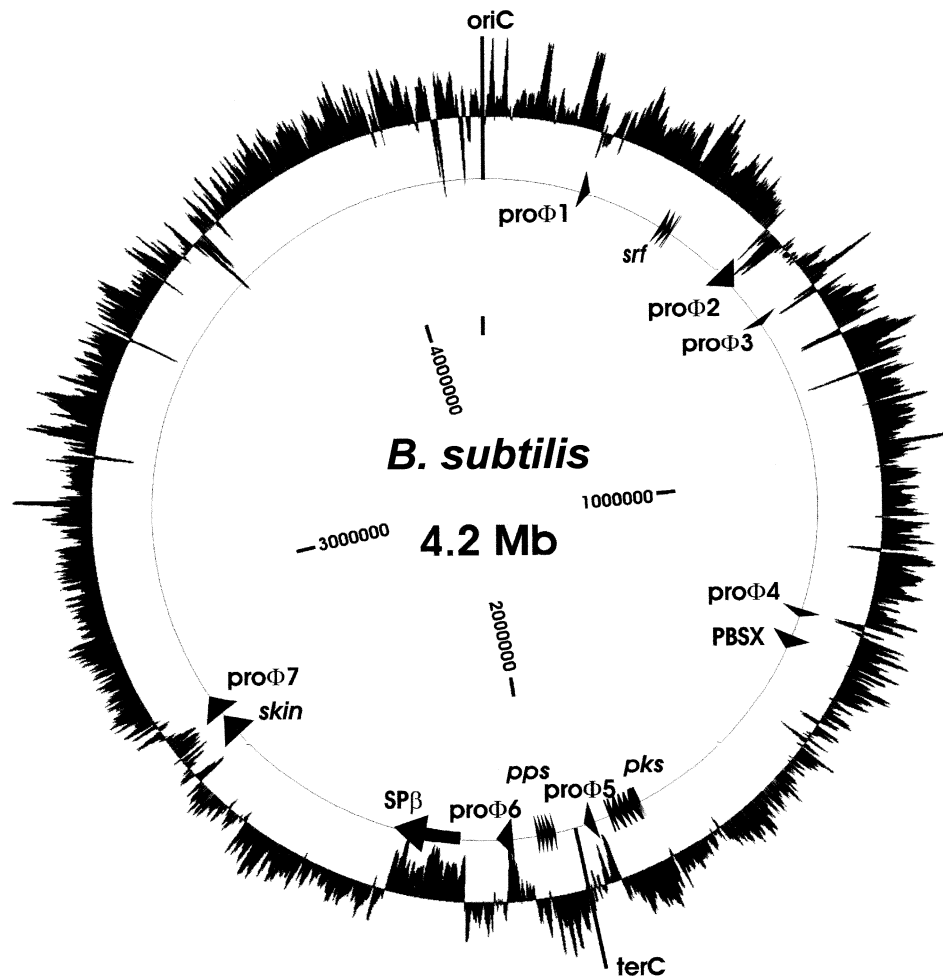


FIG. 1.—Relative location of potentially dispensable regions on the chromosome of *B. subtilis* 168. The distribution of AT-rich islands on the *B. subtilis* genome (inner side of the circle) is depicted with the Genome Viewer program (<http://www.cmbi.kun.nl/genome>) in sliding windows of 4,000 nucleotides with steps of 200 nucleotides. The outer boundary of the illustration is defined by the window of 4,000 nucleotides with the lowest A + T content, whereas the inner boundary is defined by the window with the highest A + T content. The baseline indicates the midpoint between the inner and outer A + T content boundaries. The relative location of prophages (SP β , PBSX, *skin*, pro Φ 1–7) and gene clusters involved in the synthesis of polyketide and peptide-antibiotics (*pks*, *pps*, *srf*) is indicated. Note that this plot does not mark PBSX as an AT-rich island, despite the fact that the A + T content of this prophage is higher than the average A + T content of the *B. subtilis* genome (Kunst et al. 1997).

B. subtilis chromosome is 43.5%. However, considerable variation in the GC content can be observed throughout the genome sequence. In particular, 10 relatively large AT-rich islands that represent known prophages (SP β and PBSX) and prophage-like regions are distributed over the chromosome (Zahler et al. 1977; Wood et al. 1990; Takemaru et al. 1995; Kunst et al. 1997). Together with three gene clusters involved in the synthesis of polyketide and peptide antibiotics (the polyketide synthase, fengycin, and surfactin operons), they form a group of possibly dispensable regions (fig. 1). Deletion of these regions would lead to removal of \sim 450 kb of DNA, or 12% of the chromosome. Furthermore, removal of phage and phage-like elements would result in the deletion of a number of autolysins, which can cause a severe problem of cell lysis during industrial fermentations.

The probable dispensability of the prophage, and prophage-like regions, can also be inferred from a classification of the *B. subtilis* genes on the basis of their codon preference (Kunst et al. 1997; Moszer 1998; Garcia-

Vallvé, Romeu, and Palau 2000). Class 1 comprises the majority of genes which have a weak codon bias and are constitutively expressed at a low or intermediary level. These genes are involved in processes such as carbon assimilation. In contrast, class 2 genes have a strong codon bias. They are highly expressed during exponential growth and encode proteins involved in transcription, translation, protein folding, and intermediary metabolism. The members of class 3 have codons enriched in A + T and are clustered in prophage or prophage-like regions. This specific codon preference is indicative of horizontal gene transfer events that may have occurred during the evolution of *B. subtilis* (Moszer, Rocha, and Danchin 1999; Garcia-Vallvé, Romeu, and Palau 2000). Interestingly, \sim 20% of the 345 small polypeptide-encoding open reading frames (ORFs; 85 codons or less) of *B. subtilis* reside in the prophage(-like) elements, and most of these small ORFs belong to class 3. The percentage of small ORFs is exceptionally high in the prophage SP β , where 30% of the ORFs belong to this category (Zuber 2001).

In the present studies, a sequential and cumulative approach was explored to delete large dispensable regions from *B. subtilis* strain 168. Finally, a multiple deletion strain lacking two prophages (SP β and PBSX), three prophage-like elements (prophage 1, prophage 3, and *skin*), and the polyketide synthase (*pks*) operon was constructed. Thus, the genome was reduced by 7.7% or 320 kb. The results show that this genome minimization affects neither cell viability nor the key physiological and developmental processes of *B. subtilis*.

Materials and Methods

Plasmids, Bacterial Strains, and Growth Conditions

The bacterial strains and plasmids used are listed in table 1. TY medium contained Bacto tryptone (1%), Bacto yeast extract (0.5%), and NaCl (1%). *B. subtilis* sporulation medium consisted of (per liter): 8 g nutrient broth, 0.5 mM NaOH, 1 mM MgSO₄, and 13 mM KCl. After sterilization, the following components were added to complete the sporulation medium: 1 mM Ca(NO₃)₂, 0.01 mM MnCl₂, and 0.001 mM FeSO₄. *B. subtilis* minimal salts used in transformation experiments consisted of (per liter): 2 g K₂SO₄, 10.8 g K₂HPO₄, 6 g KH₂PO₄, 1 g Na-citrate, and 0.02 g MgSO₄. After adjustment to pH 7.0 and sterilization, the following components were added (per 50 ml): 0.5% glucose, 0.02% casamino acids (Difco), 1.4 mg/ml l-tryptophane, and 2.2 mg/ml ferric ammonium citrate. Antibiotics were used in the following concentrations: chloramphenicol (Cm), 5 μ g/ml; erythromycin (Em), 100 μ g/ml (*Escherichia coli*) or 5 μ g/ml (*B. subtilis*); and kanamycin (Km), 20 μ g/ml. Aerobic batch cultures for [¹³C]-labeling experiments and physiological analyses were grown at 37°C in 500-ml baffled shake flasks with 50 ml of M9 minimal medium, consisting of the following components (per liter): 8.5 g Na₂HPO₄·2H₂O, 3 g KH₂PO₄, 0.5 g NaCl, 1 g NH₄Cl, 0.25 g MgSO₄·7H₂O, 11 mg CaCl₂, 50 mg tryptophane, and trace elements (Harwood and Archibald 1990). Filter-sterilized glucose was added to a final concentration of 5 g/L. Independent experiments were performed with either entirely [1-¹³C]glucose or a mixture of 20% [U-¹³C]₆glucose and 80% unlabeled glucose. These cultures were inoculated with 0.5% (v/v) from an overnight culture in M9 medium, supplemented with 10 g/L glucose.

DNA Techniques

Procedures for DNA purification, restriction, ligation, agarose gel electrophoresis, and transformation of competent *E. coli* cells were carried out as described by Sambrook, Fritsch, and Maniatis (1989). *B. subtilis* was transformed as described by Kunst and Rapoport (1995). The nucleotide sequences of primers used for polymerase chain reaction (PCR) are listed in table 1.

To construct the *B. subtilis* Δ 6 strain, plasmid-based chromosomal integration-excision systems were used. The strain *B. subtilis* Δ SP β (Dorenbos et al. 2002) was used to delete the *skin* element (2655129–2699959 bp SubtiList coordinates). To this purpose, the flanking regions of *skin* were PCR-amplified with the primer pairs YqcM1/YqcM2 and YqaB1/YqaB2, and cloned into the chromosomal

integration vector pG+ host4. The resulting plasmid was used to transform *B. subtilis* Δ SP β for chromosomal integration into *yqcM* or *yqaB*. Transformants (Em^r) were selected at 30°C. Next, the excision of the integrated plasmid (together with *skin*) from the chromosome was provoked by growth at 42°C, resulting in the twofold deleted strain TFC7A (Δ SP β Δ *skin*). PBSX (1312952–1347172 bp SubtiList coordinates) was deleted from TFC7A using a pG+ host4-based integration-excision plasmid that contains the flanking regions of this prophage, PCR-amplified with the primer pairs YjnA1/YjnA2 and XlyA1/XlyA2. This resulted in strain TF8A (Δ SP β Δ *skin* Δ PBSX). For deletion of prophage 1 (202092–220145 bp SubtiList coordinates) two fragments flanking the prophage 1 region were PCR-amplified with the primer pairs GlmS1/GlmS2 and YbdG1/YbdG2 and ligated into pORI280 after PCR-mediated splicing by overlap extension (Horton et al. 1989). The resulting plasmid, p280GY, was used to transform *B. subtilis* for chromosomal integration into *glmS* or *ybdG*. Transformants (Em^r and blue on TY plates with X-gal) were grown in the absence of erythromycin to obtain the fourfold deleted strain Δ 4 (Δ SP β Δ *skin* Δ PBSX Δ prophage 1; Em^s and white on TY plates with X-gal) due to spontaneous excision of the plasmid from the chromosome (together with the prophage 1 region). The largest part of the *pks* operon (1781306–1857233 SubtiList coordinates) in the Δ 4 strain was replaced with a Cm^r marker by double cross-over recombination. For this purpose, the pJM105A-based plasmid pJM Δ 80 was used, which contains a *pks* flanking region amplified with the primer pair EUP1/ELO1 (comprising the 5' end of *pksA*) and a cloned flanking region of *pks* (comprising the 5' ends of *pksR* and *pksS*). A Cm^r marker is located between the two *pks* flanking regions on pJM Δ 80. This plasmid was first used to replace the *pks* operon of *B. subtilis* 168 with the Cm^r marker and, subsequently, chromosomal DNA of the resulting strain (PB1862) was used to transform *B. subtilis* Δ 4. This resulted in the fivefold deleted strain Δ 5 (Δ SP β Δ *skin* Δ PBSX Δ prophage 1 *pks::cat*). The Δ 5 strain was used to delete prophage 3 (651866–665067 bp SubtiList coordinates). A derivative of pMTL20E (carrying an Em^r marker) was made, which contains two fragments flanking prophage 3 that were PCR-amplified with the primer pairs pre-ydiM1/pre-ydiM2 and post-gutR1/gutR2. A Km^r cassette is located between the two prophage 3 flanking regions. The resulting plasmid was used to transform *B. subtilis* for chromosomal integration. Transformants (Km^r) were transformed with a pEpUC Δ 1 derivative, which contains the same flanking regions of prophage 3 but lacks the Km^r marker between them. Single cross-over integrants (Km^r Em^r) were selected at 51°C. Finally, excision of the integrated pEpUC Δ 1 (together with the Km^r marker) was provoked by growth at 30°C. The resulting Km^s Em^s strain was named *B. subtilis* Δ 6 (Δ SP β Δ *skin* Δ PBSX Δ prophage 1 *pks::cat* Δ prophage 3). The sequential introduction of the deletions was verified by Southern hybridization and/or PCR.

Transcript Profiling

Transcript profiling was performed with *Bacillus subtilis* Panorama macro-arrays from Sigma-Genosys. Total

Table 1
Plasmids, Strains, and Primers

	Relevant Properties ^a	Reference
Plasmids		
pORI280	Vector for chromosomal integration and excision allowing construction of markerless deletions; <i>lacZ</i> , <i>ori</i> ⁺ of pWV01, replicates only in strains providing <i>repA</i> in trans; Em ^r	Leenhouts et al. 1996
pKTH10	Vector containing the <i>amyQ</i> gene of <i>B. amyloliquefaciens</i> ; Km ^r	Palva 1982
pLip2031	Vector containing the <i>lip</i> gene of <i>B. subtilis</i> ; Km ^r	Dartois et al. 1994
pJM105A	pBluescript-based integration vector for <i>B. subtilis</i> ; Ap ^r , Cm ^r	Perego 1993
pG+ host4	Vector containing a temperature-sensitive replicon for conditional integration and excision allowing construction of markerless deletions; Ap ^r , Em ^r	Biswas et al. 1993
pMTL20E	pBR322-derived chromosomal integration vector for <i>B. subtilis</i> ; <i>lacZ</i> , Ap ^r , Em ^r	Swinfield et al. 1990
pEpUCΔ1	Vector containing a temperature-sensitive replicon (based on pE194 <i>cop6</i> ; Weisblum et al. 1979) for conditional integration and excision allowing construction of markerless deletions; Ap ^r , Em ^r	C. Bruand, laboratory collection
pMutin4	pBR322-based integration vector for <i>B. subtilis</i> ; contains a multiple cloning site downstream of the Pspac promoter, and a promoter-less <i>lacZ</i> gene preceded by the RBS of the <i>spoVG</i> gene; Ap ^r , Em ^r	Vagner, Dervyn, and Ehrlich 1998
Strains		
<i>E. coli</i>		
EC1000	RepA ⁺ MC1000, carries a single copy of the pWV01 <i>repA</i> gene in the <i>glgB</i> gene; Km ^r	Leenhouts et al. 1996
<i>B. subtilis</i>		
168	<i>trpC2</i>	Kunst et al. 1997
ΔSPβ	<i>trpC2</i> ; ΔSPβ; sublancin 168-sensitive; laboratory name CBB312	Dorenbos et al. 2002
TFC7A	<i>trpC2</i> ; ΔSPβ; sublancin 168-sensitive; Δ <i>skin</i>	This article
TF8A	<i>trpC2</i> ; ΔSPβ; sublancin 168-sensitive; Δ <i>skin</i> ; ΔPBSX	This article
Δ4	<i>trpC2</i> ; ΔSPβ; sublancin 168-sensitive; Δ <i>skin</i> ; ΔPBSX; Δprophage 1	This article
Δ5	<i>trpC2</i> ; ΔSPβ; sublancin 168-sensitive; Δ <i>skin</i> ; ΔPBSX; Δprophage 1; <i>pks</i> :: Cm; Cm ^r	This article
Δ6	<i>trpC2</i> ; ΔSPβ; sublancin 168-sensitive; Δ <i>skin</i> ; ΔPBSX; Δprophage1; <i>pks</i> ::Cm; Δprophage 3; Cm ^r	This article
BFA3041	<i>trpC2</i> ; <i>htrB</i> ::pMutin4; contains a transcriptional <i>htrB-lacZ</i> fusion; Em ^r	Darmon et al. 2002
Δ6 <i>htrB</i> ::pMutin4	like Δ6; <i>htrB</i> ::pMutin4; <i>htrB-lacZ</i> ; Em ^r	This article
Primers 5'–3'		
YqcM1	CCCTGTTTCAGCAAATTC	This article
YqcM2	GATTGCTGTAGCTGTTGG	This article
YqaB1	TTCCATCTCCTTAGACGC	This article
YqaB2	GGGGCATAAAATTTCCCG	This article
YjnA1	AGCGAAGCACTCGGAATAC	This article
YjnA2	TAGTCGGGTTTTCAGGAGG	This article
XlyA1	ACCCGAATCTTATTTCGCG	This article
XlyA2	TTCCGTTGTAAACAGCGC	This article
GlmS1	GCGAGTGAATTCTCCTACAACATGCCGCTTCTGTCT	This article
GlmS2	GCAGCACATGTGAAGACCTTCTTATCCAAAGGGGTTAATTAATTATTA TACTCCACAGTAACACTCTTCGCAAGG	This article
YbdG1	GAAGGTCTTCACATGTGCTGCTAGGCTCCAAATGTACACCTTTCCGT AAGGGC	This article
YbdG2	CCCTTCATCCTAGCGAACCTTTCTC	This article
EUP1	GGGGTACCCAAAACAATGACAACCCAAAA	This article
ELO1	AGCTACCCAAGCTTCTAATGACAATCCTGCTTCCTT	This article
pre-ydiM1	AATTCGAACGATATCAACA	This article
pre-ydiM2	ATCCATCAGGATTGATTC	This article
post-gutR1	GTAGACTTTAATAAGAAAACG	This article
GutR2	CGAACTCATCACCCGC	This article

^a Em^r, erythromycin resistance marker; Km^r, kanamycin resistance marker; Ap^r, ampicillin resistance marker; Cm^r, chloramphenicol resistance marker.

RNA from *B. subtilis* 168 was isolated with the High Pure RNA isolation kit of Roche Molecular Biochemicals. For simultaneous reverse transcriptase reactions on all mRNAs in the RNA sample, 4 μg of total RNA was added to 1 pmol of ORF-specific primers (Eurogentec). This mixture was heated to 70°C for 10 min and subsequently stored on ice. Next, 10 μl of 5× first strand buffer (Invitrogen), 5 μl of 0.1 M DTT, 0.5 μl RNasin 40 U/μl (Roche Molecular Biochemicals), and 2.5 μl dNTPs (5 mM dATP, dGTP, dTTP, and 0.1 mM dCTP) were added to the RNA-primer

mix. The total reaction volume was adjusted to 42.5 μl with water treated with 0.1% diethyl pyrocarbonate (DEPC). After addition of 5 μl [α-³³P] dCTP (50 μCi) (Redivue, Amersham Biosciences) and 2.5 μl of Superscript II reverse transcriptase (Invitrogen), the reaction mix was incubated for 2 h at 42°C, followed by 15 min at 70°C. The reaction was stopped and the RNA was denatured by adding 2 μl of 0.5 M EDTA, 2 μl of 10% SDS, and 6 μl of 3 N NaOH. Upon incubation for 30 min at 68°C, the mixture was neutralized by adding 6 μl of 2 N HCl. The [α-³³P]-labeled cDNA was

purified using Sephadex G-25 columns (Roche Molecular Biochemicals). The percentage of label incorporation was checked by scintillation counting. Prior to hybridization, the hybridization bottles and arrays were washed with 2× SSPE (0.36 M NaCl, 20 mM Na-phosphate buffer pH 7.7, and 2 mM EDTA). The arrays were then pre-hybridized in hybridization solution (Sigma-Genosys) supplemented with 100 µg/ml salmon testis DNA (Sigma-Genosys) for at least 1 h. The labeled cDNA was added to the array after 10 min incubation at 90°–95°C in hybridization solution plus salmon testis DNA. Hybridization was performed for 12–18 h. After it was washed with washing solution (0.5× SSPE, 0.2% SDS), the array was wrapped in Saran wrap and exposed to phosphorimager screens (Packard Instrument Company) for 2 or 3 days. The screens were scanned by the Cyclone Imager (Packard Instrument Company). The Array-Pro Analyzer 4.0 software package (Media Cybernetics) was used to analyze the images. The signals from duplicate spots were averaged and the intensities were expressed as percentages of the total signal.

Metabolic Flux Ratio (METAFor) Analysis

Biomass aliquots of *B. subtilis* were harvested during late exponential growth at OD_{600 nm} values between 2.5 and 3.0. Biomass pellets from 2 ml of culture broth were washed once with 1 ml 0.9% (w/v) NaCl and hydrolyzed in 1.5 ml 6 M HCl at 110°C for 24 h in sealed glass tubes. The dried hydrolysate was derivatized with *N*-(*tert*-butyldimethylsilyl)-*N*-methyl-trifluoroacetamide (Fluka) and subjected to GC-MS analysis as described previously (Dauner and Sauer 2000). The GC-MS-derived mass distributions in proteino-genic amino acids were then used to calculate intracellular carbon flux ratios using probabilistic equations (Fischer and Sauer 2003) and a metabolic network model for *B. subtilis* (Sauer et al. 1996). The metabolic by-products acetate and acetoin were determined by high-performance liquid chromatography (HPLC). Glucose concentrations were measured with the Beckman Synchron CX5CE auto-analyzer using the glucose reagent kit supplied by Beckman. Maximum growth rates (μ_{\max}) were determined by log-linear regression analysis of OD_{600 nm} versus time, with μ_{\max} as the regression coefficient. Dry matter concentrations of biomass were calculated using a predetermined correlation factor of 0.33 g cellular dry weight per OD_{600 nm} unit.

Competence, Sporulation, and Spore Germination Assays

The *B. subtilis* $\Delta 6$ and the parental 168 strains were tested for competence using the two-step method as described previously (Bron and Venema 1972). The strains were transformed with chromosomal DNA of *B. subtilis* OG1 (*trp*⁺), and transformants were selected for tryptophane prototrophy on minimal agar without tryptophane. Transformability was expressed as the number of transformants relative to the total viable count. The ability of the strains to sporulate and germinate was tested by growing 25 colonies for 2 to 3 days on a sporulation medium agar plate at room temperature. The colonies were transferred onto a filter paper, after which they were ex-

posed to chloroform vapor in a vacuum chamber for 45 min. The filters were used to make a contact replica on TY plates, which were incubated overnight at 37°C. Finally, the number of colonies growing on the replica plates was counted. Sporulation was also tested by growing the cells overnight in sporulation medium, after which an aliquot of the culture was heated to 80°C for 10 min. Subsequently, the presence of viable spores was assayed by plating.

Proteomics

The *B. subtilis* $\Delta 6$ mutant strain and the parental strain 168 were grown at 37°C under vigorous agitation in 1 liter TY medium. After 1 h of post-exponential growth, cells were separated from the growth medium by centrifugation. The secreted proteins in the growth medium were collected for two-dimensional (2D) sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and subsequent matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS), as previously described (Jongbloed et al. 2002; Antelmann et al. 2001). Two-dimensional gel image analysis was performed with the Decodon Delta 2D software, which is based on dual channel image analysis (Bernhardt et al. 1999). Using this software, the master image (represented by green protein spots) is warped with the sample image (represented by red protein spots) after specific vector points have been set. Consequently, green protein spots in the dual channel image are predominantly present in the master image, whereas red protein spots are predominantly present in the sample image. Yellow protein spots are present at similar amounts in both images. After background subtraction, normalization is performed in order to equalize the gray values in each image. Each experiment was repeated at least two times.

Enzyme Activity Assays

To determine lipase (i.e., esterase) activity, the colorimetric assay as described by Lesuisse, Schanck, and Colson (1993) was applied with some modifications. In short, 180 µl of reaction buffer (0.1 M H₂KPO₄ pH 8.0, 0.1% Arabic gum, 0.36% Triton X-100) was supplemented with 10 µl of the chromophoric ligand 4-nitrophenyl caprylate (10 mM in methanol). The reaction was started by the addition of 10 µl of culture supernatant. Lipase activity was determined by measuring the increase in absorbance at 405 nm/min of incubation at room temperature, per OD₆₀₀ of the culture at the time of sampling.

Protease activity was quantified with azocasein (Sigma). Growth medium (250 ml) was mixed with 2% azocasein suspension (150 ml) in 50 mM Tris-HCl (pH 7.5), 4 mM CaCl₂, and incubated for 60 min at 25°C. The reaction was stopped by the addition of 1.2 ml 10% TCA. After centrifugation, absorbance changes (440 nm) of the supernatant were determined.

α -Amylase activity was assayed with a halo assay. Cells were grown to the stationary phase, after which they were separated from the growth medium by centrifugation. The medium fractions were spotted on Durapore membrane filters (Millipore) that were placed on TY-agar plates

Table 2
Functional Classification of Deleted Protein-Encoding Genes

Functional Classification	168 (4,100 genes)	Deleted (332 genes)
1 Cell envelope and cellular processes	876 (21%)	15 (5%)
2 Intermediary metabolism	742 (18%)	9 (3%)
3 Information pathways	482 (12%)	20 (6%)
4 Other functions	289 (7%)	93 (28%)
5 Similar to unknown proteins	667 (16%)	22 (7%)
6 No similarity	1,053 (26%)	173 (52%)

NOTE.—For the functional classification of the deleted genes of the $\Delta 6$ strain, the SubtiList database (<http://genolist.pasteur.fr/SubtiList/>) was used. The functional classification of all *B. subtilis* 168 protein-encoding genes was adapted from Moszer (1998).

containing 1% starch (Merck). The amounts of medium spotted on the filters were corrected for the OD_{600 nm} of each culture. After overnight incubation at 37°C, the plates were analyzed for starch degradation by staining with iodine vapor. Diameters of the resulting clear zones (halos) were measured.

To assay β -galactosidase activity, overnight cultures were diluted in fresh medium and samples were taken at different time intervals for OD_{600 nm} readings and β -galactosidase activity determinations. For strains containing a transcriptional *lacZ* fusion, the β -galactosidase assay and the calculation of β -galactosidase units (Miller units: nmol.OD₆₀₀⁻¹.min⁻¹) were performed as described by Hyyryläinen et al. (2001). Experiments were repeated at least twice, starting from independently obtained transformants. In all experiments, the relevant controls were performed in parallel. Although some differences were observed in the absolute β -galactosidase activities, the ratios between these activities in the various strains tested were largely constant.

Western Blotting and Immunodetection

To assay the *B. amyloliquefaciens* α -amylase (AmyQ) production levels, cells were separated from the growth medium by centrifugation. Samples for SDS-PAGE were prepared as described previously (van Dijk et al. 1991). After separation by SDS-PAGE, proteins were transferred to a Protran nitrocellulose transfer membrane (Schleicher and Schuell) as described by Kyhse-Andersen (1984). AmyQ and LipA were visualized with specific antibodies and horseradish peroxidase- or alkaline phosphatase-anti-rabbit IgG conjugates (Jackson ImmunoResearch).

Motility Plate Assay

To study the motility of *Bacillus* cells, 2 μ l aliquots of overnight cultures were transferred to a TY-agarose plate containing 0.75%, 0.5%, or 0.27% agarose. Before transfer to the plates, the OD_{600 nm} of each culture was measured and adjusted to 1. After overnight incubation at 37°C, the degree of swarming was assessed.

Results

Deletion of 332 Genes

As an initial step, candidate regions for deletion were tested for essential gene functions by constructing single deletion strains (i.e., Δ SP β , Δ PBSX, Δ skin, Δ prophage 1, Δ prophage 3, and Δ pks). The deletions were constructed in such a way that flanking ORFs remained unaffected and no novel ORFs were created. For this purpose, various chromosomal integration-excision vectors were used (table 1). All single deletions yielded viable and apparently healthy cells. Subsequently, the deletions were combined in the following order. First, bacteriophage SP β was deleted from the parental strain *B. subtilis* 168. *Skin* and PBSX were sequentially deleted from the chromosome of the Δ SP β strain, after which the prophage 1 deletion and the *pks::cat* replacement were introduced. Finally, *B. subtilis* $\Delta 6$ was obtained by the deletion of prophage 3. The absence of the deleted regions was verified by Southern hybridization and/or PCR. The fact that the $\Delta 6$ strain could be obtained confirms that the combined deletions do not encode essential gene functions for viability of *B. subtilis*, at least not when cells are grown aerobically in TY broth at 37°C.

Six functional categories have been assigned to the genes of *B. subtilis* (Kunst et al. 1997; Moszer 1998). To take an inventory of the genes that have been removed from the 168 strain, we compared the functional categories of the deleted genes (332 in total) with the functional classification of all *B. subtilis* protein-encoding genes (Kunst et al. 1997; Moszer 1998) (table 2). Compared to the average in the chromosome, the percentage of genes that specify proteins with no similarity to other proteins (functional category number 6) is much higher among the deleted genes. As was to be expected, most of the deleted genes within the “other functions category” (number 4) encode proteins with phage-related functions. To gain insight into the actual gene functions that are absent from the $\Delta 6$ strain, a search for deleted genes encoding proteins with a known function or showing similarity to known proteins (functional categories 1–4) was performed using the SubtiList database (<http://genolist.pasteur.fr/SubtiList/>) (Kunst et al. 1997; Moszer 1998). The results are documented in table 3. With respect to “health” of *B. subtilis* $\Delta 6$, it is interesting to note that this strain lacks 12 genes which have been implicated in cell lysis. Furthermore, SP β encodes the lantibiotic sublancin 168 (SunA). Deletion of SP β leads to sublancin 168 sensitivity (Dorenbos et al. 2002).

It is known that the *B. subtilis* genome contains many paralogous genes, ranging from gene doublets and triplets to large families with up to 77 members (Kunst et al. 1997). To assess how many unique genes (singlets) of the *B. subtilis* genome were actually deleted, we investigated the possible presence of paralogs for all 332 deleted genes (table 4). The results show that 68% (227) of the deleted genes encode unique functions. Apparently, none of these functions is essential for growth and viability of *B. subtilis*. Strikingly, SP β and prophages 1 and 3 consist mostly of singlets, whereas PBSX and *skin* contain relatively large numbers of genes that belong to paralogous gene families. In all, 26 doublets and 2 triplets were completely deleted (58 genes), which implies that 255 to 285 different gene

Table 3
Deleted Gene Functions of *B. subtilis* Δ6

Gene	Location	Function of Corresponding Protein
<i>alkA</i>	Prophage 1	DNA-3-methyladenine glycosylase
<i>adaA</i>	Prophage 1	Methylphosphotriester-DNA alkyltransferase and transcriptional regulator (AraC/XylS family)
<i>adaB</i>	Prophage 1	O6-methylguanine-DNA methyltransferase
<i>ndhf</i>	Prophage 1	NADH dehydrogenase (subunit 5)
<i>ybcF</i>	Prophage 1	Similar to carbonic anhydrase
<i>ybcL</i>	Prophage 1	Similar to chloramphenicol resistance protein
<i>ybcM</i>	Prophage 1	Similar to glucosamine-fructose-6-phosphate aminotransferase
<i>ybcP</i>	Prophage 1	Similar to coenzyme PQQ synthesis protein
<i>ybdA</i>	Prophage 1	Similar to ABC transporter (binding protein)
<i>ybdB</i>	Prophage 1	Similar to ABC transporter (permease)
<i>ydiO</i>	Prophage 3	Similar to DNA-methyltransferase (cytosine-specific)
<i>ydiP</i>	Prophage 3	Similar to DNA-methyltransferase (cytosine-specific)
<i>ydiS</i>	Prophage 3	Similar to DNA restriction
<i>yjoB</i>	Upstream of PBSX	Similar to cell-division protein FtsH homolog
<i>rapA</i>	Upstream of PBSX	Response regulator aspartate phosphatase
<i>phrA</i>	Upstream of PBSX	Phosphatase (RapA) inhibitor
<i>xlyB</i>	Upstream of PBSX	N-acetylmuramoyl-L-alanine amidase (PBSX prophage-mediated lysis)
<i>yjqB</i>	Upstream of PBSX	Similar to phage-related replication protein
<i>yjqC</i>	Upstream of PBSX	Similar to manganese-containing catalase
<i>xre</i>	PBSX	Transcriptional regulator
<i>xtmA</i>	PBSX	PBSX defective prophage terminase (small subunit)
<i>xtmB</i>	PBSX	PBSX defective prophage terminase (large subunit)
<i>xkdY (xepA)</i>	PBSX	PBSX prophage lytic exoenzyme
<i>xhIA</i>	PBSX	Involved in cell lysis upon induction of PBSX
<i>xhIB</i>	PBSX	Hydrolysis of 5-bromo 4-chloroindolyl phosphate (X-phos); involved in cell lysis upon induction of defective prophage PBSX (holin)
<i>xlyA</i>	PBSX	N-acetylmuramoyl-L-alanine amidase (PBSX prophage-mediated lysis)
<i>pksB</i>	<i>pks</i>	Similar to a group of hypothetical proteins similar to ATP synthase subunit 3 (involved in polyketide synthesis)
<i>pksC</i>	<i>pks</i>	Similar to AT (acyltransferase) domains or functions, consensus to the active site (involved in polyketide synthesis)
<i>pksD</i>	<i>pks</i>	Similar to Pks type I, EryA, consensus to AT acyl transferase domain (involved in polyketide synthesis)
<i>pksE</i>	<i>pks</i>	Similar to Pks of type I and II, with consensus to AT acyltransferase active site and to mitochondrial energy-transfer signature (involved in polyketide synthesis)
<i>acpK</i>	<i>pks</i>	Acyl-carrier protein
<i>pksF</i>	<i>pks</i>	Similar to <i>Rhizobium leguminosarum</i> nodulation protein E and to <i>E. coli</i> 3-oxoacyl-(acyl-carrier protein) synthase I (involved in polyketide synthesis)
<i>pksG</i>	<i>pks</i>	Similar to hamster hydroxymethylglutaryl-CoA synthase (involved in polyketide synthesis)
<i>pksH</i>	<i>pks</i>	Similar to <i>Rhodobacter capsulatus</i> enoyl-CoA hydratase (involved in polyketide synthesis)
<i>pksI</i>	<i>pks</i>	Similar to <i>Rhodobacter capsulatus</i> enoyl-CoA hydratase (involved in polyketide synthesis)
<i>pksJ (pksK)</i>	<i>pks</i>	Polyketide synthase of type I
<i>pksL</i>	<i>pks</i>	Polyketide synthase of type I
<i>pksM</i>	<i>pks</i>	Polyketide synthase
<i>pksN (pksP)</i>	<i>pks</i>	Polyketide synthase of type I
<i>pksR</i>	<i>pks</i>	Polyketide synthase
<i>sspC</i>	SPβ	Small acid-soluble spore protein (alpha/beta-type SASP)
<i>yosS</i>	SPβ	Similar to deoxyuridine 5'-triphosphate nucleotidohydrolase
<i>yosP</i>	SPβ	Similar to ribonucleoside-diphosphate reductase (beta-subunit)
<i>yosO</i>	SPβ	Similar to ribonucleoside-diphosphate reductase (alpha-subunit)
<i>yosN</i>	SPβ	Similar to ribonucleoside-diphosphate reductase (alpha-subunit)
<i>yosR</i>	SPβ	Similar to thioredoxin
<i>yosQ</i>	SPβ	Similar to phage-related endodeoxyribonuclease
<i>mtbp</i>	SPβ	Modification methylase Bsu
<i>yorL</i>	SPβ	Similar to DNA polymerase III (alpha subunit)
<i>yorK</i>	SPβ	Similar to single-strand DNA-specific exonuclease
<i>yorI</i>	SPβ	Similar to putative replicative DNA helicase
<i>yoqW</i>	SPβ	Similar to general secretion pathway protein
<i>ligB</i>	SPβ	DNA ligase
<i>yoqD</i>	SPβ	Similar to phage-related DNA-binding protein anti-repressor
<i>yonR</i>	SPβ	Similar to transcriptional regulator (phage-related) (Xre family)
<i>yonN</i>	SPβ	Similar to HU-related DNA-binding protein
<i>yomS</i>	SPβ	Similar to phage-related lytic exoenzyme
<i>yomI</i>	SPβ	Similar to phage-related immunity protein
<i>yomJ</i>	SPβ	Similar to lytic transglycosylase
<i>blyA</i>	SPβ	N-acetylmuramoyl-L-alanine amidase
<i>bhIA</i>	SPβ	Holin-like protein
<i>bhIB</i>	SPβ	Holin-like protein
<i>bdbB</i>	SPβ	Thiol-disulfide oxidoreductase
<i>yolJ</i>	SPβ	Similar to glycosyltransferase

Table 3
Continued

Gene	Location	Function of Corresponding Protein
<i>bdbA</i>	SP β	Thiol-disulfide oxidoreductase
<i>sunT</i>	SP β	Sublancin 168 lantibiotic transporter
<i>sunA</i>	SP β	Sublancin 168 lantibiotic antimicrobial precursor peptide
<i>uvrX</i>	SP β	UV-damage repair protein
<i>yokG</i>	SP β	Similar to delta-endotoxin
<i>yokF</i>	SP β	Similar to micrococcal nuclease
<i>yokD</i>	SP β	Similar to aminoglycoside N3'-acetyltransferase
<i>yokA</i>	SP β	Similar to DNA recombinase
<i>arsC</i>	<i>skin</i>	Arsenate reductase
<i>arsB</i>	<i>skin</i>	Extrusion of arsenite
<i>arsR</i>	<i>skin</i>	Arsenic resistance operon repressor (<i>arsR-yqcK-arsBC</i>)
<i>rapE</i>	<i>skin</i>	Response regulator aspartate phosphatase
<i>phrE</i>	<i>skin</i>	Regulator of the activity of phosphatase RapE
<i>cwlA</i>	<i>skin</i>	N-acetylmuramoyl-L-alanine amidase (minor autolysin)
<i>yqxG</i>	<i>skin</i>	Similar to phage-related lytic exoenzyme
<i>yqaE</i>	<i>skin</i>	Similar to transcriptional regulator (phage-related) (Xre family)
<i>yqaT</i>	<i>skin</i>	Similar to phage-related terminase large subunit
<i>yqaS</i>	<i>skin</i>	Similar to phage-related terminase small subunit

NOTE.—Functions of genes belonging to categories 1–4 were obtained from the SubtiList database (<http://genolist.pasteur.fr/SubtiList/>). However, genes that belong to sub-category 4.4 (phage-related functions), and of which no further information is known, were omitted.

functions are absent from *B. subtilis* $\Delta 6$. Forty-seven deleted genes are members of paralogous gene families that were not completely deleted. Most of the deleted doublets consist of one gene located on PBSX and another on *skin*, which is consistent with the view that these genomic regions share a common ancestor and have diverged relatively recently (Médigue et al. 1995; Krogh et al. 1996).

The AT-content of most of the prophage genes is indicative of a “foreign” origin, which may result in transcription levels that differ from those of “native” *B. subtilis* genes. Furthermore, the localization of most prophage genes near the terminus of replication of the *B. subtilis* chromosome suggests that these genes may be expressed at lower levels than genes close to the origin of replication (Rocha et al. 2000). To visualize the expression of genes of all 10 prophage and prophage-like regions, the DNA macro-array technique was used. Although DNA array analyses are generally not considered to be quantitative, the results summarized in figure 2 provide a qualitative indication that, on average, the transcription of prophage genes (black bars) is lower than that of the other *B. subtilis* genes (gray bars). Moreover, 30% of the prophage genes show a transcription level of less than 0.5% of the highest transcriptional level measured under the conditions tested.

Physiology and Metabolic Flux Patterns of *B. subtilis* $\Delta 6$

As a first approach to investigate possible effects of the six combined deletions on cell physiology, we compared the growth of *B. subtilis* 168 and *B. subtilis* $\Delta 6$ in a mineral salts medium with glucose. As shown in batch culture experiments, the pattern of the growth curves of both strains did not differ significantly. In fact, both strains exhibited identical maximum growth rates (μ_{\max}) and biomass yields. These important diagnostic parameters show that the multiple deletions have no major impact on cell physiology (table 5).

To elucidate potential effects on intracellular carbon metabolism, *B. subtilis* $\Delta 6$ and the parental strain 168 were subjected to a metabolic flux ratio (METAFor) analysis, which is based on gas chromatography and mass spectrometry (GC-MS). For this purpose, cultures were grown in minimal medium supplemented with either 100% [^{13}C]glucose, or a mixture of 20% [$^{13}\text{C}_6$]glucose and 80% unlabeled glucose. All cultures exhibited comparable extracellular physiology (table 5). GC-MS-derived mass distributions in proteinogenic amino acids from these cultures were then used to calculate intracellular carbon flux ratios (table 6). Consistent with previous flux analyses

Table 4
Relative Abundance of Paralogous Genes in the Deleted Regions of *B. subtilis* $\Delta 6$

	Prophage 1 (20 genes)	Prophage 3 (10 genes)	PBSX (42 genes)	SP β (185 genes)	<i>skin</i> (61 genes)	<i>pks</i> (14 genes)	Total (332 genes)
Singlets	75%	100%	21%	85%	43%	71%	68%
Doublets	5%	0%	60%	9%	46%	29%	23%
Triplets	10%	0%	10%	3%	5%	0%	5%
Quadruplets	0%	0%	7%	2%	3%	0%	3%

NOTE.—The BLAST2P algorithm (Altschul et al. 1997) was used for protein comparisons in the SubtiList database (<http://genolist.pasteur.fr/SubtiList/>). To define paralogous genes, the similarity (identical residues and conservative replacements) between the corresponding proteins should be $\geq 35\%$ over the entire protein length in reciprocal BLAST2P comparisons. Total numbers of annotated genes in each deleted region are indicated. Note that the percentages of singlets, doublets, triplets and quadruplets for individual regions do not sum up to 100% due to the fact that some genes belong to larger paralogous gene families.

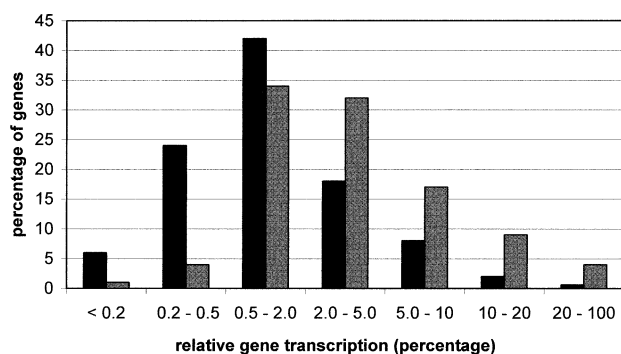


FIG. 2.—Transcription of genes in prophage-like regions. *B. subtilis* 168 was grown in TY medium at 37°C until 3 h after the end of exponential growth. Next, cells were collected and total RNA was extracted for duplicate transcript profiling experiments using DNA macro-arrays. The percentage of genes transcribed at a certain level (y-axis) is plotted against the relative level of gene transcription (x-axis). For this purpose, the transcription level of each gene represented on the array was related to the transcription level of the most highly expressed gene (100%). The analysis includes a total number of 462 prophage-like genes and 3,653 non-prophage genes (Sigma Genosys array product information). Notably, four *B. subtilis* 168 genes were left out of the analysis because no statistically significant transcription data were obtained for these genes. Black bars represent prophage genes; gray bars represent non-prophage genes.

of fast-growing *B. subtilis* cultures (Sauer et al. 1997; Dauner, Storni, and Sauer 2001), the gluconeogenic phosphoenolpyruvate [PEP] carboxykinase was inactive (PEP from oxaloacetate [OAA]) and the anaplerotic pyruvate carboxylase and the tricarboxylic acid (TCA) cycle contributed to similar extents to OAA synthesis (OAA through the TCA cycle), irrespective of the deletion of the 332 genes. Relative to the glucose uptake rate, the catabolic fluxes through the pentose phosphate (PP) pathway were determined at ~30%, which is similar to what was described for glucose-limited chemostat cultures (Dauner, Storni, and Sauer 2001). Taken together, the comparison of parental and deletion strains revealed an almost identical intracellular carbon metabolism. In particular, relative in vivo activities of the PP pathway (Serine [SER] through the PP pathway) and the TCA cycle (OAA through the TCA cycle) were identical within the resolution limits of this analysis.

B. subtilis $\Delta 6$ Has a Normal Competence and Sporulation Phenotype

The deleted parts of the chromosome do not encode proteins that are known to be involved in transformability of *B. subtilis* (table 3). To verify the absence of, thus far, unknown competence genes from the deleted regions, the transformability of *B. subtilis* $\Delta 6$ cells was analyzed. Under the conditions tested, the transformation frequency of the $\Delta 6$ strain (1.8×10^{-3}) was comparable to that of the parental strain (1.6×10^{-3}). This shows that none of the genes absent from *B. subtilis* $\Delta 6$ is required for competence development.

The *skin* (for *sigK* intervening) element is positioned within the *sigK* gene. It is excised at a particular stage of sporulation, leading to the reconstitution of *sigK* by fusion of the *spoIIIC* and *spoIVCB* genes. The sigma factor σ^K , encoded by *sigK*, is required for sporulation (Errington

Table 5
Physiological Parameters of *B. subtilis* $\Delta 6$ and the Parental Strain 168

		168	$\Delta 6$
μ_{\max}	1/h	0.52 ± 0.02	0.52 ± 0.02
q_{glucose}	mmol/g/h	9.0 ± 0.1	9.0 ± 0.1
q_{acetate}	mmol/g/h	2.1 ± 0.2	3.3 ± 0.2
q_{acetoin}	mmol/g/h	< 0.2 mM	< 0.2 mM
Biomass yield	g/g	0.28 ± 0.02	0.30 ± 0.02

NOTE.—Maximum growth rates (μ_{\max}), major extracellular fluxes (q), and biomass yields were determined as described in *Materials and Methods*.

et al. 1988; Kunkel et al. 1988; Stragier et al. 1989). In our mutant, the *skin* element has been deleted from *arsC* to *yqaB*, leaving the *spoIIIC* and *spoIVCB* genes intact. Furthermore, *skin* contains the *phrE/rapE* genes which, like the *phrA/rapA* genes from PBSX, are involved in the early stages of the sporulation process (Lazzera et al. 1999; Perego 1999). To investigate the effect of the six combined deletions on sporulation, we analyzed spore formation and germination in *B. subtilis* $\Delta 6$. None of these developmental processes was detectably influenced in this strain (data not shown). This demonstrates that the combined deletion of known determinants for sporulation encoded by *skin* and PBSX does not affect sporulation.

Changes in the Extracellular Proteome

To investigate the effects of the large deletions on protein secretion in general, the extracellular proteomes of *B. subtilis* $\Delta 6$ and 168 were compared by employing 2D PAGE-MS. A representative result is shown in figure 3A, in which dual channel imaging was used to monitor possible changes in extracellular protein composition. Specifically, the phage proteins YoaA (SP β), XlyA, XkdG, XkdK, and XkdM (PBSX) were shown to be absent from the growth medium of *B. subtilis* $\Delta 6$ (represented as green protein spots in fig. 3A). Furthermore, the amounts of the lipoproteins MntA (manganese transport) and YfiY (possibly involved in iron(III) dicitrate transport) are reproducibly detected at increased levels in the medium of the *B. subtilis* $\Delta 6$ strain (red protein spots), whereas the secreted esterase LipA was present in strongly decreased amounts (green protein spot). The latter observation was remarkable, as the *lipA* gene is still present in *B. subtilis* $\Delta 6$. To verify whether the decrease in the secretion of LipA by *B. subtilis* $\Delta 6$ is significant, this strain was transformed with pLip2031. The presence of this plasmid results in the overproduction of LipA. Next, LipA secretion was monitored both by activity assays and by Western blotting. The results showed that, under overproducing conditions, the secretion of LipA by *B. subtilis* $\Delta 6$ is reduced about threefold as compared to the parental strain 168 (data not shown).

It has to be noted that, in contrast to the MntA, YfiY, and LipA spots, the relative abundance of certain minor protein spots (labeled in green or red) varies in different experiments. Such variations must, therefore, be attributed to the natural dynamics in the composition of the extracellular proteome of *B. subtilis*. Consistent with the

Table 6
METAFor Analysis of *B. subtilis* $\Delta 6$ and Parental Strain 168

Origin of Metabolite Pool	<i>B. subtilis</i>	
	168	$\Delta 6$
Glycolysis and TCA cycle		
OAA through TCA	0.51 \pm 0.02	0.54 \pm 0.02
PEP from OAA	0.01 \pm 0.01	0.00 \pm 0.04
PYR from MAL (lower bound)	0.06 \pm 0.01	0.09 \pm 0.01
PYR from MAL (upper bound)	0.12 \pm 0.03	0.17 \pm 0.03
PP pathway		
SER through PP pathway ^a	0.35 \pm 0.02	0.33 \pm 0.01
PEP through TK	0.09 \pm 0.02	0.08 \pm 0.02
E4P through TK	0.40 \pm 0.02	0.39 \pm 0.02

NOTE.—Batch cultures contained either 100% [¹³C]glucose, or a 20/80 mixture of [¹³C₆] and unlabeled glucose. Abbreviations: E4P, erythrose-4-phosphate; MAL, malate; OAA, oxaloacetate; PEP, phosphoenolpyruvate; PP, pentose phosphate; PYR, pyruvate; SER, serine; TCA, tricarboxylic acid; TK, transketolase.

^a Indicates flux ratios that were determined from [¹³C]glucose experiments.

2D gel electrophoretic analysis, which suggests that protease secretion is not affected in the $\Delta 6$ strain, the azocasein-degrading activity of proteases in the medium of *B. subtilis* $\Delta 6$ was comparable with that of proteases secreted by the parental strain 168. Notably, the extracellular appearance of 13 intracellular proteins (e.g., RocA, KatA, and GroEL) that lack a typical secretion signal (Antelmann et al. 2001) was also unaffected by the six deletions. Moreover, no additional cytoplasmic proteins appeared on the extracellular proteome of *B. subtilis* $\Delta 6$, which shows that this engineered strain is “healthy” and not prone to increased levels of cell lysis.

High-Level Secretion of Active AmyQ by *B. subtilis* $\Delta 6$

To assess the secretion capacity of the $\Delta 6$ strain, we studied the secretion of active AmyQ of *Bacillus amyloliquefaciens* with an α -amylase plate assay. For this purpose, the 168 and $\Delta 6$ strains were transformed with plasmid pKTH10, which results in the high-level production and secretion of AmyQ (Palva 1982). The results demonstrated that the deletion strain can still secrete active AmyQ, a heterologous protein (data not shown). Importantly, *B. subtilis* $\Delta 6$ pKTH10 did not reveal a significant difference in secretion of AmyQ compared to the parental strain containing pKTH10. Likewise, the secretion of the endogenous α -amylase AmyE by the $\Delta 6$ strain was not affected, which is consistent with the 2D gel analysis (fig. 3A; yellow AmyE spot).

Previous studies have shown that the high-level production of AmyQ imposes a so-called secretion stress on cells of *B. subtilis*. This stress is sensed by the C_{ss}RS two-component regulatory system. Activation of the C_{ss}RS system results in the transcription of the *htrA* and *htrB* genes at significantly elevated levels (Hyyryläinen et al. 2001; Darmon et al. 2002). Consequently, the level of *htrA* or *htrB* transcription can be used as an indicator for secretion stress. To study the induction of secretion stress by AmyQ in *B. subtilis* $\Delta 6$ pKTH10, an *htrB-lacZ* transcriptional gene fusion was introduced in this strain. Next, β -galactosidase activities in cells grown in TY medium at 37°C were

measured as a function of time. As shown in figure 4A (closed symbols), the transcription of *htrB-lacZ* was not affected by the $\Delta 6$ mutations, demonstrating that these mutations do not trigger a secretion stress response. Moreover, *B. subtilis* $\Delta 6$ pKTH10 containing the *htrB-lacZ* fusion displayed an AmyQ-induced secretion stress response that is very similar to that of the parental strain transformed with pKTH10 (fig. 4A, open symbols). Consistent with this observation, Western blotting analyses revealed that the AmyQ-producing cells of the $\Delta 6$ strain and the parental strain 168 contained comparable amounts of the precursor and mature forms of this secretory protein (fig. 4B). Furthermore, similar amounts of mature AmyQ protein were detectable in the growth medium, which is in accordance with the results of the activity assay that was performed (fig. 4B and data not shown). Proteomics was employed to monitor possible effects of AmyQ-induced secretion stress on the composition of the extracellular proteome of *B. subtilis* $\Delta 6$. The dual-channel analysis of 2D gels (fig. 3B) revealed that the overproduction of AmyQ has only a few significant consequences for the composition of the extracellular proteome of the $\Delta 6$ strain. First, a number of AmyQ spots with a slightly different pI is detectable in the medium of *B. subtilis* $\Delta 6$ pKTH10. Furthermore, the extracellular level of HtrA, a known indicator of the AmyQ-induced secretion stress response, is significantly increased in the $\Delta 6$ -derived sample. Unexpectedly, the high-level production of AmyQ in the $\Delta 6$ strain also results in the extracellular appearance of the PtsH (or HPr) protein, a histidine-containing phosphocarrier protein of the phosphotransferase system. Taken together, these observations show that the absence of 332 genes from *B. subtilis* $\Delta 6$ has no major impact on protein secretion in general, or on the high-level secretion of the heterologous protein AmyQ in particular.

The $\Delta 6$ Strain Has a Changed Motility

As a final approach to characterize *B. subtilis* $\Delta 6$, the motility of cells of this strain was analyzed on plates containing different concentrations of agarose. No difference in motility was observed for *B. subtilis* $\Delta 6$ cells and cells of the parental strain 168 that were spotted on plates containing 0.75% agarose, but clear differences were observed on plates containing lower amounts of agarose (fig. 5). Remarkably, the motility of $\Delta 6$ cells is higher than that of 168 cells on plates containing 0.5% agarose. In contrast, the parental strain was shown to be more motile than the $\Delta 6$ strain when the agarose concentration was lowered to 0.27%, the concentration typically used in “swimming” assays for *B. subtilis*. Under these conditions a clear zone is visible between the swimming cells of both strains. This is probably due to the secretion of sublancin 168 by the parental strain, which kills the cells of the $\Delta 6$ strain. The latter cells are sensitive to sublancin 168, because they lack the SP β -specific immunity against this lantibiotic. Notably, none of the proteins encoded by the 332 deleted genes shows similarity to known pilus, flagellar, or chemotaxis proteins that could cause a changed motility (table 3). This implies that one or more of the

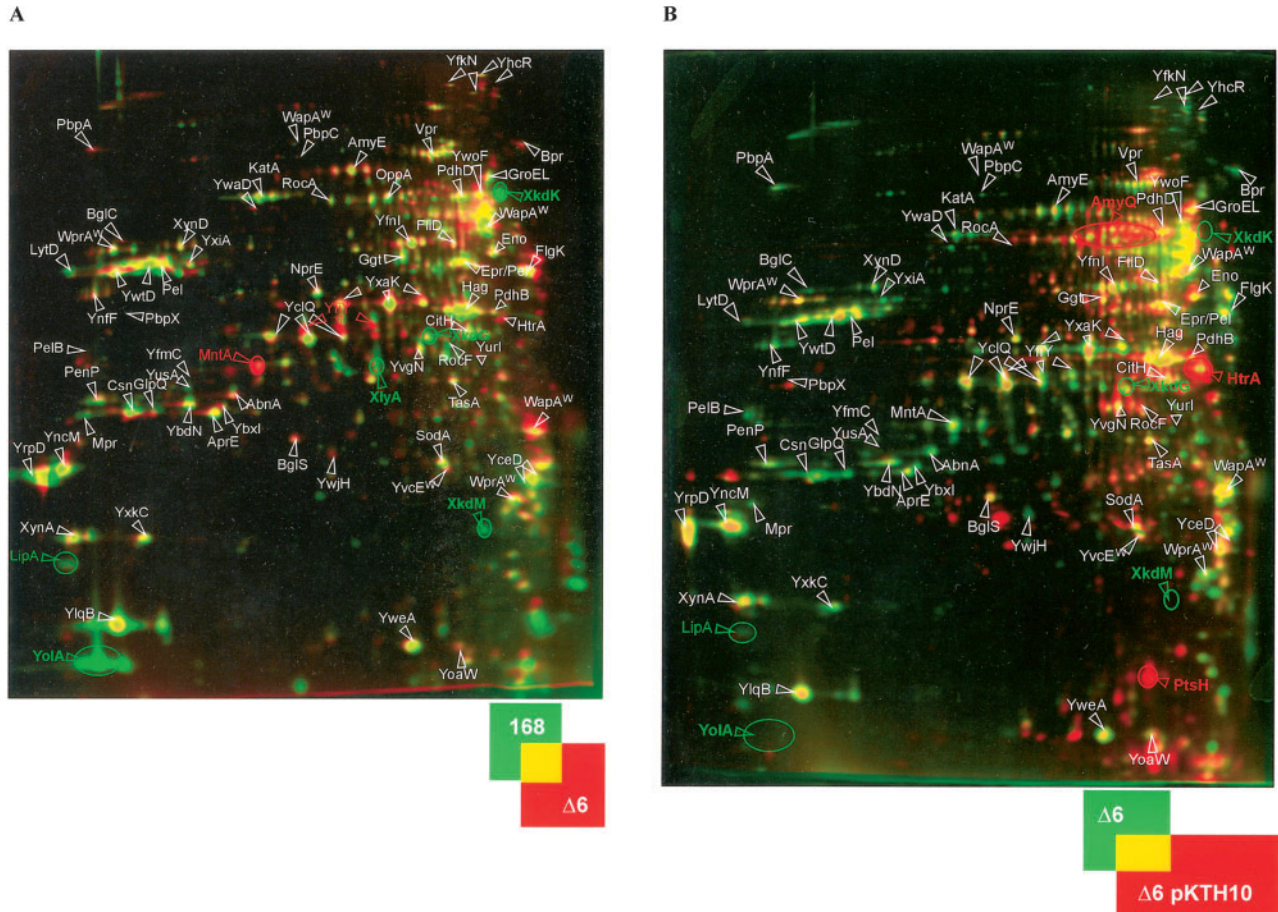


FIG. 3.—The extracellular proteome of *B. subtilis* $\Delta 6$. **A**. Comparison of the extracellular proteomes of *B. subtilis* 168 and $\Delta 6$. Cells of *B. subtilis* 168 and *B. subtilis* $\Delta 6$ were grown in TY medium and extracellular proteins were harvested 1 h after entry of the cells into the stationary phase. The extracellular proteins were analyzed by 2D PAGE, MALDI-TOF MS, and dual-channel imaging, as indicated in *Materials and Methods*. Green protein spots are predominantly present in the master image of the extracellular proteome of *B. subtilis* 168, red protein spots are predominantly present in the image of the extracellular proteome of the $\Delta 6$ strain, and yellow spots are present at similar amounts in both proteomes. The picture shown was obtained by dual channel imaging of two representative warped 2D gels on which extracellular proteins of the parental and mutant strains were separated. The proteins identified by MS are indicated on the image. **B**. The extracellular proteome of *B. subtilis* $\Delta 6$ under secretion stress conditions. *B. subtilis* $\Delta 6$ and *B. subtilis* $\Delta 6$ pKTH10 were grown in TY medium, and extracellular proteins were harvested 1 h after entry of the cells into the stationary phase. The extracellular proteins were analyzed by 2D PAGE and dual channel fluorescence imaging as indicated for panel A. Green protein spots are predominantly present in the master image of the extracellular proteome of *B. subtilis* $\Delta 6$, red protein spots are predominantly present in the image of the extracellular proteome of the *B. subtilis* $\Delta 6$ pKTH10 strain, and yellow spots are present at similar amounts in both proteomes.

deleted genes either has a direct role in cell motility or will affect the function of other motility factors.

Discussion

Most estimates on the minimal gene set required for life are based on theoretical considerations and indirect evidence from random mutagenesis or systematic gene disruption. For *B. subtilis*, a minimal set of 270 to 500 genes was predicted. However, the de facto reduction of the *B. subtilis* genome by the simultaneous deletion of large gene clusters has not been performed so far. In the present studies, we demonstrate for the first time that the consecutive deletion of large regions from the *B. subtilis* genome is possible. In total, six large regions were deleted, ranging from ~ 13 kb (prophage 3) to ~ 134 kb (SP β). This has led to a reduction in genome size of 320 kb (7.7% of the genome) and a loss of 332 protein-encoding genes (8.1% of

the *B. subtilis* genes). The genome reduction has not affected the growth and viability of the resulting $\Delta 6$ strain under laboratory conditions, which indicates that the prophage(-like) regions and *pks* have not evolved into regions encoding very important indispensable functions. This view is supported by the observation that neither the primary metabolism nor the essential process of protein secretion is detectably affected in *B. subtilis* $\Delta 6$. Furthermore, our data show that the nonessential processes of competence development and sporulation have not become dependent of prophage-like functions. The latter finding is particularly remarkable, because certain genes on *skin* and PBSX are involved in the initiation of sporulation, and because the *skin* element became inserted into the *sigK* gene. Importantly, the deleted regions and other prophage-like regions may encode functions that are beneficial for survival in the soil. For example, the *pks* operon has been implicated in antibiosis (Albertini et al. 1995). Moreover,

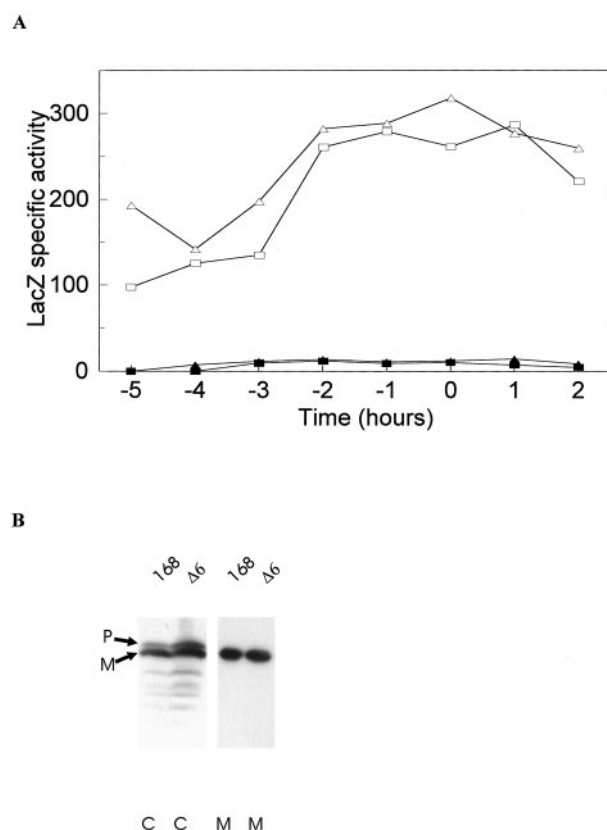


FIG. 4.—AmyQ-induced secretion stress response. A. To compare the induction of secretion stress responses by high-level AmyQ production and secretion in *B. subtilis* Δ6 and the parental strain 168, a transcriptional *htrB-lacZ* fusion was used. Time courses of *htrB-lacZ* expression were determined by analyzing β -galactosidase activity (indicated in $\text{nmol} \times \text{min}^{-1} \times \text{OD}_{600}^{-1}$) in cells grown in TY medium at 37°C. Samples were withdrawn at the times indicated; zero time is defined as the transition point between exponential and post-exponential growth. The strains used for the analyses were: *B. subtilis* 168 *htrB*::pMutin4 (BFA3041; closed rectangles); *B. subtilis* Δ6 *htrB*::pMutin4 (closed triangles); *B. subtilis* 168 *htrB*::pMutin4 pKTH10 (open rectangles); and *B. subtilis* Δ6 *htrB*::pMutin4 pKTH10 (open triangles). The parental strain 168 was used as a negative control (data not shown). B. Cells of *B. subtilis* BFA3041 (marked 168) and Δ6 *htrB*::pMutin4 (marked Δ6), both containing pKTH10 for AmyQ production, were harvested after 2 h of post-exponential growth in the experiment described for panel A. Cells (C) were separated from the growth medium (M) by centrifugation. (Pre-)AmyQ was visualized by SDS-PAGE, Western blotting, and immunodetection, using AmyQ-specific antibodies. p, pre-AmyQ; m, mature AmyQ.

the production of sublancin 168, which requires genes specified by SP β (Dorenbos et al. 2002), may give *B. subtilis* a competitive advantage in its natural habitat. Interestingly, the altered motility of *B. subtilis* Δ6 may reflect either a prophage-facilitated adaptation of *B. subtilis* to natural conditions or a property that is somehow beneficial to one or more prophages residing in this organism. Thus far, we have not been able to link the different motility phenotypes to specific prophage genes. Although transcript profiling showed that the expression of certain motility genes is affected in *B. subtilis* Δ6 when this strain is grown in batch culture (our unpublished observations), the relevance of this observation for growth on plates with different amounts of agarose remains to be determined.

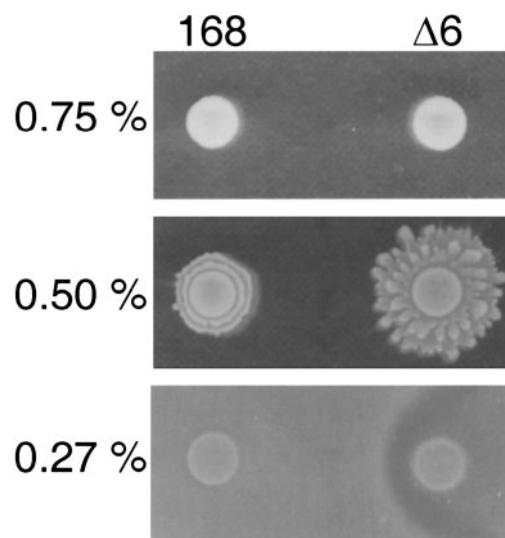


FIG. 5.—Motility of *B. subtilis* Δ6. *B. subtilis* Δ6 or *B. subtilis* 168 was grown on TY plates containing different agarose concentrations (0.75%, 0.50%, and 0.27%) to visualize the motility of the strains.

A normal maintenance and replication of the *B. subtilis* chromosome is required for successful progress through the developmental processes of competence and sporulation (Piggot and Coote 1976; Ireton and Grossman 1992; Ireton, Gunther, and Grossman 1994; Sciochetti, Piggot, and Blakely 2001). Because these processes are not disturbed in *B. subtilis* Δ6, it can be concluded that the overall chromosome “physiology” (i.e., the sum of all processes required for chromosome replication and maintenance) is not significantly disturbed by the combined deletions. Nevertheless, it remains to be determined whether specific processes, such as chromosome condensation and packaging and global distribution of transcriptional signals are completely unaffected. On the one hand, the apparent lack of effect on chromosome physiology is not surprising, because the deletions are almost evenly distributed along the chromosome: ~ 141 kb (3.3%) has been deleted from the 0–172° replicore and ~ 180 kb (4.3%) from the 172–360° replicore (fig. 1). On the other hand, competence development was seemingly not affected in the intermediate strains, in which the relative sizes of the replicores are more affected (unpublished observations). The view that the stepwise deletions created in the present studies do not interfere with chromosome physiology would be in line with a stepwise invasion of prophage(-like) regions during evolution of the *B. subtilis* chromosome.

The present transcript-profiling experiments with DNA arrays suggest that genes in the prophage(-like) regions are expressed at a relatively lower level than genes in the non-prophage regions. Although the efficiency of the DNA hybridizations in these experiments may be somewhat biased by the fact that prophage genes have a higher AT content than the remaining genes, the results are likely to be biologically relevant, because the prophages SP β and PBSX are not induced under standard laboratory conditions. Although genes of these prophages are most likely repressed, at least two other explanations

for the low transcription levels of prophage genes and genes in prophage-like regions are conceivable. First, the low transcription of most of these genes may relate to their presumed foreign origin. For example, promoters of prophage(-like) genes may be optimal for other organisms, which can result in a poor match with authentic promoter sequences of *B. subtilis*. Second, several of these genes belong to the 25% of *B. subtilis* genes that are transcribed in the opposite direction of replication fork movement (Kunst et al. 1997; Rocha et al. 2000). This may interfere with their efficient transcription. It has to be noted that, for unknown reasons, the relative transcription levels of prophage 1 and prophage 6 genes are comparable with those of the non-prophage genes of *B. subtilis*.

At present it is not clear to what extent the $\Delta 6$ strain represents an improved bacterial cell factory. Clearly, the capacity for high-level production and secretion of a heterologous model protein was neither positively nor negatively affected by the mutations in this strain. This suggests that no large energy resources were redirected toward product formation. Furthermore, only a few proteins were absent from the extracellular proteome, indicating that the $\Delta 6$ strain is only marginally improved in terms of the removal of unwanted by-products. It is presently not clear why the amounts of MntA and YfiY were significantly increased in the medium of *B. subtilis* $\Delta 6$, while the extracellular accumulation of LipA was reduced. Furthermore, no major changes were observed in the cellular proteome of *B. subtilis* $\Delta 6$, which is consistent with the fact that no prophage proteins are detectable on the cellular proteome of the parental strain (H. Antelmann and M. Hecker; unpublished data). In fact, these findings are consistent with the low transcriptional levels of the prophage-like genes. Importantly, the analysis of the extracellular proteome of *B. subtilis* $\Delta 6$ revealed that the sensitivity of this strain to cell lysis is not increased, even under conditions of severe secretion stress. The only major changes observed under these conditions concerned the presence of increased amounts of HtrA and PtsH in the medium. The increased level of extracellular HtrA is consistent with the induction of *htrA* transcription under conditions of secretion stress (Hyyryläinen et al. 2001). At present it is not known whether the elevated levels of PtsH reflect a physiological response of the $\Delta 6$ strain to protein secretion stress.

Thus far, the $\Delta 6$ strain seems to be moderately inferior to the parental strain 168 in only two aspects. First, *B. subtilis* $\Delta 6$ lacks the gene for the thiol-disulfide oxidoreductase BdbB, which plays a minor role in the heterologous secretion of the *E. coli* PhoA protein by *B. subtilis* (Bolhuis et al. 1999). Probably, BdbB supports its paralog BdbC (which is present in *B. subtilis* $\Delta 6$) in the formation of two disulfide bonds in PhoA. Second, together with the deletion of the prophages, the respective immunity regions were lost. If necessary for production purposes, the *bdbB* gene as well as the phage immunity regions can be re-inserted into the chromosome of *B. subtilis* $\Delta 6$.

In terms of cell factory engineering, *B. subtilis* $\Delta 6$ has one major advantage over conventional *B. subtilis* production strains: it lacks the *BsuM* restriction-modification system. As shown by Ohshima et al. (2002), prophage 3 encodes both the genes for *BsuM* modification (*ydiO* and

ydiP) and *BsuM* restriction (*ydiR*, *ydiS*, and *ydiA*). *BsuM* restriction has been shown to reduce the transformation efficiency of *B. subtilis* with recombinant plasmids up to 7,800-fold (Haima, Bron, and Venema 1987). Moreover, this system was found to be responsible for structural plasmid instability in *B. subtilis*, which limits the application potential of plasmids for high-level protein production. With respect to bacterial evolution for survival in the soil, it is interesting to speculate that *B. subtilis* has recruited prophage 3, specifying the *BsuM* system, in order to protect itself against invading foreign DNA.

Finally, it was recently shown that 12 K-islands could be deleted from the *E. coli* genome, resulting in an 8.1% reduced genome size (Kolisnychenko et al. 2002). Unfortunately, apart from growth experiments, which revealed no major differences with the parental strain MG1655, a detailed phenotypical analysis has so far not been documented for this engineered *E. coli* strain. In the present studies, we have taken the phenotypic characterization of the first *B. subtilis* strain with a reduced genome a few steps further by a combination of transcript profiling, metabolic flux analysis, proteomics, and dedicated assays for competence development, sporulation, protein secretion, and cell motility. We are convinced that such an integrated approach will be of utmost importance for the functional genomic characterization of organisms such as *E. coli* and *B. subtilis*. In particular, a combination of genome reduction, transcript profiling, proteomics, and metabolomics seems necessary to attribute physiological functions to the large families of paralogous genes in these organisms. This will also lead to optimized “next generation cell factories.” The deletion of two gene families with 3 paralogs, 26 families with 2 paralogs, and 227 unique genes represents an important step toward achieving these goals for *B. subtilis*.

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