

Peptidoglycan Recycling in Gram-Positive Bacteria Is Crucial for Survival in Stationary Phase

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ABSTRACT Peptidoglycan recycling is a metabolic process by which Gram-negative bacteria reutilize up to half of their cell wall within one generation during vegetative growth. Whether peptidoglycan recycling also occurs in Gram-positive bacteria has so far remained unclear. We show here that three Gram-positive model organisms, *Staphylococcus aureus*, *Bacillus subtilis*, and *Streptomyces coelicolor*, all recycle the sugar *N*-acetylmuramic acid (MurNAc) of their peptidoglycan during growth in rich medium. They possess MurNAc-6-phosphate (MurNAc-6P) etherase (MurQ in *E. coli*) enzymes, which are responsible for the intracellular conversion of MurNAc-6P to *N*-acetylglucosamine-6-phosphate and *D*-lactate. By applying mass spectrometry, we observed accumulation of MurNAc-6P in MurNAc-6P etherase deletion mutants but not in either the isogenic parental strains or complemented strains, suggesting that MurQ orthologs are required for the recycling of cell wall-derived MurNAc in these bacteria. Quantification of MurNAc-6P in Δ *murQ* cells of *S. aureus* and *B. subtilis* revealed small amounts during exponential growth phase (0.19 nmol and 0.03 nmol, respectively, per ml of cells at an optical density at 600 nm [OD₆₀₀] of 1) but large amounts during transition (0.56 nmol and 0.52 nmol) and stationary (0.53 nmol and 1.36 nmol) phases. The addition of MurNAc to Δ *murQ* cultures greatly increased the levels of intracellular MurNAc-6P in all growth phases. The Δ *murQ* mutants of *S. aureus* and *B. subtilis* showed no growth deficiency in rich medium compared to the growth of the respective parental strains, but intriguingly, they had a severe survival disadvantage in late stationary phase. Thus, although peptidoglycan recycling is apparently not essential for the growth of Gram-positive bacteria, it provides a benefit for long-term survival.

IMPORTANCE The peptidoglycan of the bacterial cell wall is turned over steadily during growth. As peptidoglycan fragments were found in large amounts in spent medium of exponentially growing Gram-positive bacteria, their ability to recycle these fragments has been questioned. We conclusively showed recycling of the peptidoglycan component MurNAc in different Gram-positive model organisms and revealed that a MurNAc-6P etherase (MurQ or MurQ ortholog) enzyme is required in this process. We further demonstrated that recycling occurs predominantly during the transition to stationary phase in *S. aureus* and *B. subtilis*, explaining why peptidoglycan fragments are found in the medium during exponential growth. We quantified the intracellular accumulation of recycling products in MurNAc-6P etherase gene mutants, revealing that about 5% and 10% of the MurNAc of the cell wall per generation is recycled in *S. aureus* and *B. subtilis*, respectively. Importantly, we showed that MurNAc recycling and salvaging does not sustain growth in these bacteria but is used to enhance survival during late stationary phase.

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Peptidoglycan (PGN) encases the bacterial cell, forming a huge, netlike, turgor-resisting and shape-maintaining envelope structure that is composed of glycan strands of two alternating β -1,4-linked sugars, *N*-acetylglucosamine (GlcNAc) and *N*-acetylmuramic acid (MurNAc), cross-linked by short peptides (1). The PGN is remarkably dynamic and is constantly remodeled, degraded, and rebuilt during bacterial growth and cell division (2, 3). As an inherent part of this process, a significant portion of the PGN is continuously excised from the cell wall by the activity of endogenous lytic enzymes (autolysins) and released into the me-

di-um in a process termed “turnover” (3–7). The reason for the continuous degradation of the PGN is still unclear, but apparently, a minimal set of autolytic enzymes is essential for bacterial growth, and PGN turnover may be intrinsically coupled with cell elongation and division (2, 5). Early studies showed that the Gram-negative bacterium *Escherichia coli* breaks down about half of its PGN within one generation during exponential growth (8, 9). Gram-negative bacteria possess a thin PGN layer embedded within an inner and an outer membrane, and thus, PGN turnover products are mostly retained within the periplasm, from where

they are instantly recovered and salvaged (7, 8). Thus, only small amounts of PGN turnover products were found in the medium, while about 45% of the mature PGN was recovered in one generation in a process called cell wall or PGN recycling (8, 9). Uehara and Park later revealed that *E. coli* continuously recycles about 30% of newly synthesized septal PGN during cell division, and they calculated that about 60% of the PGN of the sidewall is recycled, taking into account that the PGN of the cell poles is basically inert (10). To quantify PGN turnover and recycling in *E. coli*, cell walls were radioactively prelabeled and the amounts of radioactive soluble products that were released into the medium or accumulated in recycling mutants were determined (8–10).

PGN recycling is not essential, at least under laboratory conditions, and why this pathway was kept on the chromosomes of most bacteria remained enigmatic (7). The PGN recycling metabolism of Gram-negative bacteria had attracted much attention due to a connection with AmpC-type β -lactamase induction (9) and, more recently, fosfomycin antibiotic resistance (11, 12). It is now reasonably well understood (for reviews, see references 3, 7, 13, and 14). In brief, the major cell wall-recycling products of Gram-negative bacteria are anhydromuropeptides (GlcNAc- β -1,6-anhydroMurNAc peptides) that are generated by the action of endogenous lytic transglycosylases and endopeptidases (autolysins), which in part are constituents of the huge cell wall synthetic complexes of elongation and division (2, 15). Anhydromuropeptides are actively transported into the cell by the AmpG permease and are further degraded in the cytoplasm by a mechanism that involves a set of dedicated recycling enzymes, mostly discovered and characterized in the seminal work of J. T. Park and coworkers (for reviews, see references 3 and 7). The recycling enzymes *N*-acetylglucosaminidase NagZ, L,D-carboxypeptidase LdcA, *N*-acetylmuramyl-L-alanine amidase AmpD, and anhydroMurNAc kinase AnmK process anhydromuropeptides in the cytoplasm, finally yielding MurNAc-6-phosphate (MurNAc-6P), besides other products. In addition, MurNAc-6P is the product of MurNAc uptake and concomitant phosphorylation by the specific phosphotransferase system (PTS) transporter MurP, allowing *E. coli* to grow on MurNAc as a sole source of carbon (16). A distinctive recycling enzyme that *E. coli* requires in order to catabolize MurNAc, as well as anhydroMurNAc, is the MurNAc-6P etherase MurQ (17, 18). This enzyme cleaves off the lactyl ether substituent from the phosphorylated form of MurNAc, yielding GlcNAc-6-phosphate (GlcNAc-6P) and D-lactate. The *murQ* operon of *E. coli* strain K-12 consists of three genes, encoding the MurNAc-6P etherase MurQ (17), the MurNAc transporter MurP (PTS EII-BC domain) (16), and a low-affinity penicillin binding protein named PBP4B (19) (Fig. 1). The transcriptional regulator MurR is transcribed divergently from the *murQ* operon and functions as a repressor in the absence of MurNAc-6P (20). Orthologs of *murQ* are missing in some Gram-negative bacteria, including *Pseudomonas* species (11), which instead use an alternative MurNAc-6P recycling route that bypasses the *de novo* PGN biosynthesis pathway (11, 12, 21).

It has long been assumed that large amounts of PGN fragments are shed from the cell wall of Gram-positive bacteria during vegetative growth and released into the growth medium (5, 22–24). However, whether these fragments are taken up and recycled in Gram-positive bacteria is currently unclear. Gram-positive bacteria have a thick PGN layer and lack an outer membrane, and hence, PGN turnover products are lost to the medium as they

cannot be trapped in the periplasm as in Gram-negative bacteria. Applying continuous radioactive labeling and pulse-chase labeling approaches, the amount of PGN breakdown was measured by determining the radioactivity found in the growth medium relative to that remaining in the insoluble cell wall material. These studies came up with rather inconsistent rates of PGN release in Gram-positive bacteria during growth, ranging from no turnover to up to 50% turnover per generation (5, 22–25). The first reports revealed PGN turnover in the Gram-positive bacterium *Bacillus megaterium*, i.e., the release of PGN fragments into the growth medium (4), at a rate of 15 to 20% turnover per generation by measuring the loss of radiolabeled diaminopimelic acid from the prelabeled cell wall. Later, Mauck et al. calculated a rate of 30 to 50% PGN turnover per generation in *Bacillus subtilis* based on labeling studies using [¹⁴C]glucosamine and [¹⁴C]glutamine (22). *Staphylococcus aureus* strains were also described to turn over their cell wall at constant rates of up to 25% per generation (26) or about 15% per generation (27) during growth. Thus, the reported turnover rates differ to a large extent. Notably, Pooley emphasized that turnover rates determined with pulse-chase experiments often result in an overestimation of the amount of turnover, since there is a time lag between PGN synthesis and PGN degradation (25). He argued that the overall rate of turnover of the cell wall in *B. subtilis* is more likely about 8% per generation and is maintained by an approximately sixfold-higher rate of turnover of a small fraction, the old cell wall, while the major part, the newer wall, is initially resistant to turnover (25). Contradictory data, however, reported no or only very limited turnover in *B. megaterium* (28), as well as in *Streptococcus* bacteria (23). Recently, Boersma et al. confirmed that in the ovococcal *Streptococcus pneumoniae*, only minimal PGN turnover proceeds, indicated by the persistence of fluorescent D-amino acid label (29).

Still, it has not been investigated so far whether turnover fragments are recovered from the culture supernatant and recycled by Gram-positive bacteria (see reference 24 and references therein). We have previously identified putative PGN recycling genes on the chromosomes of Gram-positive bacteria and have characterized some of the encoded enzymes (24, 30). In particular, we recognized the general presence of *murQ* orthologs on the chromosomes of Gram-positive bacteria (11, 20), indicating that MurNAc recovery may occur in these organisms (Fig. 1). However, a clear proof of cell wall recycling during vegetative growth of Gram-positive bacteria is still missing, and the role of MurNAc-6P etherase enzymes in this process remains unclear. Thus, we constructed markerless MurNAc-6P etherase gene deletion mutants of the three Gram-positive model organisms *B. subtilis*, *S. aureus* and *Streptomyces coelicolor* and investigated the intracellular accumulation of MurNAc-6P in these strains during different growth phases by using mass spectrometry—hence, without the necessity to radioactively label the cell wall. Our study provides clear evidence for MurNAc recycling, i.e., the release of the sugar from the cell's own peptidoglycan wall and its uptake/scavenging, occurring predominantly during the transition to stationary growth phase. Strikingly, PGN does not affect the growth rates of *S. aureus* and *B. subtilis* but is crucial for their survival during stationary phase.

RESULTS

Identification of putative MurNAc-recycling gene clusters in Gram-positive bacteria. The organization of genomic *murQ* re-

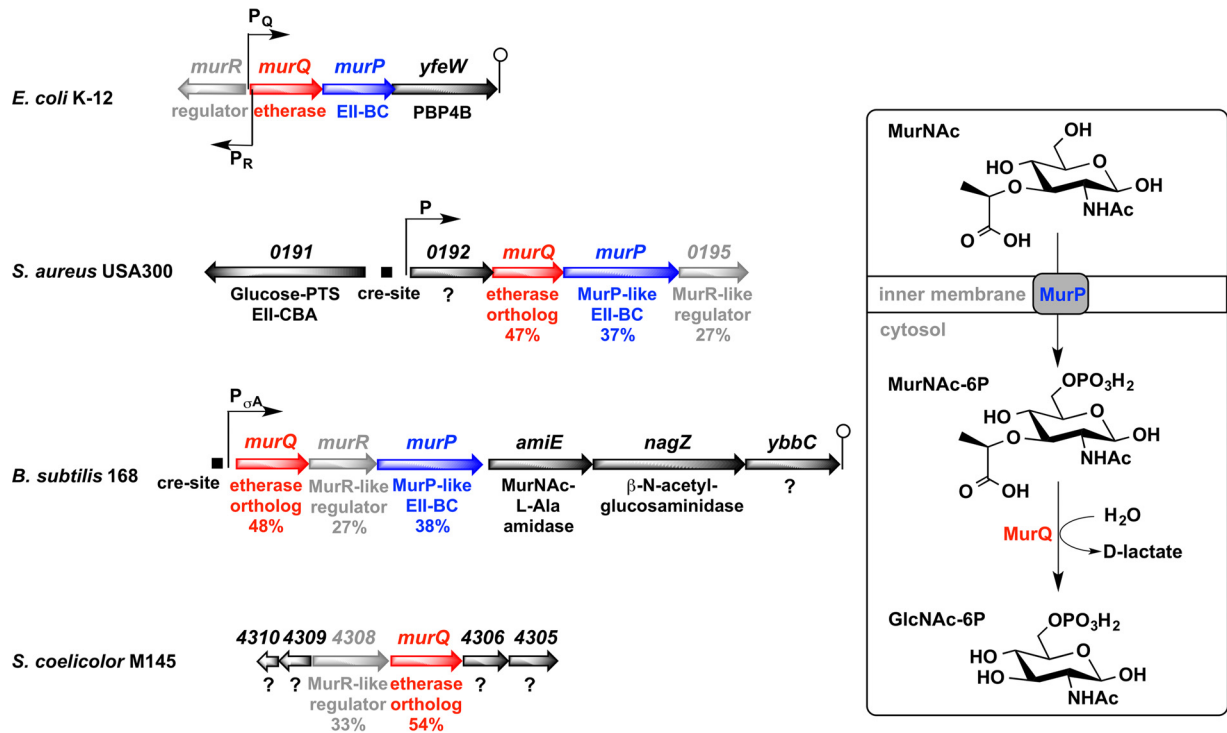


FIG 1 MurQ operon (MurNac-recycling divergon) and MurNac catabolic pathway in *E. coli* (top and right, respectively), and organization of chromosomal regions of *murQ* orthologs of the Gram-positive bacteria *S. aureus*, *B. subtilis*, and *S. coelicolor*. The schematic of the organization of the *E. coli* K-12 *murQ* operon genes includes the promoters for transcription of the *murQ* operon (P_Q) and the terminator (lollipop), according to Jaeger and Mayer (20). *murQ* encodes MurNac-6P etherase, *murP* encodes the MurNac-specific phosphotransferase system (PTS) transporter EII-BC domain, and *yfeW* encodes the low-affinity penicillin binding protein 4B (PBP4B). Upstream from the *murQ* gene and divergently transcribed is the *murR* gene, a transcriptional repressor of the MurNac recycling divergon. The schematic for *S. aureus* USA300 (NCBI Reference Sequence accession no. NC_007793.1) shows putative genes for MurNac utilization, as well as the proteins they encode. SAUSA300_0192 encodes a protein whose function is unknown, SAUSA_0193 encodes an ortholog of MurQ, SAUSA_0194 encodes a MurP-like PTS EII-BC domain protein, and SAUSA_0195 encodes a MurR-like regulator. The schematic for *B. subtilis* 168 (NCBI Reference Sequence accession no. NC_000964.3) shows the putative promoter $P_{\sigma A}$ in front of the recycling cluster of 6 genes, including *murQ* (formerly *ybbI*), *murR* (formerly *ybbH*), *murP* (formerly *ybbF*), encoding the MurP EII-BC domain, *amiE* (formerly *ybbE*), encoding the MurNac-L-alanine amidase AmiE, *nagZ* (formerly *ybbD*), encoding the N-acetylglucosaminidase NagZ, and *ybbC*, encoding a protein whose function is unknown. The schematic for *S. coelicolor* A3(2)/M145 (NCBI Reference Sequence accession no. NC_003888.3) shows a putative cluster of genes for MurNac recycling that includes SCO4308, encoding a MurR-like regulator, and SCO4307, encoding an ortholog of MurQ, as well as two open reading frames encoding proteins whose functions are unknown, SCO4305 and SCO4306. The amino acid sequence identities (%) of orthologous proteins relative to the sequences of *E. coli* MurQ, MurP, and MurR are shown. Catabolite-responsive elements (*cre* sites) were identified in the promoter regions upstream from the *murQ* genes of *B. subtilis* and *S. aureus*.

regions of selected Gram-positive bacteria, as well as of *E. coli*, and a schematic representation of the MurNac catabolic pathway in *E. coli* are depicted in Fig. 1. On the chromosome of the *S. aureus* USA300 isolate FPR3757 (hereinafter referred to as USA300), we identified orthologs of *murQ* (SAUSA300_0193), *murP* (SAUSA300_0194), and *murR* (SAUSA300_0195) of *E. coli*, organized in a putative operon along with SAUSA300_0192, whose function is unknown. SAUSA300_0193–0195 encode proteins with 47%, 37%, and 27% amino acid sequence identities to the respective *E. coli* proteins, based on analysis of the full-size proteins using the basic local alignment search tool (BLAST). A putative MurNac-recycling operon was also identified on the genome of *B. subtilis* strain 168, containing the genes *murQ*, *murR*, and *murP* (formerly *ybbI*, *ybbH*, and *ybbF*, respectively), which encode proteins with amino acid sequence identities of 48%, 27%, and 38% to the respective *E. coli* enzymes. Downstream from *murQ*, three further genes are located on the *B. subtilis* chromosome, *amiE* (formerly *ybbE*), *nagZ* (formerly *ybbD*), and *ybbC*, which encodes a protein of unknown function and has not been renamed. The first two genes encode the N-acetylmuramyl-L-alanine amidase AmiE and

N-acetylglucosaminidase NagZ of *B. subtilis*, which were shown to be involved in the sequential extracellular degradation of mucopeptides released during PGN turnover (30). On the genome of *S. coelicolor* strain A3(2)/M145, genes encoding an ortholog of the MurQ etherase (SCO4307; 54% amino acid identity to MurQ of *E. coli*) and a putative MurR-like regulator (SCO4308; 33% identity to MurR of *E. coli*) were identified by BLAST analysis; however, an ortholog of MurP is missing. These genes are organized in a putative operon along with the genes SCO4305 and SCO4306, which encode proteins whose functions are unknown (Fig. 1).

As putative orthologs of the MurNac-6P etherase MurQ of *E. coli* are present in all three Gram-positive bacteria and as *S. aureus* and *B. subtilis* also possess putative orthologs of the MurNac-PTS transporter MurP of *E. coli*, we assumed that MurNac can be catabolized and possibly recycled in these organisms according to the *E. coli* pathway depicted in Fig. 1.

MurNac-6P accumulation in Δ murQ mutants. We constructed markerless in-frame deletion mutations of the respective MurNac-6P etherase genes (referred to as Δ murQ mutants for all three species hereinafter) in *S. aureus* USA300, *B. subtilis* 168, and

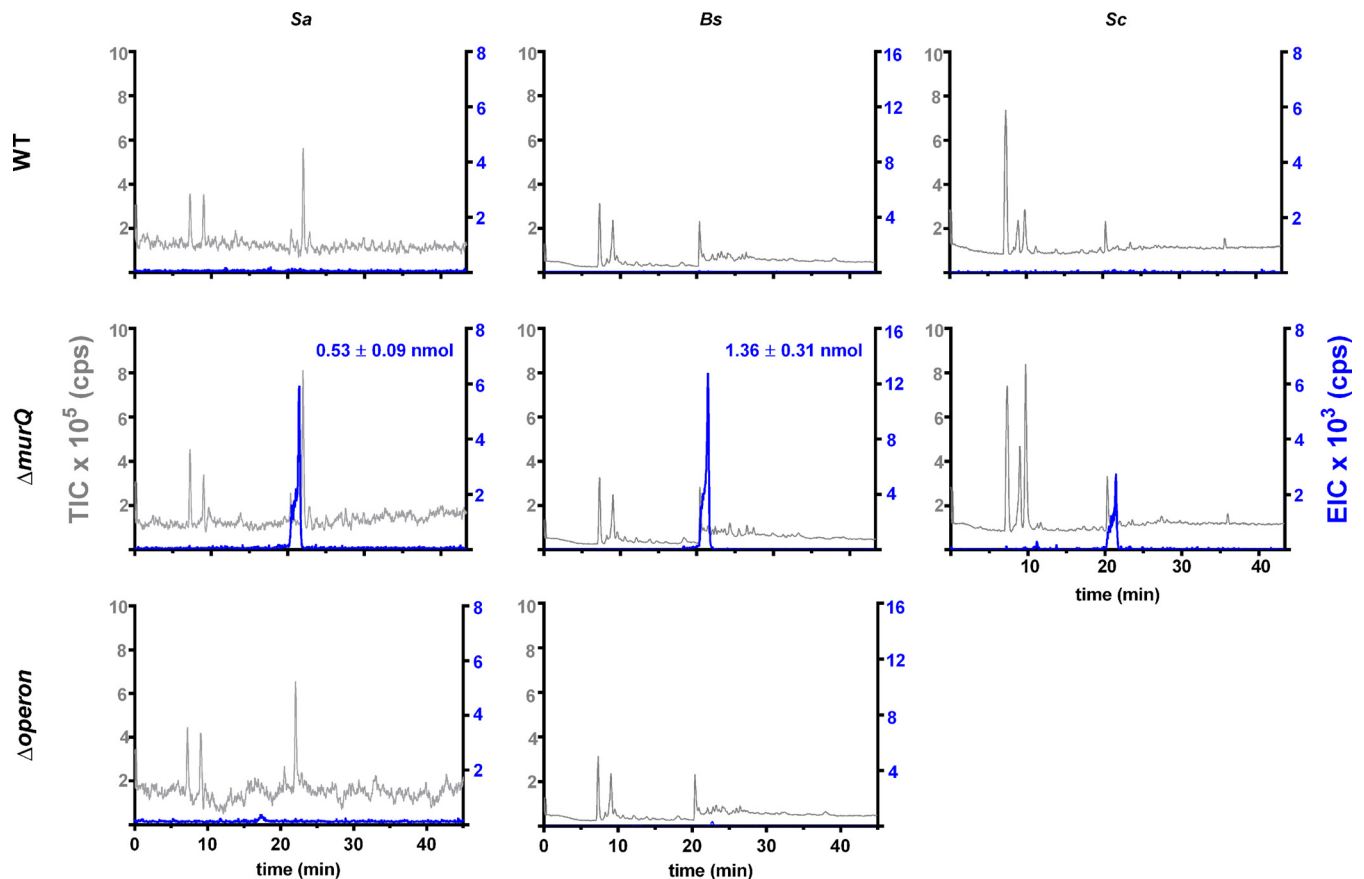


FIG 2 Accumulation of MurNac-6P in $\Delta murQ$ mutants of Gram-positive bacteria but not in the parental strains. *S. aureus* (Sa), *B. subtilis* (Bs), and *S. coelicolor* (Sc) wild-type strains (WT) and $\Delta murQ$ mutants, as well as the *S. aureus* $\Delta SAUSA_0192-0195$ and *B. subtilis* $\Delta murQRP$ mutants ($\Delta operon$), were cultured in LB medium for 24 h. Acetone extracts of cytosolic fractions were analyzed by LC-MS in negative-ion mode. Mass spectra of MurNac-6P in the investigated samples are presented with the total-ion chromatograms (TIC) ($\times 10^5$ counts per s [cps]) in gray and the extracted-ion chromatograms (EIC) ($\times 10^3$ cps) ($m/z^{-1} = 372.07$ and retention time on the HPLC column of 21 min) in blue.

S. coelicolor strain M145 to investigate whether these Gram-positive bacteria recycle MurNac derived from their own PGN, involving MurNac-6P etherase. We expected that if recycling of the MurNac content of their PGN occurred, $\Delta murQ$ cells would accumulate MurNac-6P intracellularly. In addition, we generated an *S. aureus* USA300 deletion mutant lacking the entire putative *murQPR* operon ($\Delta SAUSA_0192-0195$) and, in addition, a *B. subtilis* 168 $\Delta murQRP$ deletion mutant. Both of these mutants lack *murQ* and the genes encoding the putative MurNac PTS transporters. Thus, these mutants should not be able to take up MurNac released from the PGN wall and therefore should not accumulate MurNac-6P. Gene deletions on the chromosome were confirmed by PCR (see Fig. S1 in the supplemental material) and sequencing.

At first, we investigated the intracellular accumulation of MurNac-6P in cells grown for 24 h in nutrient-rich medium (LB medium). Cytosolic fractions were obtained by disrupting the cells with glass beads and were subsequently extracted with acetone prior to analysis by liquid chromatography-mass spectrometry (LC-MS). In the extracts of all $\Delta murQ$ mutants of *S. aureus*, *B. subtilis*, and *S. coelicolor*, we detected MurNac-6P (Fig. 2), which was identified based on identity with a standard according to retention time during high-performance liquid chromatogra-

phy (HPLC) separation and on the exact mass (retention time on the HPLC column of 21 min and m/z^{-1} of 372.07) (see Fig. S2 in the supplemental material). In contrast, MurNac-6P was not detected in any corresponding parental strain and, notably, not in *S. aureus* $\Delta murQPR$ and *B. subtilis* $\Delta murQRP$ mutant cells either, indicating that the accumulation of MurNac-6P, and hence recycling, relies on both a functional MurQ etherase and the presence of the putative MurNac PTS transporter (Fig. 2). The absence of MurNac-6P accumulation in these operon mutants further indicates that the MurQP pathway is the only route for MurNac recycling via MurNac-6P in these organisms.

$\Delta murQ$ mutants have no growth defects, and the addition of MurNac does not enhance growth in rich medium. We asked whether impairment of PGN recycling and the accumulation of MurNac-6P would affect growth. Therefore, we monitored the optical density at 600 nm (OD_{600}) during growth of wild-type and $\Delta murQ$ strains of the unicellularly growing Gram-positive bacteria *S. aureus* and *B. subtilis* in LB medium. Surprisingly, the mutant strains displayed no growth deficiencies compared to the growth of the parental strains, even during incubation in stationary phase for 72 h (Fig. 3). The growth of *S. coelicolor* by apical tip extension results in multicellular mycelial pellets composed of stationary (old compartments) and actively growing (tip compart-

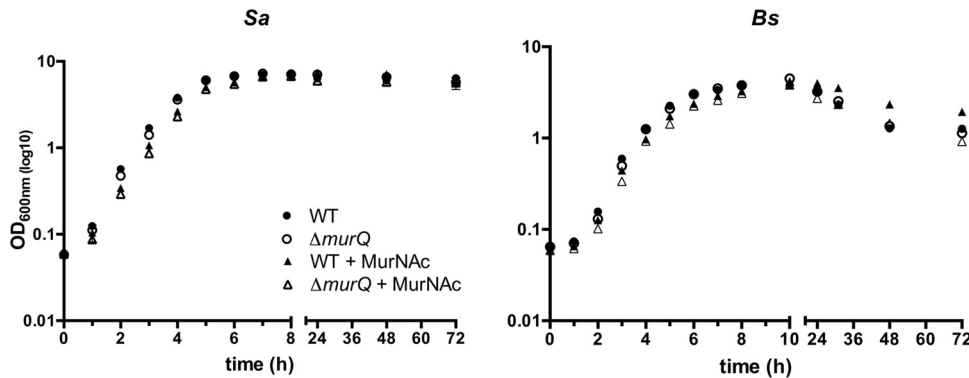


FIG 3 Growth of *S. aureus* and *B. subtilis* wild-type strains and $\Delta murQ$ mutants in rich medium with or without MurNAc. *S. aureus* (Sa) and *B. subtilis* (Bs) wild-type strains (WT, solid symbols) and the respective mutants ($\Delta murQ$, open symbols) were grown in LB medium in the absence (circles) or presence (triangles) of 0.2% MurNAc. Bacterial growth was monitored by measuring optical density at 600 nm and is presented as the mean values \pm standard errors of the means (SEM) in log₁₀ scale.

ments) mycelium. Since this precludes the separation of distinct growth phases, *S. coelicolor* was excluded from these studies.

We further asked whether MurNAc added to the medium would support growth. Therefore, 0.2% MurNAc was added to the LB medium and the growth of wild-type and $\Delta murQ$ strains of *S. aureus* and *B. subtilis* was followed during prolonged culturing by measuring the OD₆₀₀. Surprisingly, externally supplied MurNAc did not support the growth of either strain but, rather, had a weak repressive effect on growth during exponential phase. Interestingly, in late stationary phase, wild-type *B. subtilis* cells yielded a higher OD₆₀₀ in the presence of MurNAc. In contrast, wild-type *S. aureus* cells had no late-stationary-phase growth advantage in the presence of MurNAc.

Growth-phase-dependent accumulation of MurNAc-6P. We wondered in which phases of vegetative growth PGN recycling occurs. Therefore, we determined the amount of MurNAc-6P that accumulated in the *murQ* mutants in different growth phases in *S. aureus* and *B. subtilis*. The levels of MurNAc-6P accumulation were examined in extracts of the cytosolic fractions of $\Delta murQ$ mutants and parental strains (wild type) of *S. aureus* and *B. subtilis* in exponential growth phase (OD₆₀₀ of 3 and 2, respectively), transition phase (OD₆₀₀ of 7.5 and 4), and stationary phase (OD₆₀₀ of 6.5 and 3) (see Fig. S3 in the supplemental material). Quantification of the accumulation product was achieved by comparison to the MurNAc-6P standard, which was generated from MurNAc in an enzymatic synthesis reaction with MurNAc kinase from *Clostridium acetobutylicum* (31) and subsequently purified by HPLC as described elsewhere (S. Unsleber, M. Borisova, and C. Mayer, unpublished data). Dilution series of the MurNAc-6P standard were analyzed by LC-MS, and the area under the curve was determined for the product peaks (see Fig. S2 in the supplemental material) and used to quantify the amounts of recycling product that accumulated in $\Delta murQ$ mutants during the growth phases analyzed.

MurNAc-6P was absent from all wild-type samples in the three growth phases tested (Fig. 2; see also Fig. S3 in the supplemental material). The *S. aureus* $\Delta murQ$ mutant accumulated MurNAc-6P only in small amounts during exponential phase (for cell cultures with an OD₆₀₀ of 1 [OD1 cells], the amounts of MurNAc-6P were 0.19 ± 0.02 nmol/ml of OD1 cells [mean \pm standard error of the mean]); however, the amounts increased

threefold in transition phase (0.56 ± 0.1 nmol) (see Fig. S3) and stationary phase (0.53 ± 0.09 nmol), respectively (Fig. 2). Similarly, in *B. subtilis*, small amounts of MurNAc-6P were detected in the $\Delta murQ$ samples during exponential phase (0.030 ± 0.005 nmol/ml of OD1 cells), the amounts detected increased significantly in transition phase (0.52 ± 0.07 nmol) (see Fig. S3), and MurNAc-6P reached the maximum intracellular amounts (1.36 ± 0.31 nmol) in stationary phase after 24 h of culturing (Fig. 2).

We then complemented the $\Delta murQ$ mutants of *S. aureus* and *B. subtilis* and studied the effect on the accumulation of MurNAc-6P. To complement the MurQ ortholog in *S. aureus*, the $\Delta murQ$ mutant was transformed with pRB474 constitutively expressing the MurQ ortholog from *S. aureus* (pRB474-*murQ*) or with the empty pRB474 plasmid as a control. By LC-MS analysis, we observed a 65% reduction in the amount of MurNAc-6P when the *S. aureus* MurQ ortholog was expressed in *trans* (see Fig. S4 in the supplemental material). For complementation of MurQ in *B. subtilis*, either the vector pX-*murQ* or the empty vector pX (control) was integrated into the *amyE* sites in the chromosome of the *B. subtilis* $\Delta murQ$ mutant. Enzyme expression was induced with 0.3% xylose, since the expression of MurQ in pX-*murQ* is under the control of a xylose-inducible promoter. MurQ complementation in the *B. subtilis* recycling mutant resulted in a complete disappearance of the intracellular MurNAc-6P that accumulated in the $\Delta murQ$ mutant (see Fig. S4).

To relate our data obtained with *S. aureus* and *B. subtilis* to the recycling data for a Gram-negative bacterium, we measured MurNAc-6P accumulation in *E. coli* wild-type and $\Delta murQ$ cells. It has been reported that MurNAc-6P accumulates in *E. coli* *murQ* (strain TJ2e) mutant cells grown in the absence and presence of external MurNAc as assayed by thin-layer chromatography and radiodetection (17, 18). Previously, *E. coli* MurNAc-6P was quantified only in stationary phase. Here, we detected already in mid-exponential phase large amounts of MurNAc-6P in the *E. coli* $\Delta murQ$ mutant (1.06 nmol) that remained high in the transition growth phase (0.99 nmol), whereas in the parental strain, no MurNAc-6P was detected (see Fig. S3 in the supplemental material).

Thus, the accumulation of MurNAc-6P in *E. coli* was already high in exponential phase. In contrast, we obtained only minimal amounts of MurNAc-6P from exponentially grown $\Delta murQ$ cells

of Gram-positive bacteria, particularly in *B. subtilis*, but much larger amounts from cells in transition and stationary phases. We aimed to clarify whether the apparent delay of PGN recycling in Gram-positive bacteria is due to a low activity or abundance of MurQ and MurP orthologs or to the small amounts of MurNac provided from the breakdown of cell wall turnover products. Therefore, we added MurNac (0.02%) to the culture medium and determined the intracellular concentrations of MurNac-6P in *S. aureus* and *B. subtilis* wild-type and $\Delta murQ$ cells at different growth phases (Fig. 3; see also Fig. S5 in the supplemental material). MurNac-6P was generally absent in the cytosolic fractions of wild-type cells grown in LB medium supplemented with 0.02% MurNac, except for small amounts measured in the *S. aureus* cells after 24 h of growth (see Fig. S5). However, in *S. aureus* $\Delta murQ$, the intracellular concentrations of MurNac-6P in the presence of MurNac increased in exponential phase to 2.33 ± 0.32 nmol/ml of OD1 cells and in transition phase to 6.21 ± 0.66 nmol/ml of OD1 cells, and they reached a maximum in stationary growth phase of 11.56 ± 0.39 nmol/ml of OD1 cells (see Fig. S5). Thus, *S. aureus* cells tolerated the very high intracellular concentrations of MurNac-6P without showing any obvious growth defect, even with 0.2% exogenous MurNac (Fig. 3). In *B. subtilis* $\Delta murQ$ cells, the addition of MurNac caused large increases in the intracellular MurNac-6P levels, particularly in exponentially growing cells. When MurNac was added to the medium, MurNac-6P concentrations of 1.05 ± 0.05 nmol were determined in exponential phase. During transition phase, adding MurNac to the medium caused the levels of MurNac-6P to increase to 5.21 ± 0.73 nmol/ml of OD1 cells, and adding MurNac in stationary phase caused the levels to increase to 3.11 ± 0.13 nmol/ml OD1 cells. Surprisingly, the levels of MurNac-6P dropped from transition to stationary phase in *B. subtilis* $\Delta murQ$ cells grown in LB with MurNac (see Fig. S5). Reevaluation of the MS data revealed that the reduction of the amount of MurNac-6P in stationary-phase cells correlated with an increase in MurNac. This is most likely caused by an unknown phosphatase in *B. subtilis* that is responsible for dephosphorylation of MurNac-6P, thus yielding MurNac (data not shown).

Cell wall recycling is crucial for survival in stationary phase.

Although large amounts of MurNac-6P accumulate in $\Delta murQ$ cells of *S. aureus* and *B. subtilis* grown in LB and even more in the presence of MurNac, the growth rates determined by optical density measurements were more or less identical for wild-type and mutant cells for at least 24 h of growth (Fig. 3). However, in late stationary phase in LB medium supplemented with 0.2% MurNac, *B. subtilis* wild-type cultures remained at a higher OD (Fig. 3). We supposed that, although the impairment of recovery of the MurNac of the peptidoglycan wall had no effect on growth, it might still affect cell survival. To investigate whether MurNac recycling provides a survival benefit, we analyzed the viability of cells by determining the CFU counts (CFU/ml) (Fig. 4; see also Table S3 in the supplemental material). Similar cell numbers were determined for wild-type and $\Delta murQ$ strains grown to exponential phase and transition phase in medium with and without added MurNac. Intriguingly, in stationary phase (24 h of culturing), only the wild-type *S. aureus* cell cultures supplemented with exogenous MurNac maintained high cell numbers; in all other cases, the CFU counts dropped dramatically. This is not a growth but a survival effect, since the cell numbers did not increase between 8 h and 24 h of incubation of wild-type *S. aureus* cell cul-

tures in medium with MurNac but did remain at a high level, whereas cell numbers of the $\Delta murQ$ strain dropped by twofold. Notably, prolonged incubation of *S. aureus* cells in medium with MurNac for 48 h and 72 h showed that wild-type cell cultures maintained seven- and sixfold higher cell numbers (CFU/ml), respectively, than did mutant cell cultures. A slight but significant survival advantage of *S. aureus* wild-type cells compared to the survival of mutant cells was also observed upon culturing for 48 h and 72 h in LB medium without MurNac. Thus, prolonged incubation in the culture medium revealed a defect in the survival of the MurNac-recycling-mutant cells compared to the survival of wild-type cells (Fig. 4; see also Table S3).

Similar data were obtained for *B. subtilis*. We observed no significant differences in the CFU counts of wild-type and mutant cell cultures within the first 24 h of growth in LB either in the absence or presence of MurNac. However, the viability of *murQ* mutant cell cultures decreased dramatically during prolonged incubation in stationary phase (48 h and 72 h). In the presence of MurNac, surprisingly, a dramatic decrease of viability was observed, possibly due to cell lysis at the onset of sporulation. Notably, this effect was even enhanced in the recycling mutant (Fig. 4; see also Table S3).

DISCUSSION

We show here that three Gram-positive bacteria, *S. aureus*, *B. subtilis*, and *S. coelicolor*, recycle the MurNac contents of their PGN cell wall during vegetative growth in rich medium, which requires the orthologs of the MurQ enzyme of *E. coli*. Thereby, we provide for the first time direct evidence for PGN recycling in Gram-positive bacteria and proof of MurQ function in these organisms. Since these differently growing species, the elongating rod-shaped *B. subtilis*, the orthogonally dividing coccoidal *S. aureus*, and the apically growing filamentous *S. coelicolor*, all recycle the MurNac of their PGN, this pathway is presumably a general feature of Gram-positive bacteria. Indeed, orthologs of *murQ*, initially identified and related to PGN recycling in the Gram-negative model bacterium *E. coli* (17, 18), are found on the chromosomes of nearly all Gram-positive bacteria (an exception is *Streptococcus* species). We directly measured the recycling product MurNac-6P that accumulated in the *murQ* mutants by applying a mass spectrometry-based method, which avoids radioactive prelabeling of cell walls and the subsequent measurement of the radioactive fragments released but still allows sensitive detection and quantification of MurNac-6P in cell extracts within a large concentration range of 2.5 to 1,250 μM . Since the wild-type strains, as well as complemented mutants, contained no MurNac-6P or greatly reduced amounts, respectively, it is evident that the MurQ etherases are required for MurNac recycling. The possibility that MurNac-6P accumulated in $\Delta murQ$ mutants due to the presence of MurNac in the growth medium could be excluded because MurNac is absent from the LB culture medium (24, 32; data not shown). Furthermore, MurNac-6P accumulation was not detected in strains of *S. aureus* and *B. subtilis* that were defective in both MurQ and the putative MurNac transporter, indicating that the MurP MurNac phosphotransferase transporters are the only uptake systems for MurNac recycling in these organisms.

We determined the levels of MurNac-6P that accumulated in *murQ* mutants of *S. aureus* and *B. subtilis* in different growth phases. The amounts of MurNac-6P are presented as nanomoles per milliliter of OD1 cells. Assuming that 1 ml of OD1 cells con-

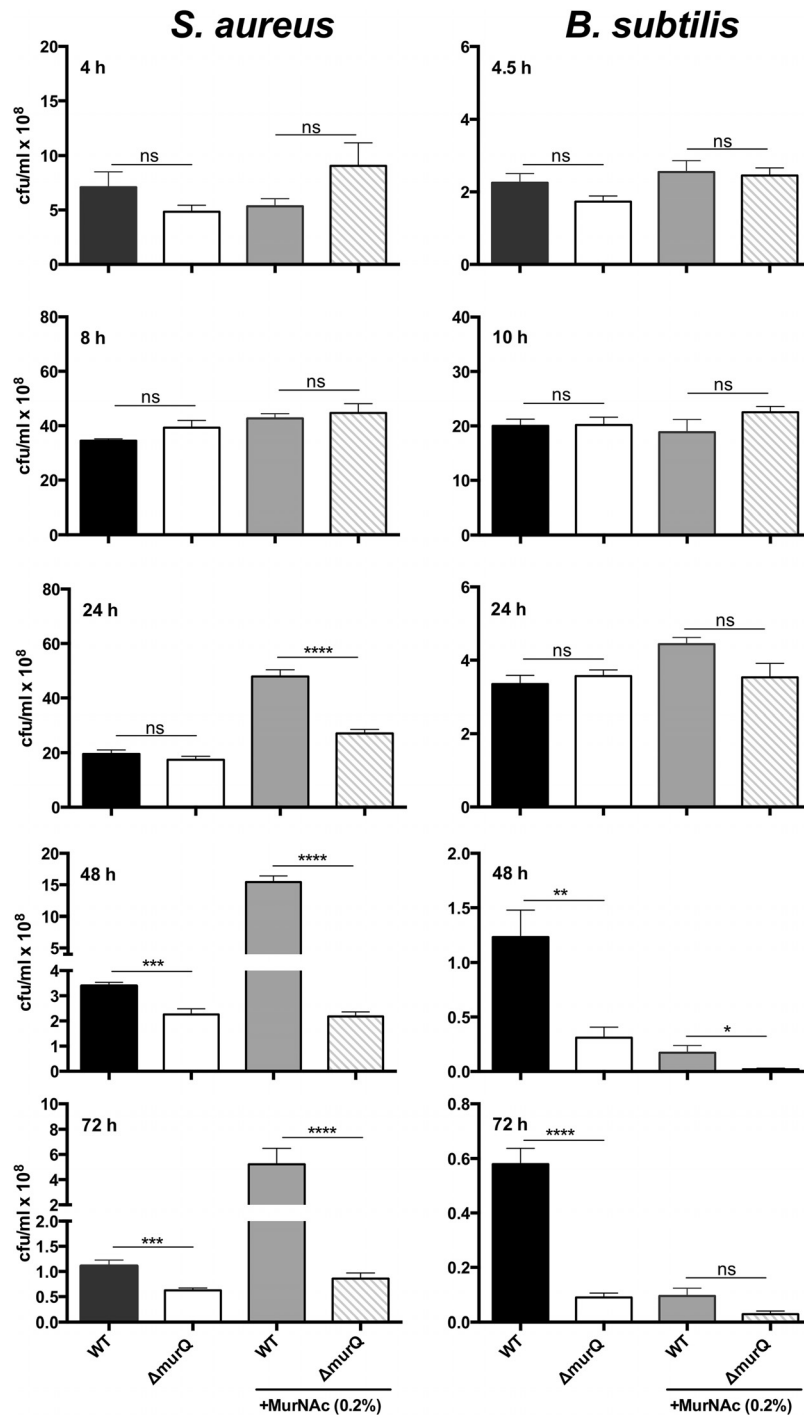


FIG 4 Determination of viable cell titers of *S. aureus* and *B. subtilis* wild-type strains (WT) and their Δ murQ mutants grown in LB medium with or without MurNAc. Wild-type and Δ murQ mutant cells of *S. aureus* and *B. subtilis* were grown in LB medium in the absence (left two bars in each graph) or presence of 0.2% MurNAc. Bacterial cultures were diluted appropriately in 0.9% NaCl solution in 96-well plates and plated on LB agar. Viable cells were determined by counting CFU/ml ($\times 10^8$) at mid-exponential (4 h for *S. aureus* and 4.5 h for *B. subtilis*), transition (8 h and 10 h, respectively), and stationary (24 h, 48 h, and 72 h) phases. Data are presented as the mean values \pm SEM from three independent biological replicates and were analyzed for statistical significance with the nonpaired *t* test. A *P* value of <0.05 was determined as statistically significant (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; ns, nonsignificant).

tains a cell volume of about 1 μ l, a value of 1 nmol would equate roughly to an intracellular accumulation of 1 mM of MurNAc-6P. We show here that MurNAc recycling is low during exponential growth and mainly occurs during transition and stationary phases

in *S. aureus* and *B. subtilis*. These results largely explain why PGN turnover products are found in abundance in the culture medium of exponentially growing Gram-positive bacteria (see reference 24 and references therein). In Gram-positive bacteria, PGN recycling

occurs not synchronous with turnover, as in *E. coli* (2), but after a delay. Turnover of the Gram-positive cell wall occurs by a so-called inside-to-outside growth mechanism (33). The PGN is first assembled near the plasma membrane and is then gradually moved to the outside, where it becomes stretched and more susceptible to the activity of autolysins. The small amounts of MurNAc-6P in exponential-phase cells, particularly in the *B. subtilis* $\Delta murQ$ mutant, can be explained by the delayed release of MurNAc from the PGN wall due to the slow autolytic processing of larger PGN turnover fragments. It is likely that MurNAc is released more slowly from the very long PGN chains present in *B. subtilis* than from the rather short PGN chains of *S. aureus* (34).

MurNAc recycling does not sustain the growth of *S. aureus* and *B. subtilis*; instead, it appears to be of major relevance for their survival capacity when growth is stalled during nutrient limitation at the onset of stationary phase and recycling may be used to overcome a shortage of nutrients and precursors for cell (wall) differentiation. Our results are in agreement with those of previous studies indicating that the transcription of *B. subtilis murQ* occurs mainly during the transition to stationary phase (35). Moreover, these results are consistent with previous findings showing that the expression of the recycling *N*-acetylglucosaminidase NagZ of *B. subtilis* is low during exponential phase but highly elevated in stationary phase (30). The enzyme releases GlcNAc from the PGN, and it is likely that besides MurNAc, the GlcNAc part of the PGN is also recycled; however, this would require uptake by a different transport system.

When MurNAc was added to the medium, the accumulation of MurNAc-6P increased greatly, indicating that the uptake and recycling of MurNAc is limited by the availability of the sugar. As mentioned above, the release of MurNAc depends on autolytic processing of PGN, which is growth phase-dependently regulated. In the proximity of *murQ* and its orthologs on the genomes of the bacteria studied, transcriptional regulator proteins were identified that, similar to the case for *E. coli* (20), may likely accelerate the transcription of the MurNAc transporter and MurQ in the presence of MurNAc-6P. Interestingly, in stationary phase, large amounts of MurNAc and MurNAc-6P were found in the cytosolic fractions of *B. subtilis* $\Delta murQ$ mutant cells grown in LB medium supplemented with MurNAc, which might be due to dephosphorylation of MurNAc-6P at high concentrations by a putative phosphatase. Notably, an enzyme with identical function was recently proposed in a *Pseudomonas* species (13).

To relate our quantitative data on MurNAc-6P accumulation in *S. aureus* and *B. subtilis* to its accumulation in the Gram-negative bacterium *E. coli*, we reexamined and quantified the MurNAc-6P accumulation in the *murQ* mutant of *E. coli* using our mass spectrometry-based method. In *E. coli murQ* mutant cells, the maximum accumulation of MurNAc-6P occurred already in mid-exponential growth phase and reached an intracellular concentration of c. 1 mM (1 nmol/ml of OD1 cells); Wientjes and colleagues reported that the PGN of one *E. coli* cell contains 3.5×10^6 molecules of diaminopimelic acid (36), and presumably, similar numbers of MurNAc molecules are present. As 1 ml of a cell culture with an OD₆₀₀ of 1 (OD1 cells) in exponential phase amounts to 5×10^8 to 1×10^9 cells, it can be calculated that one *E. coli* cell contains 1.7×10^{15} to 3.5×10^{15} molecules of MurNAc. Thus, a recycling rate of 45% per generation would lead to a MurNAc-6P accumulation of about 1.3 to 2.6 mM. The value of about 1 mM obtained in exponentially growing *E. coli* cells in this

study is a little lower than the expected concentration range. Interestingly, the amounts of the recycling product MurNAc-6P determined in *E. coli* cells during mid-log phase and in the examined Gram-positive bacteria during transition to stationary phase were similar. This indicates that, compared to the rates of PGN synthesis, the turnover and recycling of PGN appear to be quite similar in both groups of bacteria. However, related to the thickness of the Gram-positive cell wall, with approximately 5- to 10-fold-greater PGN contents than in *E. coli*, it can be estimated that only about 5 to 10% of the PGN is recycled in *S. aureus* and *B. subtilis* per generation. These values are in the range of the 8% recycling reported for *B. subtilis* by Pooley (25) but are lower than the rates of turnover reported elsewhere (22, 26, 27).

The most intriguing finding of our study was that PGN sugar recycling affects survival fitness. Apparently, the cell wall sugar MurNAc does not serve primarily as an energy source but is preferentially utilized for cell wall synthesis. The same presumably holds for GlcNAc, as the MurNAc and GlcNAc catabolic pathways merge. Amino sugar utilization and its regulation were studied in *B. subtilis* and in *S. aureus* (37) and were compared with those of *E. coli* in a recent review (38). These studies revealed that *S. aureus* and *B. subtilis* use GlcNAc preferentially for cell wall synthesis; in *B. subtilis*, about 95% of GlcNAc from the medium is usually incorporated into PGN. Our findings are consistent with these observations. Both cell wall sugars, GlcNAc and MurNAc, are presumably used in *S. aureus* and *B. subtilis* for cell wall synthesis, at least during growth in rich medium. Combined with the data presented here, one could speculate that in Gram-positive bacteria, MurNAc recycled from the PGN, as well as that salvaged from the medium, is not used to gain energy or for growth but might be utilized to stabilize the cell wall, and thus, it increases survival during prolonged starvation. Although PGN recycling is not essential, a huge set of recycling genes are maintained in almost all bacterial genomes, suggesting that the pathway must provide a benefit for bacterial cells. We show here that PGN recycling is crucial for the survival of Gram-positive bacteria during stationary phase.

MATERIALS AND METHODS

Chemicals, enzymes, and oligonucleotides. Enzymes for DNA restriction and for cloning were obtained from New England Biolabs (Ipswich, MA) or Thermo Fischer Scientific (Schwerte, Germany). The GeneJET plasmid miniprep kit, PCR purification kit, and Gene Ruler 1-kb marker were from Thermo Fisher Scientific and Qiagen (Venlo, Netherlands). *N*-Acetylmuramic acid (MurNAc) was from Bachem (Bubendorf, Switzerland), and the DNA dye NonTox was from AppliChem (Darmstadt, Germany). Oligonucleotides were purchased from MWG Eurofins (Ebersberg, Germany) and are listed in Table S1 in the supplemental material.

Bacterial strains, growth conditions, and construction of mutants and plasmids. The plasmids and bacterial strains used in this study are shown in Table S2 in the supplemental material. The construction of mutant strains and plasmids is described in Text S1 in the supplemental material. *Escherichia coli* K-12, *Bacillus subtilis* 168, and *Staphylococcus aureus* USA300 were cultured aerobically in lysogeny broth (LB; 5 g/liter yeast extract, 10 g/liter tryptone, 10 g/liter NaCl) at 37°C and under continuous shaking at 160 rpm or on solid LB supplemented with 1.5% agar. *B. subtilis* and *S. aureus* overnight cultures (~16 h) were used to inoculate fresh LB medium to yield an initial optical density at 600 nm (OD₆₀₀) of 0.05 for growth studies or the determination of intracellular accumulation of *N*-acetylmuramic acid-6 phosphate (MurNAc-6P) at different growth phases. Tryptic soy broth (TSB; Oxoid) was used to generate the markerless *S. aureus* $\Delta murQ$ mutant and for complementation experiments.

S. coelicolor M145 was grown in LB medium with constant shaking (180 rpm) at 30°C. Antibiotics were used, when appropriate, at the following concentrations: ampicillin (100 µg/ml) for *E. coli*; chloramphenicol (5 µg/ml) and erythromycin and tetracycline (10 µg/ml) for *B. subtilis*; erythromycin (5 µg/ml) and chloramphenicol (10 µg/ml) for *S. aureus*; and apramycin (50 µg/ml) for *S. coelicolor*. For induction of the *PxyLA'* promoter in *B. subtilis*, 0.3% xylose was added to the LB growth medium.

Generation of cytosol fractions. Overnight cultures of *S. aureus*, *B. subtilis*, and *E. coli*, including both the wild types and the respective $\Delta murQ$ mutants, were used to inoculate LB medium, yielding an initial OD₆₀₀ of 0.05, and cells were grown at 37°C. *S. aureus* cells were harvested at mid-exponential phase (OD₆₀₀ of 3 after growth for ~3.5 h in 100-ml cultures), at transition phase (OD₆₀₀ of 7.5 after growth for 8 h in 40-ml cultures), and at stationary phase (OD₆₀₀ of 6 after growth for 24 h in 50-ml cultures). *B. subtilis* cultures were harvested at mid-exponential phase (OD₆₀₀ of 2 after growth for ~4 h in 150-ml cultures), at early stationary phase (OD₆₀₀ of 4 after growth for 10 h in 75-ml cultures), and at stationary phase (OD₆₀₀ of 3 after growth for 24 h in 100-ml cultures). *E. coli* cells were harvested at mid-exponential phase (OD₆₀₀ of 1.5 after growth for ~3 h in 200-ml cultures) and at transition phase (OD₆₀₀ of 3.5 after growth for 6.5 h in 86-ml cultures). Bacteria were centrifuged at 3,000 × g for 10 min and washed with 20 ml deionized water, and pellets were frozen at -80°C. Frozen cell samples were thawed at room temperature and suspended in water to yield 1.2-ml cell suspensions with an OD₆₀₀ of 250.

Approximately 10⁸ spores of *S. coelicolor* M145 wild type and the $\Delta murQ$ ($\Delta SCO4307$) mutant were incubated in LB medium (50 ml) and grown for 24 h in a rotary shaker at 30°C. Fifteen-milliliter amounts of the cultures were harvested by centrifugation, washed, and frozen at -80°C until being dissolved in 1,000 µl water for further analysis.

The whole bacterial samples, suspended in the amount of water indicated above, were transferred to new tubes containing ~0.25 g glass beads (0.25 to 0.5 mm; Roth), and cells were disrupted in a FastPrep FP120 (Thermo Savant) cell disrupter at speed 6 for 35 s. This was repeated 4 times, with cooling on ice for 1 min after the second cycle. Lysates were cooled briefly and subsequently centrifuged for 10 min at maximum speed in a microcentrifuge. Two hundred microliters of the supernatant was added to 800 µl of ice-cold acetone to precipitate the remaining proteins in the supernatant. After centrifugation (12,000 × g for 10 min), the supernatant was transferred to a new tube, and samples were dried under vacuum for 2 h at 55°C and finally stored at 4°C prior to LC-MS measurements.

Analysis of MurNAc-6P accumulation by LC-MS. Sample analysis of bacterial cytosolic fractions was conducted using an electrospray ionization-time of flight (ESI-TOF) mass spectrometer (MicrO-TOF II; Bruker), operated in negative-ion mode and connected to the UltiMate 3000 high-performance liquid chromatography (HPLC) system (Dionex). Dried samples were dissolved in 100 µl of water before measurement, and 5-µl amounts were injected into a Gemini C₁₈ column (150 by 4.6 mm; Phenomenex) at 37°C. The following previously described (12) 45-min-gradient program at a flow rate of 0.2 ml/min was used. Five minutes of washing with 100% buffer A (0.1% formic acid with 0.05% ammonium formate) was followed by a linear gradient over 30 min to 40% buffer B (100% acetonitrile). A 5-min delay and 5 min of reequilibration completed the method. The mass spectra of the investigated samples are presented as total-ion chromatograms (TIC) and extracted-ion chromatograms (EIC) for MurNAc-6P, created using Data Analysis (Bruker) and Prism 6 (GraphPad) software. To quantify MurNAc-6P concentrations in cell extracts (presented as nmol/ml of OD1 cells), we generated EIC for MurNAc-6P ($m/z^{-1} = 372.07$) in each sample and determined the area under the curve of the peak obtained by using Prism 6 (baseline of 30). A dilution series of a MurNAc-6P standard (1,250 µM to 2.45 µM) was also analyzed by LC-MS, and the data are presented as EIC based on the area under the curve of the measurements obtained. The

standard curve was applied to define the intracellular MurNAc-6P concentrations in the bacterial cytosolic fractions.

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at <http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.00923-16/-/DCSupplemental>.

Text S1, DOCX file, 0.1 MB.
Figure S1, DOCX file, 1.1 MB.
Figure S2, DOCX file, 0.1 MB.
Figure S3, DOCX file, 0.4 MB.
Figure S4, DOCX file, 0.1 MB.
Figure S5, DOCX file, 0.3 MB.
Table S1, DOCX file, 0.1 MB.
Table S2, DOCX file, 0.1 MB.
Table S3, DOCX file, 0.1 MB.

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