Serine/Threonine Protein Phosphatase-Mediated Control of the Peptidoglycan Cross-Linking L,D-Transpeptidase Pathway in Enterococcus faecium

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RESEARCH ARTICLE

Serine/Threonine Protein Phosphatase-Mediated Control of the Peptidoglycan Cross-Linking L,D-Transpeptidase Pathway in *Enterococcus faecium*

Emmanuelle Sacco,[a,b,c] Mélanie Cortes,[a,b,c] Nathalie Josseaume,[a,b,c] Louis B. Rice,[d] Jean-Luc Mainardi,[a,b,c,e] Michel Arthur[a,b,c]

INSERM, U1138, LRMA, Equipe 12 du Centre de Recherche des Cordeliers, Paris, France[a]; Université Pierre et Marie Curie, UMR S 1138, Paris, France[b]; Université Paris Descartes, Sorbonne Paris Cité, UMR S 1138, Paris, France[c]; Rhode Island Hospital, Brown University, Providence, Rhode Island, USA[d]; Assistance Publique-Hôpitaux de Paris, Service de Microbiologie, Hôpital Européen Georges Pompidou, Paris, France[e]

E.S. and M.C. contributed equally to this article.

ABSTRACT **The last step of peptidoglycan polymerization involves two families of unrelated transpeptidases that are the essential targets of β-lactam antibiotics. D,D-transpeptidases of the penicillin-binding protein (PBP) family are active-site serine enzymes that use pentapeptide precursors and are the main or exclusive cross-linking enzymes in nearly all bacteria. However, peptidoglycan cross-linking is performed mainly by active-site cysteine L,D-transpeptidases that use tetrapeptides in *Mycobacterium tuberculosis*, *Clostridium difficile*, and β-lactam-resistant mutants of *Enterococcus faecium*. We have investigated reprogramming of the *E. faecium* peptidoglycan assembly pathway by a switch from pentapeptide to tetrapeptide precursors and bypass of PBPs by L,D-transpeptidase Ldt_{fm}. Mutational alterations of two signal transduction systems were necessary and sufficient for activation of the L,D-transpeptidation pathway, which is essentially cryptic in wild-type strains. The first one is a classical two-component regulatory system, DdcRS, that controls the activity of Ldt_{fm} at the substrate level. As previously described, loss of DdcS phosphatase activity leads to production of the D,D-carboxypeptidase DdcY and conversion of the pentapeptide into the tetrapeptide substrate of Ldt_{fm}. Here we show that full bypass of PBPs by Ldt_{fm} also requires increased Ser/Thr protein phosphorylation resulting from impaired activity of phosphoprotein phosphatase StpA. This enzyme negatively controlled the level of protein phosphorylation both by direct dephosphorylation of target proteins and by dephosphorylation of its cognate kinase Stk. In combination with production of DdcY, increased protein phosphorylation by this eukaryotic-enzyme-like Ser/Thr protein kinase was sufficient for activation of the L,D-transpeptidation pathway in the absence of mutational alteration of peptidoglycan synthesis enzymes.**

IMPORTANCE **The mechanism of acquisition of high-level ampicillin resistance involving bypass of the penicillin-binding proteins (PBPs) by L,D-transpeptidase Ldt_{fm} was incompletely understood, as production of tetrapeptide precursors following transcriptional activation of the *ddc* locus by the DdcRS two-component regulatory system was necessary but not sufficient for full activation of the L,D-transpeptidation pathway. Here, we identified the release of a negative control of Ser/Thr protein phosphorylation mediated by phosphatase StpA as the additional factor essential for ampicillin resistance. Thus, bypass of PBPs by Ldt_{fm} requires the modification of signal transduction regulatory systems without any gain of function by mutational alteration of peptidoglycan biosynthetic enzymes. In contrast, previously characterized mechanisms of antibiotic resistance involve horizontal gene transfer and mutational alteration of drug targets. Activation of the L,D-transpeptidation pathway reported in this study is an unprecedented mechanism of emergence of a new metabolic pathway since it involved the recruitment of preexisting functions following modifications of regulatory circuits.**

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Address correspondence to Michel Arthur, michel.arthur@crc.jussieu.fr.

Enterococcus faecium and *Enterococcus faecalis* are commensal bacteria but also important nosocomial pathogens (1). These bacteria have an unusually high capacity for resistance to antibiotics since they combine several mechanisms of intrinsic resistance with the acquisition of resistance determinants by horizontal gene transfer (2–4). Intrinsic β-lactam resistance is mediated by species-specific low-affinity penicillin-binding proteins (PBPs), such as PBP5, that are thought to be sufficient for peptidoglycan cross-linking under conditions in which the D,D-transpeptidase catalytic domain of all other PBPs is inactivated by β-lactams (5–7). Although β-lactam resistance is conveyed by a single PBP in the enterococci, expression of resistance depends upon additional host factors (8, 9). In *E. faecalis*, factors essential for PBP5-mediated resistance to β-lactams of the cephalosporin

class have been detected by genetic analyses but their functions remain largely unknown. These factors include the CroRS two-component regulatory system, which is thought to regulate genes essential for PBP5 activity, although none of the targets of CroRS-mediated transcriptional regulation identified so far has a demonstrated role in peptidoglycan synthesis or cephalosporin resistance (10–13). A serine/threonine protein kinase (IreK) was identified as essential for ceftriaxone resistance in *E. faecalis* (14). Subsequent characterization of the associated protein phosphatase, IreP, showed that the phosphorylation of one or several IreK substrates is essential for cephalosporin resistance (15). Recently, a substrate of IreK and IreP, designated IreB, has been identified and shown to negatively control cephalosporin resistance (16). Additional proteins essential for ceftriaxone resistance include bifunctional transglycosylase-transpeptidase (class A) PBPs for elongation of glycan chains, either PonA or PBPF (17, 18), one of the two *E. faecalis* MurA synthetases (UDP-*N*-acetylglucosamine 1-carboxyvinyl transferase) performing the first committed stem of peptidoglycan synthesis (19), and alanine transferase of the Fem family for synthesis of the L-Ala–L-Ala side chain of peptidoglycan precursors (20). Genome-wide identification of ampicillin resistance determinants in *E. faecium* identified a different set of proteins contributing to intrinsic β-lactam resistance, including the L,D-transpeptidase Ldt_{fm}, the D,D-carboxypeptidase DdcP, and the glycosyltransferase Pgt (21).

In previous studies, we have identified a PBP5-independent β-lactam resistance mechanism in *E. faecium* based on *in vitro* selection of mutants resistant to ampicillin (a β-lactam of the penam class) (22). We started from *E. faecium* D344S, a derivative of *E. faecium* D344R that had spontaneously lost the *pbp5* gene (22). Parental strain D344S is hypersusceptible to ampicillin because of the absence of low-affinity PBP5 (22). In five consecutive selection steps with increasing concentrations of ampicillin, we obtained five mutants, M1, M2, M3, M4, and M512, that gradually acquired high-level ampicillin resistance (22, 23). In mutant M512, 4→3 cross-links formed by PBPs were replaced by 3→3 cross-links generated by an ampicillin-insensitive L,D-transpeptidase, Ldt_{fm} (22, 24). This enzyme cleaves the L-Lys^3–D-Ala^4 peptide bond of the acyl donor and links the carbonyl group of L-Lys^3 to the acyl acceptor (24). Ldt_{fm} and PBPs were found to be structurally unrelated and to harbor different active-site residues for nucleophilic attack of the carbonyl group of the acyl donor (Cys versus Ser, respectively) (24–26). Ldts and PBPs also differ in their spectra of inactivation by β-lactams (27). PBPs are potentially inactivated by all β-lactams, including members of the penam (ampicillin), cephalosporin (ceftriaxone), carbapenem (imipenem), and monobactam (aztreonam) classes, depending upon the specific β-lactam and PBP under consideration (28). In contrast, L,D-transpeptidases are efficiently inactivated by a single class of β-lactams, the carbapenems (27, 29).

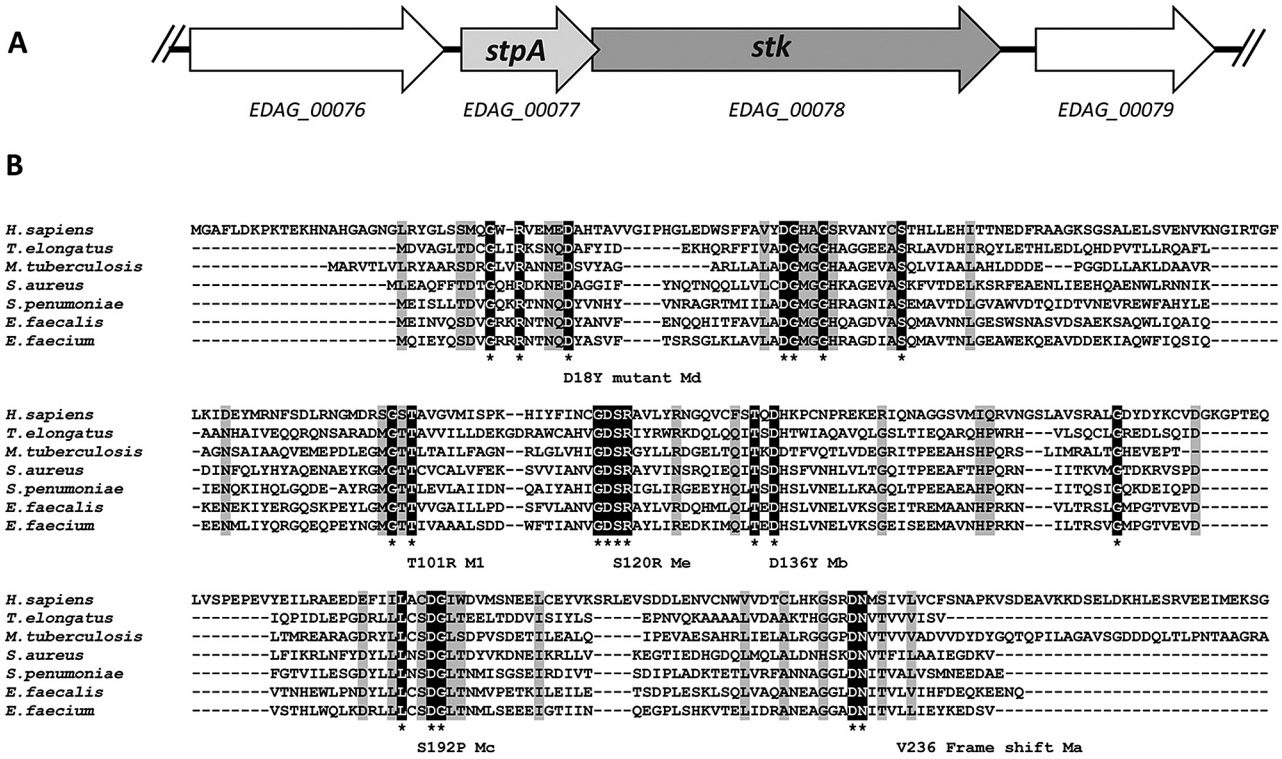
The proportion of 3→3 cross-links generated by Ldt_{fm} is very low in wild-type strains of *E. faecium* (22). Ldt_{fm} is constitutively produced by parental strain D344S (23, 30), but the participation of the L,D-transpeptidase to peptidoglycan cross-linking is marginal since this enzyme requires a tetrapeptide stem in the acyl donor substrate (24) instead of the pentapeptide stem assembled in the cytoplasm (31). Thus, activation of the L,D-transpeptidation pathway requires pentapeptide-to-tetrapeptide conversion (23). The corresponding enzyme, DdcY, was identified as a metallo-D,D-carboxypeptidase that cleaves the C-terminal residue (D-Ala^5) of a UDP-MurNAc pentapeptide to generate the precursor of the L,D-transpeptidation pathway (30). DdcY is encoded by a cryptic locus, *ddc*, that is sporadically disseminated in *E. faecium* strains and encodes a two-component regulatory system (DdcRS) in addition to DdcY (30). Activation of the *ddc* locus in M512 results from a mutation in the *ddcS* sensor kinase gene that impairs the phosphatase activity of the enzyme and leads to constitutive expression of the locus (30). In this investigation, we identified a second locus involved in the activation of the L,D-transpeptidation pathway, which encodes a eukaryotic-enzyme-like serine/threonine protein kinase (Stk) and its cognate phosphatase (StpA). We show that decreased protein dephosphorylation by StpA and the previously characterized activation of the *ddc* locus are both sufficient and necessary for high-level β-lactam resistance mediated by L,D-transpeptidation in *E. faecium*.

RESULTS

Identification of mutations necessary and sufficient for high-level ampicillin resistance. We have previously shown that acquisition of high-level resistance to ampicillin by mutant M512 requires activation of the *ddc* locus, which encodes the D,D-carboxypeptidase DdcY, for formation of the tetrapeptide-containing substrate of the L,D-transpeptidase (30). In mutant M512, activation of the locus results from a $T^{161}A$ substitution in the sensor kinase DdcS that impairs the phosphatase activity of the protein (30). Here, we show that acquisition of ampicillin resistance involves the mutational alteration of a second locus encoding a putative serine/threonine kinase (Stk) and a phosphatase (StpA) (Fig. 1A). The mutation was detected in mutant M1 (first selection step) and leads to a Thr-to-Arg substitution at position 101 of StpA, which is invariant in related phosphatases (Fig. 1B).

Acquisition of the *stpA* mutation by strain D344S was associated with a moderate increase in the MIC of ampicillin (from 0.06 to 0.5 μg/ml) (Fig. 1C). In order to evaluate the contributions of DdcY and StpA to resistance, the *ddcY* gene was cloned under the control of constitutive promoter P2 of expression vector pJEH11 and the resulting plasmid (pJEH11Ω*ddcY*) was introduced into D344S. Production of the D,D-carboxypeptidase in this host resulted in a moderate level of resistance to ampicillin (MIC, 0.5 μg/ml). In contrast, plasmid pJEH11Ω*ddcY* conferred high-level resistance on mutant M1 (MIC, >2,000 μg/ml). Thus, the $T^{101}R$ substitution in StpA and production of DdcY were both necessary and sufficient for high-level ampicillin resistance.

Selection of high-level ampicillin resistance in a D,D-carboxypeptidase-producing background. Spontaneous mutants of D344S/pJEH11Ω*ddcY* were obtained on agar plates containing ampicillin (4 or 8 μg/ml) at a frequency of ca. 5×10^{-7}. The characterization of a total of five independent mutants in each case revealed the expression of high-level ampicillin resistance (MIC, >2,000 μg/ml) and the presence of a mutation in *stpA* (mutants Ma to Me, Fig. 1B and C). The mutations led to amino acid substitutions in four mutants and to a frameshift mutation in the remaining mutant. Three of the four substitutions affected invariant residues, and the remaining substitution ($S^{192}P$) was located within a highly conserved motif (Fig. 1B). The frameshift mutation resulted from a base deletion in codon 236 located close to the 3′ end of the *stpA* gene (246 codons), which encodes a conserved region of the protein. These results indicate that mutations in *stpA* are reproducibly selected by ampicillin in a D,D-carboxypeptidase-producing background.



C

Strain	Chromosomal mutation (amino acid substitution or frame shift)		Plasmid	MIC of ampicillin (μg/ml)
D344S	none	(none)	none	0.06
D344S	none	(none)	pJEH11Ω*ddcY*	0.5
D344S	none	(none)	pJEH11Ω*stk*	0.5
M1	*stpA* $C^{302}G$	(StpA $T^{101}R$)	none	0.5
M1	*stpA* $C^{302}G$	(StpA $T^{101}R$)	pJEH11Ω*ddcY*	>2000
Ma	*stpA* ΔT^{708}	(frame shift)	pJEH11Ω*ddcY*	>2000
Mb	*stpA* $G^{406}T$	(StpA $D^{136}Y$)	pJEH11Ω*ddcY*	>2000
Mc	*stpA* $T^{574}C$	(StpA $S^{192}P$)	pJEH11Ω*ddcY*	>2000
Md	*stpA* $G^{52}T$	(StpA $D^{18}Y$)	pJEH11Ω*ddcY*	>2000
Me	*stpA* $C^{360}A$	(StpA $S^{120}R$)	pJEH11Ω*ddcY*	>2000

FIG 1 Roles of *ddcY* and *stpA* in ampicillin resistance. (A) Map of the *E. faecium* locus encoding the protein phosphatase StpA and the serine/threonine kinase Stk. (B) Sequence alignment of protein phosphatases. Identical and conserved amino acids are highlighted in black and gray, respectively. The positions of amino acid substitutions detected in the mutants are indicated below the alignment. In mutant Ma, a frameshift was detected in codon 236 of *stpA*. (C) MICs of ampicillin for *E. faecium* D344S and resistant mutants.

Modulation of the ampicillin resistance level by the balance between kinase and phosphatase activities. Overproduction of Stk in D344S following the introduction of plasmid pJEH11Ω*stk* increased the MIC of ampicillin from 0.06 to 0.5 μg/ml (Fig. 1C). Thus, the resistance phenotypes resulting from overproduction of Stk (D344S/pJEH11Ω*stk*) and impaired StpA phosphatase activity (mutant M1) were similar. This observation implies that the level of ampicillin resistance increases with the level of serine and threonine phosphorylation. In agreement, expression of a functional copy of *stpA* impaired the expression of ampicillin resistance in mutant M512 (data not shown). Thus, the level of ampicillin resistance is modulated by a balance between kinase and phosphatase activities.

***In vitro* activities of purified Stk and StpA.** A soluble fragment of the kinase Stk (residues 1 to 338) was produced in *Escherichia coli*, purified, and analyzed by Western blotting. Recombinant Stk was purified as a phosphoprotein, as shown by the detection of phosphorylated proteins (Fig. 2A) or phosphothreonine

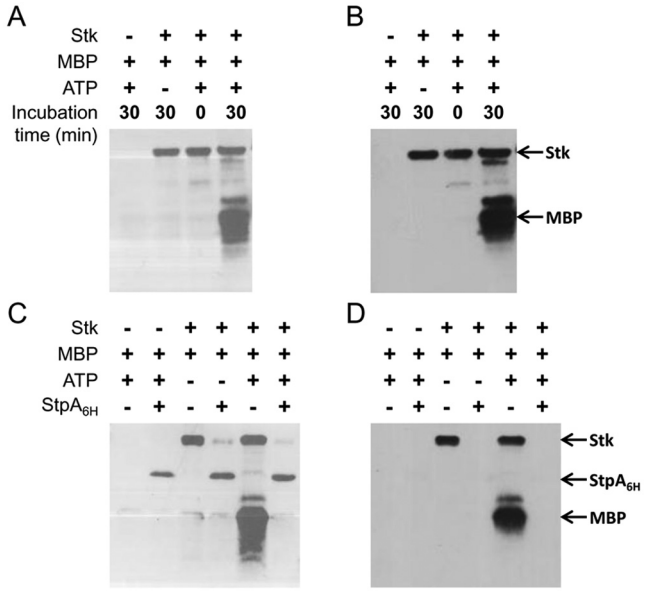


FIG 2 In vitro activities of Stk (A and B) and StpA (C and D). Phosphoproteins were revealed with a commercial kit (Pro-Q; Invitrogen) (A and C) or with phosphothreonine-specific antibodies (B and D).

residues (Fig. 2B). Mass spectrometry analyses revealed several forms of the proteins containing one to five phosphate groups (see Fig. S1A and B in the supplemental material). StpA fully dephosphorylated Stk, as shown by the complete disappearance of the phosphorylated forms of the kinase by Western blotting (Fig. 2C and D) and mass spectrometry (see Fig. S1 in the supplemental material). Upon incubation with ATP, purified Stk phosphorylated myelin basic protein (MBP) (Fig. 2A and B). The phosphoprotein bands corresponding to phospho-MBP disappeared upon the addition of the phosphatase StpA. These results indicate that StpA and Stk display the protein phosphatase and kinase activities inferred from sequence similarity. These results also indicate that kinase Stk is a substrate of StpA.

Autophosphorylation of purified Stk *in vitro*. Since recombinant Stk was purified as a phosphoprotein from *E. coli* extracts, Stk was dephosphorylated *in vitro* by the addition of StpA in a 1:10 phosphatase-to-kinase ratio (see Fig. S2 in the supplemental material). The phosphatase StpA was removed by size exclusion chromatography, and the absence of phosphate groups from purified Stk was confirmed by Western blotting and mass spectrometry analyses. Incubation of dephosphorylated Stk with ATP and Mn^{2+} resulted in the phosphorylation of Stk. The reaction led to a monophosphorylation of Stk, whereas the protein recovered from *E. coli* extracts contained multiple phosphate groups (compare Fig. S1 and S2 in the supplemental material). These results indicate that Stk catalyzes the phosphorylation of at least one of its threonine residues. The difference between the phosphorylation patterns observed *in vitro* and *in vivo* indicates that *E. coli* might produce an unknown kinase responsible for Stk phosphorylation, although this host does not produce any Stk homologue (32).

Impact of *stpA* mutations on the *in vitro* activity of the phosphatase. Recombinant forms of StpA detected in ampicillin-resistant mutants M1, Ma, Mb, Mc, Md, and Me were produced in *E. coli*. The truncated form of StpA (mutant Ma) and StpA containing the $S^{192}P$ substitution (mutant Mc) were not soluble and could not be functionally characterized. Purification of the four remaining proteins afforded soluble enzymes with reduced phosphatase activity (M1, Mb, and Me) or no detectable activity (Md) (Table 1). The amino acid substitutions detected in the former mutants mainly led to lower k_{cat} values with marginal impacts on the K_m of *para*-nitrophenylphosphate (pNPP). The values of these catalytic constants were determined in the presence of a high concentration of Mn^{2+} (2 mM). Further analysis performed with the wild-type enzyme showed that Mn^{2+} is essential for activity and that this cation cannot be replaced by Mg^{2+}, Ca^{2+}, or Zn^{2+} (data not shown). The phosphatase activity increased with the Mn^{2+} concentration, and a relatively high concentration of the cation ($\geq$2 mM) was required for maximal activity (see Fig. S3 in the supplemental material). Reduction of the Mn^{2+} concentration from 2 mM to 50 μM led to a 100-fold decrease in the catalytic efficiency of wild-type StpA (Table 1). Reduction of the Mn^{2+} concentration also reduced the activity of StpA from mutants Mb and Me. Together, these results indicated that the mutations detected in the *stpA* gene from ampicillin-resistant mutants impaired the phosphatase activity of the protein, as suggested by the positions of the mutations. Of note, sufficient Mn^{2+} is expected to be present *in vivo* for metalation of StpA since brain heart infusion (BHI) broth contains 20 μM Mn^{2+} (33) and *E. faecium* accumulates Mn^{2+} because of active uptake (34, 35).

StpA and Stk do not control expression of the *ddc* locus or ldt_{fm} L,D-transpeptidase gene. We have previously shown that the *ddc* locus encoding DdcY (D,D-carboxypeptidase) and DdcRS (two-component regulatory system) is cryptic in parental strain D344S (30). Activation of the locus in mutant M512 is due to the $T^{161}A$ substitution in DdcS, which impairs the phosphatase activ-

TABLE 1 Catalytic constants for pNPP hydrolysis by StpA from parental strain D344S and ampicillin-resistant mutants

Substitution in StpA (strain)	Catalytic constant[a] in presence of Mn^{2+} at:					
	2 mM			50 μM		
	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})	K_m (mM)	k_{cat} (min^{-1})	k_{cat}/K_m (min^{-1} mM^{-1})
None (D344S)	0.66 ± 0.09	1,200 ± 100	1,800 ± 290	0.69 ± 0.17	12 ± 1	17 ± 4.5
$T^{101}R$ (M1)	1.2 ± 0.2	0.83 ± 0.04	0.69 ± 0.12	ND[b]	ND	ND
$D^{136}Y$ (Mb)	1.8 ± 0.5	140 ± 15	80 ± 23	1.5 ± 0.4	0.09 ± 0.01	0.06 ± 0.02
$D^{18}Y$ (Md)	ND	ND	<0.003	ND	ND	ND
$S^{120}R$ (Me)	1.3 ± 0.1	28 ± 0.1	22 ± 1.6	2.6 ± 0.7	0.23 ± 0.02	0.09 ± 03

[a] Regression values ± standard errors were obtained by fitting experimental data to the Michaelis-Menten equation $V = k_{cat}\,ES/(K_m + S)$, where V is the initial velocity and E and S are the initial enzyme and substrate concentrations, respectively.
[b] ND, not determined.

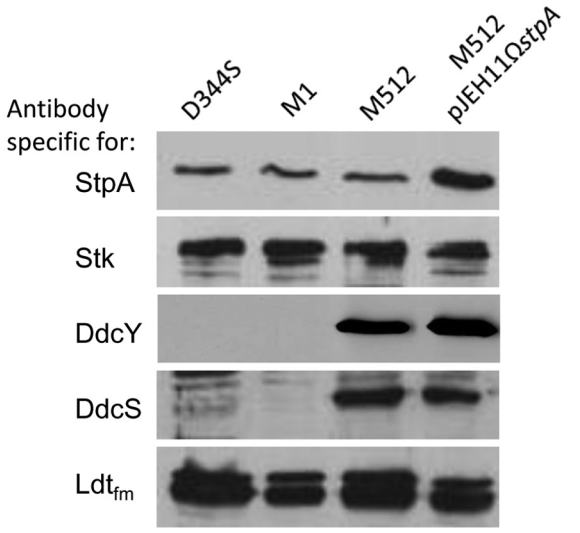


FIG 3 Level of production of proteins encoded by the *stpA-stk*, *ddc*, and ldt_{fm} loci in *E. faecium* strains. Crude *E. faecium* extracts were analyzed by Western blotting with polyclonal antibodies raised against the protein phosphatase StpA, the serine/threonine kinase Stk, the D,D-carboxypeptidase DdcY, the sensor kinase DdcS, and the L,D-transpeptidase Ldt_{fm}.

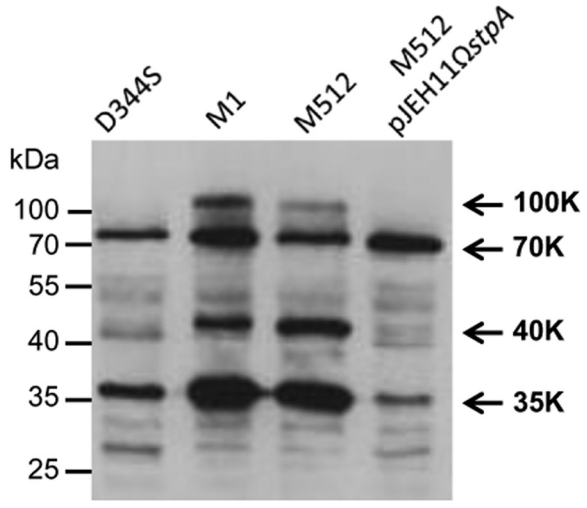


FIG 4 Western blot analysis of phosphoproteins in crude *E. faecium* extracts. Phosphoproteins were detected with antiphosphothreonine polyclonal antibodies. Arrows indicate the positions of the main phosphoproteins (100, 70, 40, and 35 kDa), which were named according to their relative electrophoretic mobility.

ity of the sensor kinase. Here we show that expression of the *ddc* locus is not under StpA control since DdcY and DdcS were not detected in extracts of D344S and M1, whereas the two proteins were produced at similar high levels by M512 and M512/pJEH11Ω*stpA* (Fig. 3). Likewise, overproduction of Stk in D344S did not lead to any increase in the levels of DdcY and DdcS production (data not shown). Production of the L,D-transpeptidase Ldt_{fm} was not affected by impaired StpA and DdcS phosphatase activities (Fig. 3) or by Stk overproduction (data not shown). These results show that the contribution of impaired StpA phosphatase activity or increased Stk activity to ampicillin resistance did not depend upon increased production of the L,D-transpeptidase Ldt_{fm} or activation of the *ddc* locus for production of the tetrapeptide substrate of this cross-linking enzyme.

***In vivo* activity of StpA.** Western blot analysis of *E. faecium* protein extracts was performed to determine whether the *in vivo* production of StpA affects the level of threonine phosphorylation. The intensity of three protein bands detected by antiphosphothreonine antibodies (100, 40, and 35 kDa) was increased in mutant M1 (StpA $T^{101}R$) in comparison to that in parental strain D344S (StpA) (Fig. 4). As expected, the phosphoprotein patterns were similar in M1 and M512, whereas production of StpA by M512/pJEH11Ω*stpA* led to a decrease in the intensity of the same set of phosphoprotein bands. These results indicate that StpA negatively controls the level of threonine phosphorylation *in vivo*.

***In vitro* dephosphorylation of phosphoproteins by purified StpA.** Comparison of the level of protein phosphorylation in crude *E. faecium* extracts (Fig. 4) does not allow determination of whether the negative control mediated by StpA relies on dephosphorylation of phosphoproteins or an indirect effect involving negative control of the kinase activity of Stk. In order to assay directly for the phosphoprotein phosphatase activity of StpA, *E. faecium* extracts were incubated with purified StpA and analyzed by Western blot assay (Fig. 5A). The assay was performed with extracts from D344S, M1, M512, and D344S/pJEH11Ω*stk*. The latter strain was included to maximize the phosphorylation of putative StpA substrates because of high-level production of the Ser/Thr kinase. Moderate overproduction of Stk by D344S/pJEH11Ω*stk* was experimentally established by the increased intensity of a 100-kDa protein band detected with anti-Stk antibodies (Fig. 5B) and detection of recombinant Stk with antipolyhistidine antibodies (Fig. 5C). The 100-kDa phosphoprotein band (Fig. 5A) had the same electrophoretic mobility as Stk (Fig. 5B) and recombinant Stk containing a 6×His tag (Fig. 5C). Purification of Stk from *E. faecium* D344S/pJEH11Ω*stk* (see Fig. S4 in the supplemental material) indicated that the 100-kDa phosphoprotein band corresponds to Stk.

Incubation of the extracts with purified StpA resulted in a decrease in the intensity of the Stk 100-kDa phosphoprotein band (Fig. 5A). This decrease resulted from dephosphorylation of phospho-Stk by StpA, as previously shown for purified StpA and Stk (Fig. 2). Dephosphorylation of the phosphoprotein bands at 40 and 35 kDa by StpA was not detected (Fig. 5A). Thus, modulation of the phosphorylation levels of these proteins by StpA is indirect and may involve a negative control of the kinase activity of Stk. Upon incubation with StpA, the intensity of the 70-kDa phosphoprotein band decreased in all four extracts. Thus, the phosphoprotein band at 70 kDa contains a substrate of StpA. These results suggest that a subset of the proteins phosphorylated by Stk can be dephosphorylated by StpA.

DISCUSSION

The last step of peptidoglycan polymerization involves two families of unrelated transpeptidases of DD and LD specificities that catalyze the formation of 4→3 and 3→3 cross-links (36). The relative contributions of the two enzyme types greatly vary among bacterial species and may also vary along the cell cycle. In *E. coli*, two L,D-transpeptidases catalyze the formation of a minority (ca. 5%) of the cross-links during the exponential growth phase but the contribution of L,D-transpeptidases to peptidoglycan cross-linking increases in the stationary phase (13%) (37, 38). In *Myco-*

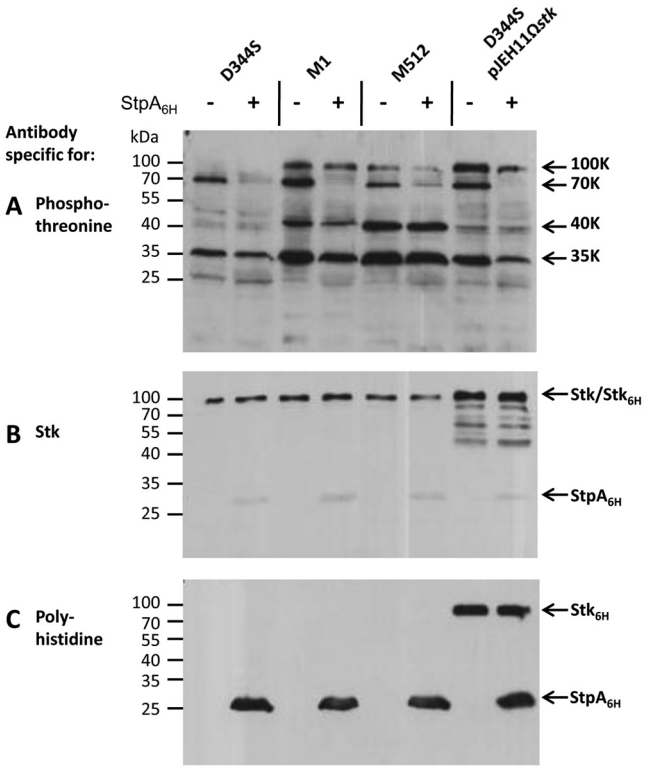


FIG 5 Dephosphorylation of *E. faecium* phosphoproteins by purified StpA. Proteins from crude cell extracts (10 μg) were incubated with (+) or without (−) purified phosphatase StpA (3 μg) for 30 min at room temperature. Proteins were separated by SDS-PAGE and transferred onto PVDF membranes. Phosphoproteins were detected with antiphosphothreonine (A), anti-Stk (B), and antipolyhistidine (C) antibodies. Arrows indicate the positions of the main phosphoproteins (100, 70, 40, and 35 kDa).

bacterium tuberculosis (39, 40) and *M. abscessus* (41), L,D-transpeptidation is the predominant mode of peptidoglycan cross-linking in both growth phases (70 to 80%), although L,D-transpeptidase paralogs appear to be differently regulated during the cell cycle (42, 43). In wild-type *E. faecium*, the L,D-transpeptidase Ldt_{fm} is constitutively produced but the enzyme makes a marginal contribution to peptidoglycan cross-linking (3%) (23, 30). The activity of Ldt_{fm} is controlled at the substrate level by the production of the D,D-carboxypeptidase DdcY, which cleaves the C-terminal D-Ala of the stem pentapeptide to form the essential tetrapeptide donor of the L,D-transpeptidation reaction (23, 30). In contrast to the ldt_{fm} gene, which is present in all of the *E. faecium* isolates that have been examined (24), the *ddcY* and upstream *ddcRS* genes are present in only a minority of the isolates belonging to this species (30). The *ddc* locus is cryptic, and its activation results from the loss of the phosphatase activity of the DdcS sensor kinase in response to selection for ampicillin resistance (30). Production of DdcY is not sufficient for the complete bypass of PBPs by Ldt_{fm} since the production of DdcY was associated with an only moderate (8-fold) increase in the ampicillin MIC (Fig. 1C). In this study, we show that the full bypass of PBPs also requires an increase in Ser/Thr protein phosphorylation resulting from impaired StpA phosphatase activity. Association of a mutation in *stpA* with expression of *ddcY* was necessary and sufficient for high-level ampicillin resistance (Fig. 1C). In support of this conclusion, we constructed strains that sequentially acquired a mutation in *stpA* and a plasmid encoding DdcY in both orders (Fig. 1C). The *stpA* mutations led to impaired phosphatase activity, as inferred from their nature and positions (Fig. 1B) and a direct assay of StpA phosphatase activity (Table 1). In agreement, homologous residues of the phosphatase Stp from *Streptococcus agalactiae* were located in the enzyme-active site (44) (see Fig. S5 in the supplemental material). Amino acid substitutions in StpA from *E. faecium* mutants led to >200-fold reductions in k_{cat} for hydrolysis of pNPP in the presence of 50 μM $MnCl_2$. The impact of the $D^{136}Y$ and $S^{120}R$ substitutions was less (43-fold and 7-fold reductions, respectively) at the nonphysiological Mn^{2+} concentration of 2 mM. This observation suggests that substitutions $D^{136}Y$ and $S^{120}R$ impaired Mn^{2+} binding. Together, these results indicate that amino acid substitutions in StpA affected the chemical step of the dephosphorylation reaction rather than substrate recognition. Thus, decreased protein dephosphorylation was essential for acquisition of ampicillin resistance.

Several assays were used to study the protein kinase and phosphatase activities of purified Stk and StpA. First, we showed that Stk catalyzes its own phosphorylation on Thr residues and subsequently transfers the phosphate group to a model protein (MBP) (Fig. 2; see Fig. S2 in the supplemental material). Phospho-Stk and phospho-MBP were dephosphorylated upon the addition of StpA (Fig. 2). This first assay established that Stk and StpA display protein kinase and phosphatase activities, respectively, as expected from sequence similarity to previously characterized enzymes (45). In a second assay, we compared the level of protein phosphorylation in crude cell extracts from *E. faecium* strains harboring different alleles of *stpA* (Fig. 4). This analysis revealed that impaired StpA activity increased the phosphorylation of several phosphoprotein bands (Fig. 4), including Stk (Fig. 5), indicating that StpA negatively modulates the level of protein phosphorylation *in vivo*. A third assay was used to determine whether StpA directly dephosphorylates proteins. In this assay, crude cell extracts were prepared from an *E. faecium* strain harboring various alleles of *stpA* and from an additional Stk-overproducing strain to maximize protein phosphorylation (Fig. 5). Incubation of the extracts resulted in the decreased phosphorylation of three phosphoprotein bands (100, 70, and 35 kDa). The extent of phosphorylation of a 40-kDa protein was greater in mutants with impaired phosphatase activity, but the intensity of the phosphoprotein band did not decrease upon incubation of the extract with purified StpA *in vitro*. For this protein band, modulation of the level of phosphorylation appears to depend upon the negative control of Stk kinase activity by StpA. Other phosphoproteins were directly dephosphorylated by StpA. For these phosphoproteins, negative control of the level of phosphorylation mediated by StpA may involve both protein dephosphorylation and negative control of the kinase Stk.

Together, our results show that the mutational alteration of two signal transduction systems is necessary and sufficient for reprogramming of the peptidoglycan assembly pathway by the production of tetrapeptide-containing precursors. The first signal transduction system is a classical two-component regulatory system, DdcRS, that controls the production of DdcY for conversion of pentapeptide into tetrapeptide precursors as previously described (30). The second system, StpA-Stk, controls the level of phosphorylation of several proteins. Several lines of evidence indicate that increased protein phosphorylation resulting from the

mutation of *stpA* was required for resistance although this did not affect the level of DdcY production. First, expression of *ddcY* under the control of a heterologous promoter in plasmid pJEH11 was sufficient for high-level ampicillin resistance (MIC, >2,000 g/ml) in mutant M1, which produced a derivative of StpA with reduced phosphatase activity, but not in the parental strain D344S, which harbors a wild-type copy of *stpA* (Fig. 1C). Thus, production of DdcY and impaired phosphatase activity were both required for ampicillin resistance. Second, Western blot analysis indicated that DdcY was produced at the same level by derivatives of D344S and M1 harboring pJEH11Ω*ddcY*, indicating, as might have been expected, that the heterologous promoter of vector pJEH11 is functional in both hosts independently of the *stpA* allele (see Fig. S6 in the supplemental material). Conversely, DdcY was not produced by D344S and M1, indicating that the *stpA* mutation present in M1 did not lead to transcriptional activation of the chromosomal copy of *ddcY*. Thus, the essential role of the *stpA* mutation did not depend upon DdcY production. In agreement, derivatives of D344S/pJEH11Ω*ddcY* selected on ampicillin (mutants Ma to Me) harbored mutations in *stpA* (Fig. 1C) that impaired the phosphatase activity of StpA (Fig. 1B and Table 1) and did not affect the level of DdcY production (see Fig. S6 in the supplemental material). Third, acquisition of a mutation in *stpA* by M1 and mutants Ma to Me did not result in the production of DdcS (Fig. 3; see Fig. S6 in the supplemental material). Thus, the chromosomal locus *ddc* was not regulated in response to modulation of protein phosphorylation by StpA. Together, these results establish that alterations of DdcRS and StpA-Stk are both required for resistance and that these signal transduction systems control the activity of distinct sets of proteins.

Production of DdcY resulting from the introduction of plasmid pJEH11Ω*ddcY* into M1 or loss of DdcS phosphatase activity by M512 resulted in similar resistance phenotypes. Thus, DdcY is the only target of DdcRS-mediated regulation that is essential for ampicillin resistance. In contrast, StpA may affect the level of phosphorylation of multiple proteins potentially involved in resistance, as shown by modulation of the level of phosphorylation of the 100-, 70-, 40-, and 35-kDa protein bands (Fig. 4 and 5). Furthermore, additional fainter phosphoprotein bands were detected by anti-phospho-Thr antibodies upon prolonged exposure of Western blots (data not shown) and Ser phosphorylation was not investigated. In agreement, phosphoproteome analyses of *Firmicutes* suggest that the number of Ser/Thr phosphoproteins may be on the order of 100 to 200 in *E. faecium* (46, 47). The role of protein phosphorylation in peptidoglycan synthesis and ampicillin resistance remains to be determined.

It is striking that activation of the L,D-transpeptidation pathway in *E. faecium* involves modifications of representatives of the two main signal transduction pathways of prokaryotes, the two-component regulatory system relying on His-to-Asp phosphotransfer (48) and the one-component eukaryotic-enzyme-like Ser/Thr phosphorylation system (45), in the absence of modification of cell wall biosynthesis proteins. In contrast, previously characterized mechanisms of acquisition of antibiotic resistance involve horizontal gene transfer, for example, *van* clusters for vancomycin resistance (49), or mutational alteration of an antibiotic target, for example, PBP5, in *E. faecium* (8, 9). Acquisition of high-level ampicillin resistance resulting from bypass of the PBPs by Ldt_{fm} was found here to depend upon the alteration of regulatory circuits without any gain of function through gene transfer or modification biosynthetic enzymes. This is an unprecedented example of the emergence of a new metabolic pathway through the recruitment of functions via the modification of regulatory circuits.

MATERIALS AND METHODS

Bacterial strains, selection of mutants, and antibiotic susceptibility assay. All cultures were performed at 37°C in BHI agar or broth (Difco Laboratories). Mutants M1 and M512 were derived from *E. faecium* D344S by serial selection on medium containing increasing concentrations of ampicillin (22, 23). Derivatives of pJEH11 were introduced into strains of *E. faecium* by conjugation (17) and electroporation (18) as previously described, except that a field of 1 kV (2-mm gap) and a shunt resistance of 1,000 Ω were used for the latter technique. Transformants and transconjugants were selected on gentamicin (128 μg/ml). Mutants Ma to Me were obtained by plating D344S/pJEH11Ω*ddcY* on agar containing ampicillin at 4 or 8 μg/ml. Colonies appeared after 5 days of incubation at a frequency of about 5×10^{-7}. MICs of ampicillin (Bristol-Myers, Paris, France) were determined by the agar dilution method after 48 h of incubation (23).

Plasmid construction. For a description of the construction of recombinant plasmids encoding the *E. faecium* D,D-carboxypeptidase DdcY, serine/threonine kinase Stk, serine/threonine phosphatase StpA, see Text S1 in the supplemental material.

Production and purification of kinase Stk and phosphatase StpA. Recombinant Stk and StpA containing a C-terminal 6×His tag were produced in *E. coli* BL21(DE3). Bacteria were grown in BHI broth containing ampicillin (100 μg/ml) at 37°C to an optical density at 600 nm of 0.9. Protein production was induced with 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 19 h at 16°C. Cells were disrupted by sonication in 40 ml of 50 mM Tris-HCl (pH 8.0) containing 300 mM NaCl, and cells debris was removed by centrifugation. Proteins were purified by affinity chromatography on Ni^{2+}-nitrilotriacetate (Sigma). Elution was performed with 500 mM imidazole in 50 mM Tris buffer (pH 8.0) containing 300 mM NaCl. Proteins were further purified by size exclusion chromatography on a Superdex 75 HL26/60 column (GE Healthcare) equilibrated with 50 mM Tris-HCl (pH 8.0) containing 150 mM NaCl. Stk and StpA eluted as single peaks corresponding to monomers. Proteins were stored at −20°C in the same buffer supplemented with 50% glycerol.

Western blot analysis. Polyclonal anti-DdcY and anti-DdcS rabbit antisera were previously described (30). Anti-Ldt_{fm}, anti-Stk, and anti-StpA antibodies were obtained by three subcutaneous injections at 2-week intervals of 500 μg of purified protein (24) into rats for Ldt_{fm} or into rabbits for Stk and Stp. Antiphosphothreonine rabbit antibodies were purchased from Invitrogen. Bacteria were lysed in 100 mM Tris-HCl (pH 7.0) with 0.18-μm glass beads (6 × 30 s; FastPrep; QBIOgene, Illkirch, France). Proteins were separated by SDS-PAGE, electrotransferred to a nitrocellulose membrane (Hybond, Amersham Biosciences, Little Chalfont, United Kingdom), and incubated with antisera at dilutions of 1/1,000 (Anti-DdcY), 1/200 (anti-DdcS and anti-Ldt_{fm}), 1/2,000 (anti-Stk), 1/1,000 (anti-StpA), and 1/2,500 (antipolyhistidine from Sigma). Antiphosphothreonine antibodies were used at dilutions of 1/250 and 1/1,000 for analyses of crude cell extracts and purified proteins, respectively. Western blot assays were incubated in Tris-buffered saline (TBS)-Tween (10 mM Tris [pH 7.5], 150 mM NaCl, 0,025% Tween 20, 2.5% nonfat dry milk) for all antibodies except for antiphosphothreonine antibodies (20 mM Tris [pH 7.5], 137 mM NaCl, 0.1% Tween 20, 3% bovine serum albumin). Goat anti-rabbit or anti-rat IgGs coupled to peroxidase (SouthernBiotech, Birmingham, AL) were used as secondary antibodies, and proteins were detected by chemiluminescence (ECL kit; Pierce, Amersham Biosciences).

Kinase and phosphatase assays. Autophosphorylation of Stk and phosphorylation of MBP (Sigma) were tested in kinase buffer (50 mM Tris-HCl [pH 7.5], 25 mM NaCl, 1 mM $MnCl_2$, 1 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM EDTA) in the presence or absence of ATP (2 mM)

at room temperature. Dephosphorylation of Stk and MBP (Sigma) by StpA was tested by adding purified StpA (4 μM) to the kinase assay mixture. For dephosphorylation of phosphoproteins from *E. faecium* crude cell extract, purified StpA (3 μg) was incubated with *E. faecium* crude cell extract (10 μg) in 20 μl of 100 mM phosphatase buffer (pH 6.4) containing 2 mM $MnCl_2$ for 30 min at room temperature. Reactions were quenched with Laemmli SDS sample buffer and boiled for 5 min. Proteins were separated by 12% SDS-PAGE and transferred to polyvinylidene difluoride (PVDF) transfer membrane (Immobilon-FL; Millipore). Phosphorylated proteins were detected with a Pro-Q Diamond phosphoprotein blot stain kit (Invitrogen) according to the manufacturer's instructions. Blots were washed in TBS-Tween for serial detection of phosphoproteins with the Pro-Q kit and antiphosphothreonine antibodies.

Hydrolysis of pNPP (Sigma) by StpA was determined at 37°C in 50 mM Tris-HCl (pH 8.0) containing various concentrations of $MnCl_2$. Reactions were initiated by the addition of $MnCl_2$, and absorbance at 405 nm (ε = 12,500 M^{-1} cm^{-1}) was monitored with a Cary 100 Bio spectrophotometer (Varian).

SUPPLEMENTAL MATERIAL

Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01446-14/-/DCSupplemental.

Figure S1, TIF file, 7.8 MB.
Figure S2, TIF file, 21.1 MB.
Figure S3, TIF file, 1.7 MB.
Figure S4, TIF file, 1.9 MB.
Figure S5, TIF file, 12.6 MB.
Figure S6, TIF file, 5.3 MB.
Text S1, DOCX file, 0 MB.

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