Localization of the *Bacillus subtilis* murB Gene within the dcw Cluster Is Important for Growth and Sporulation

Gonçalo Real and Adriano O. Henriques*

Instituto de Tecnologia Química e Biológica, Universidade Nova de Lisboa, Avenida da República, Apartado 127, 2781-901 Oeiras Codex, Portugal

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The *Bacillus subtilis* murB gene, encoding UDP-N-acetylenolpyruvylglucosamine reductase, a key enzyme in the peptidoglycan (PG) biosynthetic pathway, is embedded in the *dcw* (for "division and cell wall") cluster immediately upstream of *divIB*. Previous attempts to inactivate murB were unsuccessful, suggesting its essentiality. Here we show that the cell morphology, growth rate, and resistance to cell wall-active antibiotics of murB conditional mutants is a function of the expression level of murB. In one mutant, in which murB was transcriptionally inactivated in a merodiploid bearing a second xylose-inducible *PxyA-murB* allele, DivIB levels were reduced and a normal growth rate was achieved only if MurB levels were threefold that of the wild-type strain. However, expression of an extra copy of *divIB* restored normal growth at wild-type levels of MurB. In contrast, DivIB levels were normal in a second mutant containing an in-frame deletion of *murB* (*ΔmurB*) in the presence of the *PxyA-murB* gene. Furthermore, this strain grew normally with wild-type levels of MurB. During sporulation, the levels of MurB were highest at the time of synthesis of the spore cortex PG. Interestingly, the *ΔmurB PxyA-murB* mutant did not sporulate efficiently even at high concentrations of inducer. Since high levels of inducer did not interfere with sporulation of a *murB*/*PxyA-murB* strain, it appears that ectopic expression of murB fails to support efficient sporulation. These data suggest that coordinate expression of *divIB* and *murB* is important for growth and sporulation. The genetic context of the *murB* gene within the *dcw* cluster is unique to the *Bacillus* group and, taken together with our data, suggests that in these species it contributes to the optimal expression of cell division and PG biosynthetic functions during both vegetative growth and spore development.

Peptidoglycan (PG) is a critical component of the eubacterial cell envelope, providing mechanical resistance to withstand osmotic pressure (55) and functioning as a major determinant of cell shape, which ultimately also relies on topological information imposed by the actin cytoskeleton (6, 10, 33, 54). PG is also intimately involved in the cell division process and, in spore-forming bacteria, is additionally required for a cytoskeleton-like role during engulfment of the prespore by the mother cell, as well as for the formation of the spore cortex, a modified layer of PG, essential for spore heat resistance (reviewed in reference 17).

In all eubacteria examined to date, many of the genes involved in PG biosynthesis and cell division are grouped in the highly conserved *dcw* (for "division and cell wall") cluster (Fig. 1A). A correlation between bacterial cell shape and the arrangement of genes within the *dcw* cluster has been suggested (51). Specifically, it appears that rod-shaped bacteria have retained a more conserved and compact *dcw* cluster, and that transitions to other bacterial shapes have involved rearrangements and loss of gene order conservation within the cluster (51). Other features of the region appear to be associated with the biology of certain groups. Examples are the duplication of the *pbpB* gene to originate the *spoVD* gene in spore formers of the genus *Bacillus* (9,11,12) and the presence of a sporulation-specific gene, *spoVE*, instead of *ftsW*, which is located in a different chromosomal location. Also, the *spoVE-murG-murB-divIB* unit (Fig. 1A) appears restricted to this group and, among the *Bacillus* species whose genomes have been sequenced, is only absent from *B. clausii* (34, 35). Both *spoVE* and *spoVD* are transcribed during sporulation in the mother cell, from σE-dependent promoters located just upstream of their coding regions, and both are dispensable for growth but essential for cortex biogenesis during spore formation (29,42,53).

In *B. subtilis* the *dcw* cluster is located in the 133° to 135° region of the chromosome (29). During vegetative growth, several genes in the cluster are cotranscribed in the form of long polycistronic messages, which appear to originate from promoters located just upstream of the *murE* gene, or even further upstream in the cluster (12, 25, 29). However, individual vegetative promoters have also been identified for specific genes or groups of genes, such as the *divIB* gene or the *pbpB* operon (12,25). The *divIB* gene codes for a protein required for septum formation during both medial (vegetative) and asymmetric (sporulation) cell division (reference 45 and references therein). *divIB* is transcribed mainly from a promoter located upstream of *murE* but also from a weak σE-type promoter located just 93 bp upstream of the *divIB* coding sequence (25,45). Cells in which *murB* is separated from *divIB* by an integrational plasmid exhibit a defect in nucleoid structure and segregation and fail to activate Spo0A-dependent gene expression at the onset of sporulation (45). Moreover, they show a slight growth defect that is exacerbated upon expression...
of a second copy of the upstream murB gene (45). Together with the observation that the spoVE-murG-murB-divIB unit is conserved within bacteria of the genus Bacillus (Fig. 1), this observation suggests that coordinated expression of these genes may be important for normal growth. Moreover, because murG and murB appear to be cotranscribed from the \(/H9268\)E-dependent promoter of spoVE, this linkage may also be important during sporulation (15).

Here, we have examined whether the chromosomal position of murB is important for its function. The murB gene codes for UDP-N-acetylenolpyruvoylglucosamine reductase, which is required at an early step in the PG biosynthetic pathway. The first step in PG biosynthesis involves the MurA enzyme, which catalyzes the production of UDP-N-acetylenolpyruvoylglucosamine (UDPGlcNAcEP) from UDP-N-acetylglucosamine (UDP-GlcNAc) and phosphoenolpyruvate. The enolpyruvyl moiety is then reduced by MurB, yielding UDP-N-acetylmuramic acid (UDPMurNAc). A pentapeptide chain is then added onto UDPMurNAc by the membrane acceptor (undecaprenylphosphate) by the MraY transferase to form lipid I, and then N-acetylglucosamine (GlcNAc) is added by another transferase, MurG, to form lipid II. The GlcNAc-MurNAc(pentapeptide)-pyrophosphoryl-undecaprenol is next translocated across the membrane, where it is incorporated into nascent PG by the transglycosylase and transpeptidation reactions catalyzed by several penicillin binding proteins (PBPs) (18).

The murB gene is essential in Escherichia coli and Staphylococcus aureus, as demonstrated by the isolation of temperature-sensitive mutants (38, 41, 44). Unsuccessful inactivation attempts also suggested its essentiality in B. subtilis (46). In this work, we have constructed and characterized a murB conditional mutant and have examined the effects of the ectopic expression of murB. We show that the murB gene is essential for normal growth, cell morphology, and resistance to cell wall-active antibiotics. We also show that normal expression of murB is required at the stage of spore cortex synthesis for the formation of heat-resistant spores. The evidence suggests that expression of murB from its normal locus within the dcw cluster is required for normal growth and efficient spore formation.

MATERIALS AND METHODS

Bacterial strains, media, and general methods. All of the B. subtilis strains used in this study are congenic derivatives of strain MB24 (trpC2 metC3) (Table 1). The E. coli strain DH5α (BRL) was used for plasmid construction and propagation (Table 1). Growth, selection, and maintenance of drug-resistant transformants of E. coli or B. subtilis was as described previously (27, 28). The high-fidelity Pfu polymerase (Stratagene, La Jolla, Calif.) was used to generate PCR fragments for cloning, which were sequenced to ensure that no mutations were introduced. Sporulation was induced by exhaustion in Difco sporulation medium (DSM) (8, 50), and its frequency was expressed as the percentage of heat-
resistant CFU relative to the total cell count (27). Spores were purified on step gradients of Gastrografin (Schering) (26, 27, 47).

**Purification of MurB and DivIB fusion proteins for antibody production.** The murB gene was PCR amplified from MB24 using primers murB-235D (5'-ATG GAGAAATGTGTAAGG-3') and murB-1165R (5'-GCTTGTCTGAGAC TGTAATCGCAGG-3'). The 930-bp product was cut with BglII and inserted between the BamHI and EcoRI sites of pMAL-c2 to create pH11032. The 12% or 15% polyacrylamide gels containing sodium dodecyl sulfate were used at the following dilutions: anti-MurB, dilution of 1:1,000; anti-DivIB, 1:250. A rabbit secondary antibody conjugated to horseradish peroxidase (Sigma) and a mouse secondary antibody were used at dilutions of 1:10,000 and 1:200, respectively. The immunobLOTS were developed with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

**Construction of a murB conditional mutant.** For construction of a murB conditional mutant, first a PCR fragment carrying the xylA promoter was produced using primers syr-61D (5'-CATATTGTATCCCTTTTCTATGAG-3') and xylR-1558R (5'-GGAATGATCTGAGGATCTGATG-3') and digested with SacI and BsmBI and inserted into the BamHI and EcoRI sites of pMAL-c2 (New England Biolabs) to yield pGR28. The divIB gene (lacking the sequence for its transmembrane domain) was PCR amplified from MB24 using primers divIB-77D (5'-CTGATTGAAGCACTA-3') and divIB-908R (5'-CCAAGCTTCCTGTTCGCGCCG-3'). The 669-bp product was digested with BglII and EcoRI and inserted between the BamHI and EcoRI sites of pMAL-c2 to create pH11002 to nmr resistance (Cmr) and subjected to immunoblot analysis as described previously (48). Antibodies were used at the following dilutions: anti-MurB, dilution of 1:1,000; anti-DivIB, 1:100; anti-β' subunit of RNA polymerase (a gift from Bill Haldenwang), 1:250. The transformation of MB24 to neomycin resistance (Nm r) with pGR43, containing the 3' end of murB was made by inserting an NruI- and Spcl-cut PCR product obtained with primers murB-213D (5'-GCAAGATCTAATGCGAGGA CACTGTCGGG-3') and murB-1597R (5'-GCTTTTGGTGTATGCTGGCTT-3'), resulted from the transformation of MB24 to neomycin resistance (Nm r) with the pGEM T Easy cloning vector, was digested in lysis buffer. Column washing and elution were as described by the manufacturer. Purified MurB and DivIB fusion proteins were used to raise rabbit polyclonal antibodies (Eurogentec, Herstal, Belgium).

**Immunoblot analysis.** Samples (10 μL) of LB or DSM cultures were collected and lysed as described before (48). Samples (20 μg) of total protein were resolved on 12% or 15% polyacrylamide gels containing sodium dodecyl sulfate and subjected to immunoblot analysis as described previously (48). Antibodies were used at the following dilutions: anti-MurB, dilution of 1:1,000; anti-DivIB, 1:100; anti-β' subunit of RNA polymerase (a gift from Bill Haldenwang), 1:250. A rabbit secondary antibody conjugated to horseradish peroxidase (Sigma) and a mouse secondary antibody were used at dilutions of 1:10,000 and 1:200, respectively. The immunobLOTS were developed with enhanced chemiluminescence reagents (Amersham Pharmacia Biotech).

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of 0.1% xylose. This produced AH1746, in which inactivation of murB within the dcw region was verified by PCR.

Construction of an inducible divIB allele. The Pspac-divIB-lacI region in pGR90 (45) was PCR-amplified with primers pH435D (5'-GAAGATGTCAGACGTTTGTTTACGGTAAGTGC-3') and pH1658R (5'-GAGATATCTATGCTGACAGC-3'), and the product was cut with EcoRI and BglII and inserted between the EcoRI and BamHI sites of pDG1664 (24), to yield pGR163. Plasmid pGR163 was used to transform MB24 selecting for Emr, to produce AH3303 (Table 1) in which Pspac-divIB-lacI resides at thrC, as verified by PCR. Chromosomal DNA from AH3303 was used to transform AH1746 selecting for Emr. A transformant was selected and named AH3356 (Table 1).

In-frame deletion of murB. First, two PCR products of 600 bp encompassing the 5' and 3' regions of murB were produced using primers murB-77D (see above) and murBaseR(BglII) (5'-GCTCATATGCAAGCGTGAACG-3') and primers murBaseD(BglII) (5'-GCTGAAAGATCTGATCAGATGGATCG-3') and divIB430R(EcoRI) (5'-CTGAGGCAATTACAGCATTCATGTC-3'). The two products were digested with BglII, ligated together, and inserted into pGEM-Teasy (Promega). This created pGR194 (Fig. 1B), which was used with pDG1731 (24) to cotransform AH1745 to spectino- mycin resistance (Sp'). One erythromycin-susceptible transformant (AH3497) was dependent on xylose for growth and had a murB::Pspac-divIB-lacI::Pspac-divIB allele.

The Pspac-divIB-lacI::Pspac-divIB region in MB24 (AH3497) was subject to immunoblot analysis using anti-MurB, anti-DivIB, and anti-β′ RNA polymerase subunit antisera (see Materials and Methods). (E) Lane 1, MB24 (wild type); lane 2, AH3497 grown in the presence of 0.0025% xylose. (G) Lane 1, MB24 (wild type); lane 2, AH3497 grown in the presence of 0.0025% xylose, as verified by PCR and sequence analysis.

Microscopy. Culture samples were collected and stained with the membrane dye FM4-64 (Molecular Probes), as described previously (45). Phase-contrast or fluorescence images were acquired in a Leica DMRA2 microscope coupled to a CoolSNAP HQ Photometrics camera (Roper Scientific).

Antimicrobial susceptibility. Susceptibility to antibiotics was determined by measuring bacterial growth on square gradient plates (12 cm by 12 cm) as described previously (2, 7). Briefly, each plate contained LB agar supplemented with 0.001%, 0.005%, 0.01%, or 0.1% xylose (50 ml in each of the top and bottom layers). Antibiotics used for the top layer were as follows: vancomycin, 0.25 μg ml⁻¹; oxacillin, 0.2 μg ml⁻¹; fosfomycin, 20 μg ml⁻¹; D-cycloserine, 10 μg ml⁻¹; cephalosporin, 10 μg ml⁻¹. Strains were grown in LB (supplemented with 0.0025% xylose when required) for ~16 h, diluted 100-fold into LB supple-
mented with 0.01% xylose, and incubated at 37°C to an OD₆₀₀ of 0.2. Cell plating was as described previously (1, 22, 23). Confluent growth along the gradient was scored after 18 h at 37°C.

RESULTS

murB is essential in B. subtilis. Previous unsuccessful attempts to insertionally inactivate the murB gene in B. subtilis were interpreted as indicating its essentiality (46). Here, we constructed a murB conditional mutant by first inserting a copy of murB under the control of the xylose-inducible PxyI promoter at the nonessential amyE locus and then by using the murB merodiploid (AH1745) to disrupt the murB gene at its normal locus, by insertion of a Nm⁰ cassette within the gene’s coding sequence. Attempts to obtain Nm⁰ transformants of AH1745 in the absence of xylose failed, but those readily arose when the medium was supplemented with 0.1% xylose. One, in which the murB gene was shown to be inactivated, was designated AH1746 (Table 1). To test the effect of varying the expression level of murB on cell growth, overnight cultures of AH1746 grown in the presence of 0.05% xylose were centrifuged, washed with LB medium, resuspended in xylose-free medium, and diluted to an OD₆₀₀ of 0.02 in prewarmed LB medium without xylose or supplemented with increasing concentrations of inducer. The growth rate of the cultures was determined by monitoring the rate of increase in the OD₆₀₀. In parallel, we monitored the levels of MurB in cells of the conditional mutant using an anti-MurB antibody (see Materials and Methods).

As shown in Fig. 2A, the growth rate of the murB conditional mutant (AH1746) was proportional to the concentration of xylose in the medium. The wild-type strain exhibited a doubling time of about 30 min in LB medium at 37°C in the absence or in the presence of 1% xylose, whereas the doubling time of the murB conditional mutant in the absence of inducer was of 93.1 min (Table 2). Residual growth of the mutant in the absence of xylose is presumably caused by leakiness of the PxyI promoter, allowing MurB to accumulate to about 30% of wild-type levels (see above). The results in Fig. 2E indicate that the levels of DivIB in strain AH1746 during the exponential phase of growth in the presence of 0.0025% xylose, which permits MurB to accumulate to wild-type levels (see above). The results in Fig. 2E indicate that the levels of DivIB in AH1746 were slightly reduced (by about 10%) relative to MB24 (wild type), while the levels of MurB or of the β′ subunit of RNA polymerase remained unchanged in the two strains (Fig. 2E). The reduction in the levels of DivIB was not sufficient to cause any discernible effect on cell division, as the cell length distribution was similar to that of the wild-type strain (data not shown; see also reference 45).

To test whether the requirement for higher than wild-type levels of MurB for normal growth of AH1746 was due to reduced levels of DivIB, we placed a second copy of divIB under the control of the IPTG-inducible Pspac promoter at the nonessential thrC locus. The resulting strain, AH3356 (∆murB::neo amyE::PxyI-murB thrC::Pspac-divIB (+)), was, like its parent, xylose dependent for growth (not shown). However, in the presence of 0.5 μM IPTG, a wild-type doubling time was observed for AH3356 at a xylose concentration of only 0.0025%; that is, four times lower than for the parental strain AH1746 (Table 2; see also above). Thus, the need for increased levels of MurB to sustain a normal growth rate in AH1746 results from the re-
duction in DivIB levels; conversely, increased expression of divIB reduces the requirement for MurB.

A nonpolar murB in-frame deletion mutant. Because of the effect of the murB::neo allele on the levels of DivIB, we constructed an in-frame deletion of the murB gene (see Materials and Methods) and transferred the new allele into strain AH1745. The new conditional mutant, AH3497, exhibited xylose dependency for growth (Table 2). AH3497 grew slowly, with a doubling time of 88.1 min in the absence of inducer (Table 2 and Fig. 2C). However, in contrast to AH1746 (murB::neo), which grew normally at a xylose concentration of 0.01%, AH3497 showed the same doubling time as the wild type in the presence of only 0.0025% xylose (Table 2 and Fig. 2C), a concentration at which AH3356 (murB::neo Pspac-divIB) also exhibited normal growth. As for AH1746, the level of MurB, but not that of the β′ subunit of RNA polymerase, increased with the concentration of xylose in the growth medium (Fig. 2F). For AH3497 growing in the presence of 0.0025% xylose, MurB accumulated to wild-type levels (Fig. 2F, lanes 1 and 4, and Fig. 2G), and the doubling time was 30.6 min, similar to that of the wild type (30.1 min; Fig. 2C and Table 2). Moreover, under these conditions, DivIB accumulated to wild-type levels, as shown by the immunoblot analysis docu-

FIG. 3. MurB depletion causes a defect in cell morphology. The figure depicts the morphology of cells in LB cultures of the wild-type (wt) strain MB24 and AH3497 (∆murB amyE::PxyA-murB). (A/a through D/d) Cells from strain AH3497 grown in the absence of xylose (A/a) or in the presence of 0.001% xylose (B/b), 0.01% xylose (C/c), or 0.1% xylose (D/d). (E/e) MB24 cells. The cells were harvested during the exponential phase of growth and stained with the membrane dye FM4-64 to allow visualization of membranes and septa for observation by phase-contrast microscopy (A to E) and by fluorescence microscopy (a to e). Arrowheads point to bulges close to division septa. Scale bars, 2 μm.
mented in Fig. 2G, where probing of the same membranes with an anti-MurB and with an anti-RNA polymerase/h9252/h11032 subunit antibody confirmed equivalent loadings. The results show that the in-frame deletion of murB has no impact on the levels of DivIB, and accordingly the conditional mutant exhibited a normal growth rate at wild-type levels of MurB.

Morphology of the murB conditional mutant. To examine whether growth of AH3497 in the absence of xylose or in the presence of low levels of inducer caused alterations in cell shape, cells in mid-log-phase cultures of the conditional mutant were examined by fluorescence and phase-contrast microscopy. We found that in the absence of inducer, approximately 30% of the cells were slightly elongated and showed bulges, often at one end of the cell, close to a division septum (Fig. 3A and a, arrowheads). Occasionally, what looked like empty vesicles bulged out from the cells, and many cells were lysed (data not shown). Induction of the xylose promoter with 0.001% xylose reduced the frequency of bulge formation to 17%, and when these appeared (Fig. 3B and b, arrowheads), they were not as pronounced as in the absence of inducer. Cell swelling has been observed for other B. subtilis mutants with lesions affecting the expression of genes essential for PG biosynthesis. For example, low levels of expression of the rodA morphogene, essential for cell elongation, causes swelling and lysis (30), as does inactivation of the genes coding for PBP2a and PBPH, which are partially redundant functional homologues of the E. coli elongation-specific PBP2 (11, 57, 58).

Xylose concentrations of 0.0025% (not shown) or higher restored normal length and cell shape to the murB conditional mutant AH3497 (Fig. 3C through d). Addition of 0.01% xylose to cells growing in the absence of inducer restored normal cell morphology, suggesting that the cell envelope deficiency could be overcome if expression of murB was restored (Fig. 3C through d). The average cell length of the population in the presence of 0.01% (3.12 ± 0.75) or 0.1% (3.26 ± 0.65) xylose, concentrations at which no alteration of cell shape was noticed, did not significantly differ from that of a wild-type population (3.55 ± 0.88). We infer from these results that expression of murB is required for normal cell shape. Also, in agreement with earlier work (10, 30, 33, 56, 57), the results also suggest a correlation between a normal growth rate and the acquisition of rod-shaped morphology by B. subtilis cells.

Cells depleted for MurB show increased susceptibility to antibiotics. Strains engineered for low-level expression of single essential genes, and thus rendered hypersensitive to inhibitors of the corresponding gene product, have been used in screens for new antimicrobial compounds (13). No MurB inhibitors with antimicrobial activity were available to us (49), but we tested whether MurB depletion could cause hypersensitivity to several other cell wall-active antibiotics. The tests were performed on gradient plates (2, 7) containing the antibiotics fosfomycin, D-cycloserine, oxacillin, vancomycin, and cephalosporin, supplemented with increasing concentrations of xylose (from 0.001 to 0.1%; see Materials and Methods). Under these conditions, the extent of the zone of growth is a function of the resistance level of the strain (Fig. 4A), and the MIC is defined as the lowest concentration of antibiotic that prevents growth (37). The results in Fig. 4B show that while...
xylose did not influence the resistance of the wild-type strain MB24, the murB conditional mutant AH3497 was more susceptible to the antibiotics fosfomycin, oxacillin, D-cycloserine, and cephalosporin at all the concentrations of xylose tested. However, while the resistance to oxacillin and cephalosporin clearly augmented in parallel with the increase in xylose concentration in the medium, the resistance of AH3497 to fosfomycin and D-cycloserine was much less pronounced. Also, even at a xylose concentration of 0.1%, which permits the accumulation of MurB to levels almost 15 times higher than in the wild type (Fig. 2E; also see above), wild-type levels of resistance were never achieved (Fig. 4A and B). In contrast, the murB conditional mutant was found to be less susceptible to vancomycin, chloramphenicol, or tetracycline at any xylose concentration tested (data not shown). Fosfomycin is an inhibitor of MurA, the UDP-GlcNAc enolpyruvyl transferase that catalyzes the first committed step in the synthesis of PG, and acts just upstream of MurB in the pathway (16, 49). MurA and MurB act sequentially to convert UDP-GlcNac into UDP-MurNAc. D-Cycloserine is a competitive inhibitor of alanine racemase (Alr) and of d-alanine-d-alanine ligase (Ddl) (16, 49). Alr and Ddl act sequentially to produce d-aladala, which is then added to UDP-MurNAc tripeptide by the MurF lyase (16, 49). Oxacillin and cephalosporin are β-lactam antibiotics that interfere with PG synthesis by inhibiting the final transpeptidation needed for the cross-linking of the PG molecules (49). Thus, a reduction in the level of expression of the murB gene does not cause a generalized hypersensitivity of the cells to antibiotics.

Rather, the enzymatic steps catalyzed by MurA, and by Alr or Ddl in the cytoplasm, and the transpeptidation step that takes place at the cell wall become more sensitive to inhibition by specific antibiotics, when the level of expression of murB is altered.

MurB levels throughout sporulation. Formation of heat-resistant spores involves the synthesis of a layer of modified PG known as the spore cortex (3, 18, 43). Synthesis of the spore cortex is likely to involve most of the vegetative components of the PG-synthesizing machinery, although it also requires the expression of several sporulation-specific genes (3, 18). Here, we used an anti-MurB antibody to examine the profile of accumulation of MurB during sporulation. For that we grew the wild-type strain MB24 in DSM medium, in which sporulation is induced by nutrient exhaustion, and took samples during growth, at the onset of the stationary phase of growth (defined as the initiation of sporulation), and at various intervals thereafter for immunoblot analysis. The results in Fig. 5B show that the levels of MurB appeared to decrease as cells approach the end of the exponential phase of growth in DSM and that the decline in MurB levels persisted until about 1 h after the onset of sporulation (Fig. 5B). The levels of MurB then appear to steadily increase until at least hour 6 of sporulation (Fig. 5B). Synthesis of the spore cortex follows the activation of the late mother cell regulator σK, which takes place around hour 4 of sporulation (17, 18, 43). Thus, the levels of MurB appear to coincide with the time of synthesis of the spore cortex. The increase in the level of MurB during sporulation is consistent with the results of a recent study suggesting that the transcrip-
tion of both murG and murB is enhanced during sporulation, presumably from the sporulation-specific spoVE promoter (14, 15) (Fig. 1). Presumably, increased transcription of murB is required to increase the levels of MurB at the time of cortex formation.

We then examined the impact of varying the expression level of murB from the PsylA promoter on the efficiency of sporulation in DSM. Under the conditions used, the Spo+ strain MB24 produced 6.7 × 10^8 to 7.0 × 10^8 spores per ml of culture, independently of the level of xylose present in the sporulation medium (Table 3). In the absence of xylose, AH3497 (ΔmurB PsylA-murB) was still able to grow, albeit slower than the wild type, entered stationary phase at an OD_600 slightly lower than a culture of the wild type (Fig. 5A), and formed only about 10^4 spores per ml of culture (Table 3). At xylose concentrations of 0.0005% or higher (up to 0.1%), AH3497 grew slower than the wild type in DSM, but at xylose concentrations of 0.0025% or higher (up to 0.1%), AH3497 showed a wild-type growth rate in DSM (Fig. 5A). However, at all xylose concentrations tested, AH3497 was impaired in the formation of heat-resistant spores, with titers in the range of 10^4 per ml of concentrations tested, AH3497 was impaired in the formation type growth rate in DSM (Fig. 5A). However, at all xylose concentrations of 0.0025% or higher (up to 0.1%), AH3497 showed a wild-type growth rate in DSM, but at xylose concentrations of 0.0005% or 0.001%, AH3497 grew slower than the wild type, entered stationary phase at an OD_600 slightly lower than a culture of the wild type (Fig. 5A), and formed only about 10^4 spores per ml of culture (Table 3). At xylose concentrations of 0.0005% or 0.001%, AH3497 grew slower than the wild type in DSM, but at xylose concentrations of 0.0025% or higher (up to 0.1%), AH3497 showed a wild-type growth rate in DSM (Fig. 5A). However, at all xylose concentrations tested, AH3497 was impaired in the formation of heat-resistant spores, with titers in the range of 10^4 per ml of culture (Table 3). The reduced sporulation efficiency was not caused by addition of xylose, because a murB’ strain (AH1745) harboring an extra copy of murB under the control of PsylA at amyE sporulated at wild-type levels regardless of the xylose concentration in the medium (Table 3).

To test whether the failure of AH3497 to sporulate efficiently could be due to insufficient levels of MurB, samples of DSM cultures in the absence or in the presence of 0.1% xylose (the highest concentration tested) were collected throughout sporulation, and the levels of MurB were analyzed by immunoblot analysis. The results in Fig. 5E show that no MurB could be detected throughout sporulation of AH3497 in DSM in the absence of inducer. In the presence of 0.1% xylose, however, the levels of MurB at the onset of sporulation (T0) were much higher than for the wild type and remained higher than for the wild type throughout sporulation (Fig. 5F). Therefore, the failure of AH3497 to sporulate efficiently in the presence of 0.1% xylose is not due to reduced levels of MurB during sporulation. Conversely, the sporulation phenotype of AH3497 also does not appear to be due to increased levels of MurB. We base this inference on the observation that in AH3497 (murB’ PsylA-murB) grown in the presence of 0.1% xylose, because a murB’ strain (AH1745) harboring an extra copy of murB under the control of PsylA at amyE sporulated at wild-type levels regardless of the xylose concentration in the medium (Table 3).

AH1745 (murB’ PsylA-murB) grown in the presence of 0.1% xylose (a condition under which the strain sporulates efficiently; Table 3), the levels of MurB during sporulation were also elevated relative to the wild type (Fig. 5D), at least to the same extent of the increase in AH3497 (Fig. 5F). We propose that the sporulation phenotype of AH3497 is due to the ectopic expression of murB in this strain.

**DISCUSSION**

In an extension of earlier work (46), the results herein presented indicate that expression of the murB gene is essential for normal growth and cell shape in *B. subtilis*. We base this conclusion on the analysis of two different murB conditional mutants, in which the only functional copy of the gene was placed under the control of the xylose-inducible PsylA promoter at the nonessential amyE locus. In one mutant (AH1746), the murB gene present in the *dcw* cluster was insertionally inactivated. In the second (AH3497), an in-frame deletion was introduced into *murB* in the *dcw* cluster. The two strains exhibited slow growth in the absence of inducer and greatly altered cell...
morphology, and their growth rate was proportional to the level of murB expression.

However, the two strains showed different requirements for MurB for normal growth in liquid medium. The murB::neo insertional mutant AH1746 required a level of MurB at least three times higher than a congenic wild-type strain for normal growth. The mutant also showed a slight reduction in the level of DivIB, encoded by the downstream divIB gene, although not sufficient to perturb normal cell division. However, a second copy of the divIB gene permitted normal growth at wild-type levels of MurB, suggesting that DivIB somehow contributes to the normal growth of the murB conditional mutant. In agreement with this suggestion, the conditional mutant AH3497, in which the murB gene at the dcw cluster was deleted in frame, showed normal levels of DivIB and a normal growth rate at wild-type levels of MurB. Separation of murB from divIB by an integrational plasmid resulted in a strain with reduced levels of DivIB but essentially normal cell division and with a slow growth phenotype (45). The reason for this slow growth phenotype was not clear, but it was not due to a polar effect on genes located downstream of divIB, and while a copy of divIB in trans corrected the slow growth phenotype of the mutant, an extra copy of the murB gene aggravated the phenotype (45). This suggested that at least in this strain, a reduction in the levels of DivIB may not be accompanied by a reduction in the levels of MurB. In any case, these observations suggested that murB and divIB have to be coordinately expressed. Cell division mutants have been reported to show a normal growth rate before cell lysis occurs. However, the function of divIB may not be restricted to cell division (45). The suggestion that coordinated expression of murB and divIB is important for normal growth is in agreement with the presence of the two cistrons in long polycistronic messages that transverse most of the dcw cluster of B. subtilis during growth (25) and with the observation that the spoVE-murG-murB-divIB unit within the dcw cluster appears to be unique to spore formers of the genus Bacillus. It could be that in Bacillus species, the coordination between synthesis of cell wall precursors and cell division is exerted at least in part at the level of transcription of the murG-murB-divIB unit (see also below).

Synthesis of the spore cortex PG is essential for spore heat resistance (3, 17, 18). The spore cortex is formed between the two membranes that surround the prespore at an intermediate stage of development, from precursors that may be produced mainly in the mother cell (3, 17, 18). The activity of the sporulation-specific σE factor is confined to the mother cell and is required for transcription of several genes involved in synthesis of the spore cortex PG (e.g., see references 9 and 53). However, synthesis of the spore cortex commences only following the complete engulfment of the prespore by the mother cell, with the concomitant activation of the late mother cell regulator σK (3, 17, 18, 43). Activation of σK takes place around hour 4 of sporulation, and synthesis of the spore cortex ensues, in parallel with the appearance of bright-phase spores (39). It seems that expression of murB during sporulation is required for synthesis of the spore cortex. Increased transcription of the murG and murB genes is seen during sporulation (15), presumably from the σE-dependent promoter of the upstream spoVE gene (53), and the levels of MurB increase coincidentally with the period of cortex formation (this work).

Surprisingly, in cells with murB absent from the dcw cluster, expression of the PsyLA-murB inducible allele from an ectopic position drastically reduced the frequency of heat-resistant spores and resulted in the accumulation of over 99% dark-

FIG. 6. MurB depletion causes the production of dark-phase spores. The figure depicts spores produced by the wild-type strain MB24 (A) and the murB conditional mutant AH3497 (ΔmurB amyE::PsyLA-murB) (B through F) in DSM medium in the absence of xylose (A and B) or in the presence of the following xylose concentrations: 0.001% (C), 0.005% (D), 0.01% (E), 0.1% (F). The spores were collected and purified as described in Material and Methods and visualized under phase-contrast microscopy. Open and filled arrowheads point to free spores or to spores still inside the mother cell, respectively. Bars, 2 μm.
phase spores, a sign of incomplete cortex formation (29, 43). Remarkably, ectopic expression of PylA-murB in cells that kept murB at its normal position within the dcw cluster did not interfere in any detectable way with the frequency of sporulation of the formation of refractile spores. Hence, displacement of the murB gene from its normal location in the dcw cluster appears detrimental for cortex formation during sporulation. This is in keeping with the idea that the spoVE-murG-murB-divIB unit within the dcw region, which is confined to Bacillus spore formers (Fig. 1), is of biological importance for this group of organisms. Both spoVE and murG appear to be involved in the membrane-linked steps of PG synthesis (4, 31). It is tempting to speculate that the clustering of murG, murB, and divIB would contribute to the coordination between synthesis of PG precursors and cell division during the vegetative life cycle and that linkage of at least the murG-murB unit to spoVE would coordinate PG precursor synthesis to their translocation across the spore membrane for the formation of the spore cortex.

Assembly of the PG molecule involves the sequential addition of l-alanine, d-glutamic acid, diaminopimelic acid, and d-alanyl-d-alanine to the UDP-MurNAc. The observation that the murB conditional mutant (AH3497) is more sensitive to d-cycloserine, an inhibitor of the d-alanine racemase (Alr) and of d-alanyl-d-alanine ligase (Ddl), suggests that a reduction in the efficiency of the MurB-catalyzed step of the PG biosynthetic pathway will lead to a rapid depletion of downstream precursors, and as a consequence it will make these cells more sensitive to any antibiotic acting downstream of that step. At suboptimal concentrations of inducer, the increased susceptibility of the mutant to d-cycloserine and to the β-lactam antibiotics oxacillin and cephalosporin suggests that its cell wall may accumulate tripeptide muropeptides. If so, a further decrease in cross-linking caused by β-lactam antibiotics could compromise the structural stability of the cell wall. That resistance to the β-lactams cephalexin and oxacillin increases with increased expression of murB is reminiscent of the situation with a Pspac-murE conditional mutant of S. aureus (19). At suboptimal levels of inducer, the mutant shows increased susceptibility to oxacillin (19).

In B. subtilis, cell wall-active antibiotics induce the extracytoplasmic sigma factors σW and σM possibly via a chemical or physical signal related to a defect in the structure or composition of the cell wall (5, 52). Significantly, vancomycin, which acts as a potent inducer of sigW, and of a large number of σW-dependent genes, results in induction of the contiguous murG, murB, divIB, and yakW genes (5). We note that the murB conditional mutant is not more susceptible to vancomycin. However, a sigW mutant is not significantly affected in its sensitivity to vancomycin (5). It is possible that in B. subtilis, increased expression of murB in the conditional mutant somehow induces expression of the genes for specific PBPs. However, the pool of soluble PG precursors, or the composition of the cell wall in the murB conditional mutant, has not yet been examined, and it is unknown whether σW is induced in the PylA-murB mutant.

The murB conditional mutant is also more sensitive to fosfomycin, an inhibitor of MurA (16, 49). MurA catalyzes the step immediately upstream of MurB, the production of UDPGlcNAcEP. In vitro experiments have shown that the activity of MurA is inhibited by its reaction product and indicated that this reaction is an important control step in the PG biosynthetic pathway (40). Increased susceptibility to fosfomycin under conditions where MurB levels are low could be caused by accumulation of UDPGlcNAcEP and inhibition of MurA, or simply to the combined effect of inhibition of two consecutive steps in the pathway. In any event, because different steps in the PG biosynthetic pathway appear to be more sensitive to known antibiotics in a way that depends on the expression of murB, we suggest that the murB conditional mutant could be used as a tool in screening for new antibacterial compounds that act at the level of cell wall biogenesis (13).

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