Identification of Multiple Genes Encoding Membrane Proteins with Undecaprenyl Pyrophosphate Phosphatase (UppP) Activity in Escherichia coli*

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The bacA gene product of Escherichia coli was recently purified to near homogeneity and identified as an undecaprenyl pyrophosphate phosphatase activity (El Ghachi, M., Bouhss, A., Blanot, D., and Mengin-Lecreulx, D. (2004) J. Biol. Chem. 279, 30106–30113). The enzyme function is to synthesize the carrier lipid undecaprenyl phosphate that is essential for the biosynthesis of peptidoglycan and other cell wall components. The inactivation of the chromosomal bacA gene was not lethal but led to a significant, but not total, depletion of undecaprenyl pyrophosphate phosphatase activity in E. coli membranes, suggesting that other(s) protein(s) should exist and account for the residual activity and viability of the mutant strain. Here we report that inactivation of two additional genes, ybjG and pgpB, is required to abolish growth of the bacA mutant strain. Overexpression of either of these genes, or of a fourth identified one, yeiU, is shown to result in bacitracin resistance and increased levels of undecaprenyl pyrophosphate phosphatase activity, as previously observed for bacA. A thermostable conditional triple mutant ΔbacA,ΔybjG,ΔpgpB in which the expression of bacA is impaired at 42 °C was constructed. This strain was shown to accumulate soluble peptidoglycan nucleotide precursors and to lyse when grown at the restrictive temperature, due to the depletion of the pool of undecaprenyl phosphate and consequent arrest of cell wall synthesis. This work provides evidence that two different classes of proteins exhibit undecaprenyl pyrophosphate phosphatase activity in E. coli and probably other bacterial species; they are the BacA enzyme and several members from a superfamily of phosphatases that, different from BacA, share in common a characteristic phosphatase sequence motif.

An essential carrier lipid, undecaprenyl phosphate (C55-P), is required for the synthesis of various bacterial cell wall polymers such as peptidoglycan, lipopolysaccharides, and teichoic acids (1–5) (Scheme 1). It is synthesized as a pyrophosphate precursor (C55-PP) by the addition of eight isoprene units to farnesyl pyrophosphate, a reaction catalyzed by the well-characterized cis-prenyl-pyrophosphate synthase UppS (6–10). However, genes and enzymes involved in subsequent steps of C55-P synthesis and recycling still remained to be identified. Bacitracin is a dodecapeptide antibiotic known to specifically block this metabolism by forming a specific complex with C55-PP. As a result, cell wall biosynthesis is inhibited and cell lysis finally occurs (11–14). Bacillus licheniformis strains that produce bacitracin are resistant to this antibiotic due to the presence of an appropriate ABC transporter efflux system (15, 16). Several mutations leading to bacitracin resistance were identified in Escherichia coli and other Gram-negative bacteria. Interestingly, all these mutations were shown to block the synthesis of non-essential cell envelope polymers such as osmoregulated periplasmic glycans and capsule polysaccharides that also require the C55-P carrier lipid for their formation (17, 18). The reduced in vivo requirements for C55-P resulting in higher availability of the carrier lipid for the synthesis of essential polymers (peptidoglycan, lipopolysaccharides) most probably explain the decreased mutant cell sensitivity to bacitracin. Not only mutations but also gene overexpression were earlier reported to be associated with bacitracin resistance (19, 20). In particular, we recently demonstrated that one such E. coli gene, named bacA (19), encoded a membrane-bound C55-PP phosphatase activity (21). Overexpression of a C55-PP phosphatase activity might very likely accelerate the conversion of the pool of C55-PP, the bacitracin target, to C55-P, resulting in an increased resistance of the cell to this antibiotic.

The dephosphorylation of C55-PP to C55-P was expected to be an essential step in the metabolism of the carrier lipid. The fact that the bacA gene could be disrupted in the chromosome without apparent growth defect in E. coli, Staphylococcus aureus, and Streptococcus pneumoniae (21, 22) was thus quite surprising. As no bacA homologous gene could be identified in the genome of these bacterial species, either the involvement of other phosphatase(s) or a metabolic bypass of this dephosphorylation step could be envisioned. As a residual 25% C55-PP phosphatase activity was still detected in the bacA mutant strain, other protein species might account for this activity. These other C55-PP phosphatases could be specific to C55-PP or

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The abbreviations used are: C55-P, undecaprenyl phosphate; C55-PP, undecaprenyl pyrophosphate; C55-OH, undecaprenol; IPTG, isopropyl-β-D-thiogalactopyranoside; GlcNAc, N-acetylglucosamine; MurNAc, N-acetylmuramic acid; UDP-MurNAc-pentapeptide, UDP-MurNAc-t-Ala-γ-D-Glu-meso-diaminopimeloyl-d-Ala-d-Ala.

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have a distinct function and act non-specifically on this substrate at a sufficient rate to sustain cell growth requirements. We have demonstrated here that multiple genes encoding proteins with \( C_{55}-\text{PP} \) phosphatase activity exist in \( E. coli \) and that the inactivation of three of them is required to block cell wall synthesis and provoke cell lysis.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—The \( E. coli \) strains DH5\( \alpha \) (Invitrogen), JM83 (23), and C43(DE3) (Avidis-France) were used as hosts for plasmids (Table I). The plasmid vector \( pTrc99A \) was obtained from Amerham Biosiences, and the \( pMAK705 \) plasmid bearing a thermosensitive replica was a kind gift from S. R. Kushner (24). The BW25113 strain and the \( pKD3 \), \( pKD4 \), \( pKD46 \), and \( pCP20 \) plasmids used for gene disruption experiments (25) were kindly provided by S. R. Kushner (24). The BW25113 \( \Delta \text{bacA}::\text{Cam}^R \) strain was previously described (21), and other mutant strains constructed in this study are listed in Table I. 2YT medium (26) was used for growing cells, and growth was monitored at 600 nm with a Shimadzu UV-1601 spectrophotometer. For strains carrying drug resistance genes, ampicillin, kanamycin, and chloramphenicol were used at 100, 25, and 25 \( \mu \)g ml\(^{-1} \), respectively.

**General DNA Techniques and \( E. coli \) Cell Transformation**—Polymerase chain reaction (PCR) amplification of genes from the \( E. coli \) chromosome was performed in a Thermocycler 60 apparatus (Bio-med) using the Expand-Fidelity polymerase (Roche Applied Science). The DNA fragments were purified using the Wizard PCR Preps DNA purification kit (Promega), and standard procedures for endonuclease digestion, agarose electrophoresis, ligation, and plasmid purification were used (27). \( E. coli \) cells were transformed with plasmid DNA by the method of Dagar and Ehrlich (28) or by electroporation.

**Generation of Chromosomal Gene Deletions**—The \( E. coli \) strain BW25113 \( \Delta \text{bacA} \) carrying a chromosomal deletion of the \( baca \) gene was constructed recently (21) using the efficient method of Datsenko and Wanner (25). The same procedure was used to generate deletions of the \( ybjG \), \( yeiU \), \( pgpA \), and \( pggB \) genes. In each case, \( \text{Inac1} \) and \( \text{Inac2} \) oligonucleotides (Table II) were used for PCR amplification of either the chromophenolic resistance gene from \( pKD3 \) or the kanamycin resistance gene from \( pKD4 \), flanked by sequences designed for specific disruption of the gene of interest. The PCR products were transformed by electroporation into the mutant \( E. coli \) cells expressing the phage lambda Red recombinase (25). Antibiotic-resistant colonies were isolated, and the disruption of the \( baca \), \( ybjG \), \( yeiU \), \( pgpA \), and \( pggB \) genes in the chromosome was verified by PCR using \( BacA1 \) and \( BacA2 \), \( YbjG1 \) and \( YbjG2 \), \( YeilU1 \) and \( YeilU2 \), \( PgpA1 \) and \( PgpA2 \), and \( PggB1 \) and \( PggB3 \) as primers, respectively (Table II). Excision of the chromophenolic resistance genes from the chromosomal mutant strains was then obtained by transformation with the \( pCP20 \) plasmid expressing the Flp recombinase, as described previously (25). Antibiotic-sensitive colonies were isolated, and the excision of the cassette was confirmed by PCR analysis as described above. Strains carrying multiple chromosomal gene deletions were generated by transduction of antibiotic resistance markers with phage P1 (26), followed by excision of the cassettes from the chromosome.

**Construction of Plasmids**—The plasmid \( pTrcBac1 \) allowing expression of the \( baca \) gene under control of the isopropyl-\( \beta \)-D-thiogalactopyranoside (IPTG)-dependent \( trc \) promoter has been previously described (21). Plasmids for expression of the \( ybjG \), \( yeiU \), and \( pggB \) genes were similarly constructed as follows. The \( ybjG \) gene was amplified from the chromosome using oligonucleotides \( YbjG1 \) and \( YbjG3 \) (Table II), and the resulting fragment was treated with \( BspHI \) and \( HindIII \) and cloned between the compatible \( NcoI \) and \( HindIII \) sites of vector \( pTrc99A \), generating \( pTrcYbjG \). The \( yeiU \) gene was amplified from the chromosome using oligonucleotides \( YeilU1 \) and \( YeilU2 \), the resulting fragment was treated with \( BspHI \) and \( HindIII \) and cloned into the compatible \( NcoI \) and \( HindIII \) sites of vector \( pTrc99A \), generating \( pTrcYeilU \). A plasmid allowing conditional (thermosensitive) expression of \( baca \) was constructed as follows: the \( baca \) gene and its proximal region were amplified using oligonucleotides \( BacA1 \) and \( BacA3 \), and the resulting DNA fragment was cut with BamHI and ScaI and cloned between the same sites of plasmid vector \( pMAK705 \) whose replication is thermosensitive (24), generating \( pMAKbaca \).

**Preparation of Crude Membrane Extracts**—The different strains described in this work were grown in 2YT medium (0.5-liter cultures) at either 30, 37, or 42 °C. For strains carrying plasmids, growth was performed in the presence of the corresponding antibiotic. When the optical density of the culture reached 0.6, 1 ml IPTG was added for induction of gene overexpression, and growth was continued for 3 h. In all cases, cells were harvested and washed with 40 vol of cold 0.5 M potassium phosphate buffer, pH 7.2, containing 2 mM \( \beta \)-mercaptoethanol (buffer A). The cell pellet was suspended in 12 ml of the same buffer, and cells were disrupted by sonication in the cold (Bioblock VibraCell sonicator model 72142). The resulting suspension was centrifuged at 4 °C for 30 min at 200,000 \( \times \) g. The pellet consisting of membranes and associated proteins was washed twice with buffer A and finally resuspended in buffer A supplemented with 150 mM NaCl, 1% \( \text{n-dodecyl-\( \beta \)- maltoside} \), and 20% glycerol.

**Undecaprenyl Phosphatase Phosphatase Assay**—The assay was performed in a reaction mixture (20 ml) containing 100 mM Tris-HCl buffer, pH 7.5, 10 mM MgCl\(_2\), 3.9 mM \( \text{n-dodecyl-\( \beta \)- maltoside} \), and 5 \( \mu \)M \( [\text{I}^3\text{C}]\text{C}_{55}-\text{PP} \) (2,035 Bq), and enzyme. The reaction mixture was incubated for 30 min at 37 °C, and the reaction was stopped by heating at 100 °C for 1 min. As described previously (21), the substrate (\( \text{C}_{55}-\text{PP} \)) and reaction product (\( \text{C}_{55}-\text{P} \)) were separated by TLC on precoated plates of silica gel 60 (Merck) using diisobutyl ketone/acetic acid/water (8:5:1, \( \text{v/v/v} \)) as a mobile phase (R\(_f\) values of \( \text{C}_{55}-\text{PP} \) and \( \text{C}_{55}-\text{P} \) were 0.36 and 0.5, respectively). The radioactive spots were located and quantified with a radioactivity scanner (model Multi-Tracermaster LB285; Berthold-France). One unit of enzyme activity corresponds to one nanomole of product formed/min.

**Pool Levels of Peptidoglycan Precursors**—Cells of the wild-type strain BW25113 and of the thermosensitive mutant strain BWTbacA (0.8-liter cultures) were grown exponentially at 30 °C in 2YT medium. At the appropriate cell density (\( \sim 10^8 \) cells/ml), the temperature of the culture was either maintained at 30 °C or increased to 42 °C. Incubation was continued until the first effects on the growth of the mutant
strain at 42 °C were observed, −2−3 h later. At this time, cells were rapidly chilled to 0 °C and harvested in the cold. The analytical procedures used for the extraction, isolation, and quantitation of the peptidoglycan nucleotide precursors were as previously described (29−31).

Isolation and Quantification of Peptidoglycan—Exponential phase cells of the wild-type BW25113 and mutant BWTsaA strains were grown at 30 °C, or first at 30 and then 42 °C as described above. Harvested cells were washed with a cold 0.85% NaCl solution and then resuspended with vigorous stirring in 20 ml of a hot (95−100 °C) aqueous 4% sodium dodecyl sulfate solution for 30 min. After standing overnight at room temperature, suspensions were centrifuged for 20 min at 200,000 × g, and the pellets were washed several times with water. Final suspensions made in 2.5 ml of water were homogenized by cultures (10²−10⁵ cells) were spread onto 2YT plates containing bacitracin from New England Biolabs, and oligonucleotides were from Sigma.

Protein Monitoring—Protein concentrations were determined by using either the Bradford procedure (33) or the QuantiPro BCA assay kit (Sigma) with bovine serum albumin as a standard.

Chemicals—DNA restriction and modification enzymes were obtained from New England Biolabs, and oligonucleotides were from MWG-Biotech. DNA purification kits were from Promega, C₅₅-OH, C₆₀-P, and C₆₀-PP were provided by the Institute of Biochemistry and Biophysics of the Polish Academy of Sciences. Pure BacA protein, [¹⁴C]C₅₅-PP (1.5−2.2 GBq/mmol⁻¹), and UDP-MurNAc peptides were prepared as described previously (21, 29). n-Dodecyl-β-D-maltoside was from Anatrace, and antibiotics and reagents were from Sigma.

RESULTS

Identification of Putative C₅₅-PP Phosphatase Genes—The gene encoding C₅₅-PP phosphatase activity was expected to be an essential gene, as the function of this enzyme is to generate the carrier lipid C₅₅-P that is absolutely required for the biosynthesis of peptidoglycan and other essential cell wall polymers (Scheme 1). The observation that a bacA (uppP) null mutant did not exhibit particular morphological changes or growth defects suggested that other proteins might exhibit such an activity in vivo (21). However, no bacA homologue was identified in the chromosome of E. coli and other bacterial species, namely S. aureus and S. pneumoniae, in which this gene was also proved to be non-essential (21, 22). Other putative genes for this activity were therefore searched for in bacterial genomes based essentially on sequence similarity with already known phosphatase activities. Phosphatases of unknown function that had been previously characterized to some extent biochemically were also considered as potential candidates.

A membrane-bound phosphatase of rather broad substrate specificity was earlier purified from Sulfolobus acidocaldarius (34). The bacitracin sensitivity of S. acidocaldarius and the catalytic properties of this enzyme made the authors speculate that it could be a dolichol-PP phosphatase. At that time, only the N-terminal amino acid sequence of the protein was determined, which allowed us to identify the corresponding gene in the S. solfataricus sequenced genome. The product of this gene was predicted to be a highly hydrophobic protein of 220 amino acid residues exhibiting sequence similarity with, and characteristic signature sequences of, members of the phosphatidic acid-phosphatase family. A BLAST search further showed that it presented the highest similarity (~30% residue identity) with proteins BcrC from B. licheniformis, YbjG from E. coli, and YwoA from Bacillus subtilis. Interestingly, the BcrC protein whose function is yet unknown was identified as one of the three components of the ABC transporter system responsible for the self protection of B. licheniformis against the antibiotic it produces, bacitracin (15, 16). Furthermore, overexpression of the ybjG gene in E. coli and ywoA gene in B. subtilis was recently reported to increase the resistance to bacitracin of these respective bacterial species (20, 35−37). Other E. coli genes encoding members of the phosphatidic acid-phosphatase family were yeiU, of unknown function, and ppgB, encoding phosphatidylglycerolphosphate phosphatase (38). It was previously shown that two genes, ppgA and ppgB, could sustain phosphatidylglycerolphosphate phosphatase activity in E. coli (38, 39); however, their inactivation did not result in the loss of viability but only in some thermosensitivity of growth. As shown in Fig. 1, sequences identical or quite similar to the common conserved phosphatase motif KX₃R-(X₉₋₁₅)-PSGH-(X₃₁₋₅₄)-SRX₂HX₆D, previously identified by Stuckey and Carman (40) and Neuwald (41), could effectively be found in the PpgB, YeiU, and YbjG proteins as well as in the B. subtilis YwoA and B. licheniformis BcrC proteins. Interestingly, this con-

### Table II

| Oligonucleotides | Sequence<sup>a</sup> |
|------------------|----------------------|
| Inact1-ybG       | GGCTTCACATTTGCTTCTCCTAGGTATGAGCTGTCTGCAATGAAATATCTCTCTTAG |
| Inact2-ybG       | GGAGTTTTTTATCTCAAGAAGATGTCTACGCTTGGCTGCTCGCAATGAAATATCTCTCTTAG |
| Inact1-yeiU      | CGCTCGGGTCTTCTTCCTGATATCCCTATCTGCTCTGCAATGAAATATCTCTCTTAG |
| Inact2-yeiU      | TACAGCTTCTACGAGACACTGATACTGCTATCGAAACGTGCAATGAAATATCTCTCTTAG |
| Inact1-pgpA      | CTCGGAAAATAACCCCGGCACTGCTACGTTGCTGCTCGCAATGAAATATCTCTCTTAG |
| Inact2-pgpA      | CCGTGCACTTCTTCTTCCTGATATCCCTATCTGCTCTGCAATGAAATATCTCTCTTAG |
| Inact1-pgpB      | TGGCCCAAAATTCTCTGCAAGCCACTTGGCTGCTCGCAATGAAATATCTCTCTTAG |
| Inact2-pgpB      | GGGAGCTCCATTTGCTTCTGAGCTGTCTGCAATGAAATATCTCTCTTAG |

<sup>a</sup> Restriction sites (in bold) introduced in oligonucleotides are indicated in parentheses.
Multiple Undecaprenyl Pyrophosphate Phosphatases in *E. coli*

**Scheme 1.** Biosynthesis of undecaprenyl phosphate (C\(_{55}\)-P) and its use for cell wall peptidoglycan synthesis. The C\(_{55}\)-PP precursor synthesized by the UppS synthetase is dephosphorylated by the UppP enzyme(s) into C\(_{55}\)-P. The MraY and MurG enzymes catalyze the successive transfers of the MurNAc-pentapeptide (M-pep) and GlcNAc (G) motifs from the peptidoglycan nucleotide precursors onto the C\(_{55}\)-P lipid, generating the lipid I and lipid II intermediates, respectively. The lipid II is then translocated to the outer side of the membrane where polymerization reactions catalyzed by the penicillin-binding proteins (PBPs) occur. At the end of this process, the carrier lipid is released in C\(_{55}\)-PP form and should be dephosphorylated before being reused for de novo peptidoglycan (or other cell wall components) synthesis. It is at present not known whether the regeneration of C\(_{55}\)-P occurs at the outer side of the membrane or only after translocation (flip-flop?) of the C\(_{55}\)-PP to the inner side.

| Protein | Sequence | (length/mass) |
|---------|----------|---------------|
| PgpB    | 96-KDVQYQBDY-52-PGSH-36-SSLLGKMKGRD-43 | (252) |
| YeiU    | 81-KENAPGRG-54-PGSH-32-PRWGMGANWTD-43 | (235) |
| YdjG    | 74-GFLQPPHD-16-PGSH-32-SRVAGVLWYLD-49 | (196) |
| YwoA    | 70-TLVYPPFR-16-PGSH-31-SRNWWGNYTD-40 | (191) |
| BcrC    | 75-GSLHSTQG-17-PGSH-31-SRWSGGYVLD-53 | (201) |

**Consensus**

| **C** | **X** | **C** | **P** | **C** | **X** | **P** |
|-------|-------|-------|-------|-------|-------|-------|

**Proposed Phosphatase Motifs**

- **PgpB** (Pseudomonas aeruginosa)
- **YeiU** (Escherichia coli)
- **YdjG** (Escherichia coli)
- **YwoA** (Bacillus subtilis)
- **BcrC** (Bacillus subtilis)

**Fig. 1.** Phosphatase motifs detected in the amino acid sequences of demonstrated (*E. coli* PgpB, YbjG, and YeIU proteins) or putative (*B. subtilis* YwoA and *B. licheniformis* BcrC proteins) C\(_{55}\)-PP phosphatase activities, as compared with the consensus sequence XX\(_{55}\)-PP亨(XX\(_{35}\)P)-PSGH(XX\(_{35}\)P)-SRX\(_{30}\)HX\(_{30}\) previously reported by Stuckey and Carman (40). Numbers indicate the lengths of amino acid sequences preceding and following the phosphatase motifs in the protein sequences. The total number of amino acids and molecular mass of each protein are indicated in parentheses.

served phosphatase motif was not detected in the sequence of BacA, and the absence of significant sequence homology between BacA and the above-mentioned proteins clearly indicated that they belonged to two different classes of proteins. All of them were predicted to be integral membrane proteins having five (YeiU), six (YbjG, YggB), and eight (BacA) transmembrane segments, respectively.

**Inactivation of All Three Genes, bacA, ybjG, and pgpB, Is Lethal—**An *E. coli* strain with the chromosomal bacA gene disrupted was recently constructed and shown to be viable (21). Membranes from wild-type and \(\Delta\text{bacA}:\text{Cam}\) \(^{R}\) strains were prepared, and enzymatic assays showed a 75% decrease of C\(_{55}\)-PP phosphatase activity in the mutant strain (21). This result confirmed the identification of the bacA gene product as a C\(_{55}\)-PP phosphatase and revealed its important, but not exclusive, contribution to the total activity detected in a wild-type strain. Following the efficient mutagenesis procedure of Datsenko and Wanner (25), other putative genes for this function, ybjG, yeiU, pgpB, and pggA, were inactivated on the chromosome. Strains with either of these four genes deleted and replaced by an antibiotic resistance cartridge were obtained, indicating that none of them was essential for growth, as previously observed for bacA (21). Strains carrying multiple gene deletions were then constructed by successive rounds of transduction by phage P1 of the integrated antibiotic resistance markers, followed by excision of the latter markers from the chromosome by using the FLP recombinase. Although most of the different combinations of multiple mutations were generated without any difficulty (Table I), those involving inactiva-
The growth of BW25113 and BWTsbacA at 42 °C, respectively, increases of the latter activity, by depletion of the pool of C55-P. UDP-GlcNAc also somewhat was clearly correlated to the arrest of synthesis of BacA and cells, following a temperature shift to 42 °C (Table III). This (Scheme 1), was observed in mutant cells, but not in wild-type strain.

30 °C in 2YT medium. At a cell density of ~10^7 cells/ml (zero time, A at 600 nm of 0.02), the temperature of the culture was either maintained at 30 °C or shifted to 42 °C. Growth of both strains at 30 °C, A, [] growth of BW25113 and BWTsbacA at 42 °C, respectively.

As shown in Table III, both wild-type and mutant strains contained similar levels of the two main nucleotide peptidoglycan precursors, UDP-GlcNAc and UDP-MurNAc-pentapeptide, when grown at 30 °C. However, a significant accumulation of the pool of UDP-MurNAc-pentapeptide, the last cytoplasmic peptidoglycan precursor and substrate of the C55-PP-dependent MraY translocase activity (Scheme 1), was observed in mutant cells, but not in wild-type cells, following a temperature shift to 42 °C (Table III). This was clearly correlated to the arrest of synthesis of BacA and depletion of the pool of C55-P. UDP-GlcNAc also somewhat accumulated after growth at 42 °C, but this was similarly observed in both types of cells and consequently not due to the mutation. It should be noted that UDP-GlcNAc is involved in other pathways and is in particular the substrate of the C55-P-dependent WeeA enzyme involved in O-antigen synthesis. The fact that UDP-GlcNAc did not accumulate to a greater extent in mutant cells was likely explained by its unrestricted consumption for the synthesis of the other cytoplasmic peptidoglycan precursors. Furthermore, we showed that the peptidoglycan content of the triple mutant cells was decreased by ~20% after growth at the restrictive temperature (Table III), demonstrating that the lytic phenotype was effectively due to the inhibition of the biosynthesis of at least this essential cell envelope component.

**Overexpression of ybjG, yeiU, pgpB, and bacA Genes Results in Increased Bacitracin Resistance**—As compared with Gram-positive species, E. coli and other Gram-negative species are not very sensitive to bacitracin, most likely because of the presence of the additional outer membrane barrier and limited uptake (20–22). For this reason, only limited variations were expected in sensitivity to this antibiotic of the different strains generated in this work. Growth of the wild-type strain BW25113 was unaffected for concentrations of bacitracin up to 90 units/ml; concentrations of 120 and 160 units/ml led to 5 and 0% survival, respectively. The different ybjG, yeiU, pgpB, and bacA single mutants and the ΔbacAΔpgpB double mutant did not show modified sensitivity to the antibiotic. The two other double mutants tested, ΔbacAΔybjG and ΔpgpBΔybjG, appeared slightly more susceptible than the parental strain because they resisted only up to 60 units of bacitracin/ml. Further introduction of the ΔyeiU mutation in the double mutant ΔbacAΔpgpB decreased its level of resistance from 90 to 60 units/ml. The effects of the overexpression of these different genes from the pTrc99A plasmid vector were also investigated. In the absence of IPTG, cells harboring either the plasmid vector or its derivatives carrying the genes ybjG, yeiU, pgpB, or bacA behaved similarly (5% survival at 120 units/ml). However, induction with 1 mM IPTG allowed cells overexpressing the genes ybjG, pgpB, or bacA to resist up to 200 units of bacitracin/ml (100% survival was observed in all cases at 160 units/ml, and 17, 20, and 70% survival was observed at 200 units/ml, respectively).

In the case of the pTrcYeiuU plasmid, an arrest of growth immediately followed the addition of IPTG, precluding analyses of the sensitivity of the corresponding strain to the antibiotic. The correlation existing between the levels of C55-PP phosphatase activity and the cell sensitivity to bacitracin was thus clearly confirmed and validated with the products of the three genes whose co-inactivation was required to affect E. coli cell viability, namely bacA, ybjG, and pgpB.

**Table III**

| Strain     | UDP-GlcNac | UDP-MurNAc-pentapeptide | Peptidoglycan |
|------------|------------|-------------------------|---------------|
| BW25113    | 230        | 625                     | 790           |
| BWTsbacA   | 350        | 680                     | 790           |
| 30 °C      | 42 °C      | 30 °C                   | 42 °C         |
| 10600      | 10500      | 8500                    | 8500          |

FIG. 2. Lytic phenotype of the thermosensitive triple mutant strain. BW25113 and BWTsbacA cells were grown exponentially at 30 °C in 2YT medium. At a cell density of ~10^7 cells/ml (zero time, A at 600 nm of 0.02), the temperature of the culture was either maintained at 30 °C or shifted to 42 °C. Growth of both strains at 30 °C, A, [] growth of BW25113 and BWTsbacA at 42 °C, respectively.
Multiple Undecaprenyl Pyrophosphate Phosphatases in E. coli

In bacteria, the C_{55}-P carrier lipid is required for the synthesis of a variety of cell wall polymers such as peptidoglycan, lipopolysaccharides, and teichoic acids (1–5), but curiously, its metabolism has been only partially elucidated to date. A cis-prenyl pyrophosphate synthase, UppS, first catalyzes the formation of its precursor, C_{55}-PP. Bacitracin, a cyclic polypeptide antibiotic, is known to inhibit this reaction through sequestration of the substrate C_{55}-PP, thereby provoking an arrest of cell wall synthesis and finally cell lysis (11–14). The first gene encoding a C_{55}-PP phosphatase was only recently identified (21). This gene had initially been named bacA by Cain et al. (19) because its overexpression resulted in bacitracin resistance, and these authors had hypothesized that it could encode a C_{55}-OH kinase activity. In fact, the recent purification to homogeneity and characterization of the BacA protein allowed us to unambiguously show that it exhibited C_{55}-PP phosphatase activity, but not C_{55}-OH kinase activity. We therefore proposed to rename this gene uppP for Undecaprenyl PyroPhosphate Phosphatase (21).

Considering the indispensable function of the bacA gene product, which is to generate the essential C_{55}-P lipid, the observation that this gene could be deleted from the chromosome without apparent effect on cell morphology and cell growth was clearly unexpected. In fact, we observed that the C_{55}-PP phosphatase activity was not completely abolished but reduced by 75% in a bacA mutant (21). This finding validated the newly established function of BacA and suggested that other protein species might be involved to catalyze such an activity and sustain the in vivo requirements for C_{55}-P molecules of cell wall synthesis. It could be hypothesized that such additional phosphatase(s) either are specific to C_{55}-PP or could have a different cellular function and act non-specifically on this substrate. Several genes encoding putative candidate proteins for this function were identified in the genome of E. coli.

All of these proteins were predicted to be integral membrane proteins and contained a characteristic phosphatase signature (40, 41) in their amino acid sequences (see Fig. 1). Two of these genes, ybjG and yeiU, had unknown functions to date, but the ppdB gene was already known to encode one of the two main phosphatidylglycerolphosphate phosphatases of E. coli (38, 39). PpgA, the other phosphatidylglycerolphosphate phosphatase, did not exhibit the same phosphatase motif. All of these different genes were disrupted individually in the chromosome without apparent effect on cell growth, and the construction of all combinations of double to quadruple mutants allowed us to demonstrate that the co-inactivation of genes bacA, ybjG, and ppdB was lethal. A thermosensitive conditional triple mutant, these three proteins in the synthesis of the essential C_{55}-P lipid, was lethal. A thermosensitive conditional triple mutant

As observed previously with bacA, the overexpression of genes ybjG and ppdB led to an increased level of C_{55}-PP phosphatase activity in E. coli cells, confirming that the products of these two genes effectively catalyze such an activity. This was in agreement with the finding that not only BacA, but also YbjG and PpgB proteins, participated in the total activity detected in wild-type cells and could efficiently complement a bacA mutation. The presence of an intact chromosomal copy of only one of the bacA, ybjG, and ppdB genes was sufficient to allow cell growth. Although the YeU protein also appeared to exhibit C_{55}-PP phosphatase activity, the inactivation experiments clearly showed that this gene alone was not able to sustain the cell growth requirements under the conditions tested. The purification and characterization of these different proteins is now required to determine and compare their respective specific activities and substrate specificity. This will help to discern phosphatase activities specifically involved in C_{55}-P metabolism from non- or less specific phosphatases of distinct metabolic function that can also use C_{55}-PP as a substrate. This also raises the question of how many specific C_{55}-PP phosphatases are expected to be involved in this process. C_{55}-PP is initially synthesized at the inner side of the cytoplasmic membrane, but it is also released at the outer side of the membrane during late polymerization steps of peptidoglycan biosynthesis (Scheme 1). Whether the dephosphorylation of C_{55}-PP occurs on both membrane sides or only on one side remains to be elucidated. Depending on the model considered, the involvement of two phosphatases with catalytic sites orientated toward the cytoplasm and the periplasm or of a single phosphatase could be envisaged. In both cases, a trans-bilayer movement of the carrier lipid should occur that could be mediated by a putative associated flippase.

As observed previously with bacA (19, 21), the overexpression of the different genes identified here was correlated to an increased resistance to bacitracin. As compared with Gram-positive bacterial species, E. coli is not very susceptible to this antibiotic, probably owing to the presence of the outer membrane and limited uptake. However, an increase of resistance was observed when cells were transformed with plasmids carrying the ybjG and ppdB genes. It had earlier been reported that the overexpression of the E. coli ybjG gene was associated with bacitracin resistance, but the C_{55}-PP activity of its product was not suspected at that time (20). Interestingly, two very close homologues of the ybjG gene product (∼52% identity) are encoded by the bcrC gene of B. licheniformis, the bacitracin-producing strain, and the ywoA gene of B. subtilis. The bcrC gene belongs to the bcrABC cluster of genes involved in the natural resistance of the strain to the antibiotic it produces (15, 16). It was demonstrated to have an essential role in the resistance process, but its function has never been elucidated (16). Similarly, the function of the B. subtilis ywoA gene product is unknown, but its overexpression was also shown to result in bacitracin resistance (35–37), and a hypothesis was recently made that this gene could encode a C_{55}-PP phosphatase activity (36). As both BcrC and YwoA proteins are predicted to be integral membrane proteins and carry the characteristic phosphatase signature, our results with YbjG clearly suggest that these two proteins should also display C_{55}-PP phosphatase activity.

Our data prove the involvement of more than one C_{55}-PP phosphatase in E. coli. It is not clear whether this situation will be similarly observed in other bacterial species. In this search, we identified genes of unknown function as well as genes with already assigned related function, all of them encoding putative or validated phosphatases. The need to inactivate several genes to block C_{55}-P synthesis does not indicate that all this machinery was dedicated to this function but only reveals that several enzymes can do the job at a rate sufficient to sustain growth of E. coli. Most probably, the number of C_{55}-PP phosphatase genes identified in a given bacterial species will be dependent on the specific activity and, more importantly, substrate specificity of the encoded enzymes. The PpgB phosphatidylglycerolphosphate phosphatase is here demonstrated to catalyze dephosphorylation of C_{55}-PP. This enzyme has a broad
substrate specificity, as it was earlier shown to also act on phosphatidic and lysophosphatidic acid (38, 39) as well as on diacylglycerol pyrophosphate (42). This raises the question of its exact primary physiological function. The other differential phosphatidylglycerolphosphate phosphatase, PgpA, is apparently more specific (38, 39). The fact that the pppA gene is intact in the thermosensitive triple mutant strain suggests that its product might not exhibit significant C55-PP phosphatase activity, if any. It should be noted that the amino acid sequence of PgpA does not contain the characteristic phosphatase motif or exhibit any obvious homology with that of PgpB, indicating that these two proteins are in fact quite different. As observed in E. coli, the bacA gene was demonstrated to be dispensable in S. aureus and S. pneumoniae (22), suggesting the involvement of additional C55-PP phosphatases also in these bacterial species. The absence of BacA in the latter pathogenic species was not, however, without any effect because a hypersensitivity to bacitracin and a reduced virulence in a mouse model of infection were observed. A decrease of the C55-PP phosphatase activity was consistent with the bacitracin hypersensitivity, and the reduced virulence of the mutant was likely because of a slowdown of the synthesis of some cell envelope components.

Our data indicate that two different classes of proteins can catalyze the reaction of dephosphorylation of C55-PP into C55-P in E. coli and probably other bacterial species: the BacA enzyme, on one hand, and several members from a family of phosphatases, on the other hand, all of which appear to be integral membrane proteins. The number of the latter could vary from one bacterial species to another, depending on their substrate specificity and ability to catalyze the formation of the carrier lipid at a rate compatible with cell viability. This essential step of C55-P metabolism was expected to be an interesting target in a search for new antibiotics. The present demonstration that different classes of enzymes participate in this process could make this search more problematic. The purification and further characterization of these different Upp enzymes will help to elucidate their respective physiological role in C55-P metabolism.

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