

The RimL Transacetylase Provides Resistance to Translation Inhibitor Microcin C

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Peptide-nucleotide antibiotic microcin C (McC) is produced by some *Escherichia coli* strains. Inside a sensitive cell, McC is processed, releasing a nonhydrolyzable analog of aspartyl-adenylate, which inhibits aspartyl-tRNA synthetase. The product of *mccE*, a gene from the plasmid-borne McC biosynthetic cluster, acetylates processed McC, converting it into a nontoxic compound. MccE is homologous to chromosomally encoded acetyltransferases RimI, RimJ, and RimL, which acetylate, correspondingly, the N termini of ribosomal proteins S18, S5, and L12. Here, we show that *E. coli* RimL, but not other Rim acetyltransferases, provides a basal level of resistance to McC and various toxic nonhydrolyzable aminoacyl adenylates. RimL acts by acetylating processed McC, which along with ribosomal protein L12 should be considered a natural RimL substrate. When overproduced, RimL also makes cells resistant to albomycin, an antibiotic that upon intracellular processing gives rise to a seryl-thioribosyl pyrimidine that targets seryl-tRNA synthetase. We further show that *E. coli* YhhY, a protein related to Rim acetyltransferases but without a known function, is also able to detoxify several nonhydrolyzable aminoacyl adenylates but not processed McC. We propose that RimL and YhhY protect bacteria from various toxic aminoacyl nucleotides, either exogenous or those generated inside the cell during normal metabolism.

Peptide-nucleotide antibiotic microcin C (McC) consists of an MRTGNAD heptapeptide with a modified AMP attached to the α carboxyl group of the aspartate through an *N*-acyl phosphoramidate linkage (Fig. 1). The N-terminal methionine is formylated; the phosphate group is decorated with a propylamine group. The peptide moiety of McC is encoded by a 21-bp-long *mccA* gene (1, 2). The remaining genes of the *mcc* cluster are responsible for the synthesis of antibiotic (*mccB*, *mccD*, and *mccE*) and immunity of the producing cell to both endogenous and exogenous McC (*mccC*, *mccE*, and *mccF*).

Escherichia coli cells actively import McC through the action of the YejABEF inner membrane transporter (3). Inside the cell, the N-terminal formyl group of McC is removed by peptide deformylase Pdf, and the peptide part is next degraded by one of the three cellular aminopeptidases—A, B, or N (4). As a result, processed McC is generated (Fig. 1). Processed McC is a nonhydrolyzable analog of aspartyl-adenylate (Fig. 1), an intermediate of a tRNA^{Asp} aminoacylation reaction catalyzed by aspartyl-tRNA synthetase (AspRS) (5). Processed McC binds to AspRS and inhibits it, leading to the cessation of production of Asp-tRNA^{Asp}, a stringent response, and the cessation of protein synthesis. Processed McC generated internally within the producing cell is detoxified by the acetyltransferase activity of the C-terminal domain of the MccE protein (MccE^{CTD}). MccE^{CTD} acetylates the primary amino group of the aminoacyl moiety of processed McC; acetylated processed McC no longer inhibits AspRS (6). MccE^{CTD} also acetylates (and, therefore, provides resistance to) a wide spectrum of nonhydrolyzable synthetic aminoacyl sulfamoyl adenylates (aaSAs, where “aa” denotes an amino acid in a single-letter code) such as DSA, shown in Fig. 1.

The MccE acetyltransferase is homologous to bacterial N-ter-

минаl acetyltransferases (NATs) of the Rim family. The *E. coli* genome encodes three Rim proteins, RimI, RimJ, and RimL, which acetylate ribosomal proteins S18, S5, and L12, respectively (7, 8). The physiological functions of these NATs and the significance of ribosomal protein acetylation for cell physiology are not entirely clear. Here, we investigated whether sequence similarity of MccE^{CTD} with Rim family NATs leads to functional similarity and allows Rim NATs to play a role in McC resistance. Our results indicate that RimL indeed contributes to the basal level of McC resistance through the same mechanism as the MccE acetyltransferase. In contrast, RimI and RimJ do not recognize processed McC or its analogs and play no role in McC resistance. We also demonstrate that an additional *E. coli* NAT, the product of the *yhhY* gene, acetylates some nonhydrolyzable aminoacyl adenylates (but not processed McC) and increases cellular resistance to these compounds.

MATERIALS AND METHODS

Bacterial strains and reagents. *E. coli* NovaBlue Singles competent cells (Novagen) were used for cloning and plasmid propagation. *E. coli* BL21(DE3) isolates were used for recombinant protein overproduction. *E. coli* K-12 strain BW25113 [*lacI*p4000 (*lacI*^q) *rrnB3* Δ *lacZ4787* *hsdR541* Δ (*araBAD*)567 Δ (*rhaBAD*)568 *rhp-1*] (9) was used as a parental strain to

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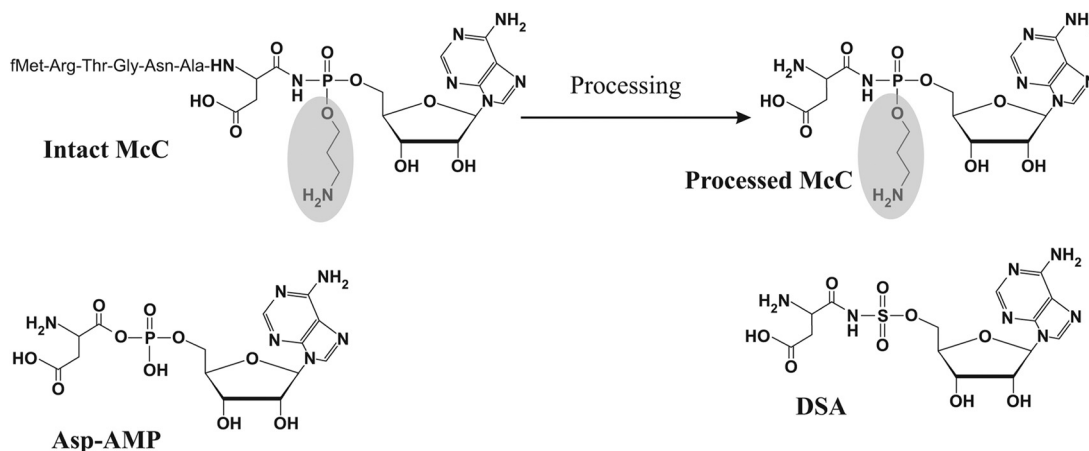


FIG 1 Microcin C and the mechanism of its action. The structures of intact McC, processed McC, aspartyl-adenylate, an intermediate of reaction catalyzed by AspRS, and aspartyl sulfamoyl adenylate DSA are shown. The propylamine group (shaded) is absent from McC(1120) and its processing product.

generate *rim* knockouts. The YhhY open reading frame (ORF) clone from the ASKA library (10) was used for purification of the YhhY protein. All restriction enzymes, Vent DNA polymerase, *Taq* DNA polymerase, DNA, and protein molecular weight markers were purchased from New England BioLabs Inc. T4 DNA ligase was from Fermentas. All oligonucleotide primers were synthesized by Integrated DNA Technologies.

Microcin C and its maturation derivative McC(1120) were purified as described elsewhere (11). Albomycin was a gift of G. Katrukha (Gause Institute of Antibiotics, Moscow, Russia). Aminoacyl sulfamoyl adenylates and synthetic McC analogs were synthesized as described previously (12).

Cloning and protein expression. The *N*^{acetyl}transferase genes—*rimI*, *rimJ*, and *rimL*—were amplified from *E. coli* BW25113 genomic DNA with appropriate primers containing engineered *NdeI* and *EcoRI* restriction sites for subsequent cloning. PCR products were blunt-end cloned into pT7Blue vector (Novagen), excised with *NdeI* and *EcoRI* enzymes, and recloned into pET28a expression vector (Novagen). All plasmids were confirmed by DNA sequencing.

E. coli BL21(DE3) cells harboring the pET28a-*rimI* (or *rimJ* or *rimL*) plasmids were grown in 1 liter of LB medium, supplemented with 25 μ g/ml kanamycin, at 37°C until an optical density at 600 nm (OD_{600}) of ~ 1 was reached. Cultures were cooled down to 16°C, expression of plasmid-borne *rim* genes was induced by the addition of 0.2 mM isopropyl 1-thio- β -D-galactopyranoside, and growth at 16°C was continued for 20 to 24 h. Cells were harvested by centrifugation and stored at -80°C before use. An ASKA library clone overproducing YhhY was grown and induced according to a protocol published elsewhere (10).

In vivo sensitivity tests. *E. coli* BL21(DE3) cells carrying pET28a, pET28a-*rimL*, pET28a-*rimI*, pET28a-*rimJ*, or pET28a-*yhhY* plasmids were grown in 5 ml of LB medium supplemented with kanamycin (50 μ g/ml) and isopropyl β -D-thiogalactopyranoside (0.1 mM) at 30°C overnight. One hundred microliters of culture was mixed with melted LB top (0.7 g/liter) agar and poured onto the surface of an LB agar plate supplemented with kanamycin (50 μ g/ml). Drops (5 μ l) of solutions of the antibiotics being tested were deposited on the solidified top agar layer. The concentration of McC, McC(1120), and albomycin was 100 μ M, the concentration of synthetic McC analog solutions was 350 μ M, and the concentration of aaSA solutions was 10 mM. Plates were incubated for several hours at 30°C. The sizes of growth inhibition zones around the drops of antibiotic solutions were monitored and recorded. Each experiment was repeated at least three times.

Tests with cells lacking *rim* genes were performed similarly, except that the LB medium contained no antibiotics and plates were incubated at 37°C.

Protein purification. To purify RimI or RimL, the frozen cell mass was thawed and sonicated in a water-ice bath in buffer A containing 20 mM Tris-HCl (pH 8.0), 0.5 M NaCl, 2 mM 2- β -mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride (PMSF). The lysate was clarified by centrifugation for 30 min at $15,000 \times g$. The supernatant was supplemented with 5 mM imidazole and loaded onto a 5-ml Ni HiTrap column (GE Healthcare) equilibrated with buffer A containing 5 mM imidazole. Bound protein was step eluted with 20, 50, 100, and 200 mM imidazole in buffer A. Fractions containing pure Rim proteins (as revealed by Coomassie blue staining of SDS gels) were pooled and dialyzed against two exchanges of 500 volumes of 40 mM Tris-HCl (pH 8.0), 100 mM NaCl, 0.5 mM EDTA, 0.5 mM 2- β -mercaptoethanol, and 10% glycerol. Protein samples were supplemented with glycerol up to 50% stored at -20°C . To purify RimJ protein, a similar protocol was used but with pH 7.0 phosphate buffer instead of Tris buffer. The YhhY protein was purified on HIS-Select nickel affinity gel (Sigma) under denaturing conditions using 8 M urea for protein solubilization and chromatography according to the manufacturer's protocol. Fractions with pure protein were pooled and dialyzed against reconstitution buffer containing 40 mM Tris-HCl (pH 8.0), 200 mM NaCl, 0.5 mM EDTA, 0.5 mM 2- β -mercaptoethanol followed by dialysis against the storage buffer (reconstitution buffer containing 50% glycerol).

Preparation of *E. coli* S30 cell extracts. *E. coli* cells were grown to an OD_{600} of ~ 0.8 in LB medium. Cells were collected by centrifugation and washed with 40 mM Tris-HCl (pH 7.6), 10 mM $\text{Mg}(\text{OAc})_2$, 50 mM potassium acetate, 0.1 mM EDTA, and 1 mM dithiothreitol (DTT). The cell pellet was resuspended in an equal volume of the same buffer and disrupted using a French press (pressure, 10^8 Pa [1,000 bar]). The lysate was next centrifuged at $30,000 \times g$ for 30 min. Then, supernatant was aliquoted and either directly used in tRNA aminoacylation reactions or stored at -80°C until further use.

tRNA aminoacylation reaction. Aminoacylation reactions were carried out according to the method described in reference 5 with some modifications. To 1 μ l of solution containing an inhibitor being tested, 3 μ l of *E. coli* S30 extract was added. Next, 16 μ l of aminoacylation mixture (30 mM Tris-HCl [pH 8.0], 1 mM DTT, 5 g/liter bulk *E. coli* tRNA [Sigma], 3 mM ATP, 30 mM KCl, 8 mM MgCl_2 , and 40 μ M desired labeled amino acid) was added, and reaction mixtures were incubated at room temperature for 5 min. The reactions were terminated by the addition of cold 10% trichloroacetic acid (TCA), and precipitated reaction products were collected on Whatman 3MM paper filters. After thorough washing with cold 10% TCA, the filters were washed twice with acetone and dried on a heating plate. Following the addition of scintillation liquid, the amount of radioactivity was determined in a scintillation counter.

N-Acetyltransferase activity assay. The N-acetyltransferase activity assay was performed in 20 μ l of 100 mM Tris-HCl (pH 8.0), 0.1 mM EDTA, 1 mM acetyl coenzyme A (acetyl-CoA), and 0.5 to 1 mM substrate for 10 min at 25°C. Reactions were initiated by the addition of 0.07, 0.2, 1, and 2 μ M purified recombinant YhhY, RimI, RimL, and RimJ, respectively, and were terminated by the addition of 500 μ l of 0.05 mM 4,4'-dithiodipyridine (DTDP) (Aldrich) (13). Enzyme activity was determined by measuring the increase in absorbance at 324 nm due to the formation of 4-thiopyridone in the reaction between DTDP and the sulfhydryl group (SH) of the CoA-SH produced after the acetyl transfer reaction, and specific activity was calculated as the amount of substrate (μ mol) converted into CoA-SH product by 1 mg of enzyme per minute of reaction.

To verify the acetyltransferase activity of recombinant Rim proteins, synthetic peptides (GenScript) matching the first 10 N-terminal residues of *E. coli* ribosomal proteins S18, S5, and L12 were used as the substrates (acetyl group acceptors) for RimI, RimJ (7), and RimL (8), respectively. Earlier, such an approach had been applied by the Blanchard group to analyze bisubstrate inhibition of RimI from *Salmonella enterica* serovar Typhimurium (14, 15) and during crystal structure determination of this enzyme (16). The recombinant enzyme preparations of RimI, RimJ, and RimL used in this work gave, respectively, the following specific activities toward chosen cognate synthetic substrates: 0.51, 0.021, and 0.052 μ mol/min/mg. No activity to noncognate substrates was detected.

MALDI-TOF MS. Samples for mass spectrometric (MS) analysis were dissolved in acetonitrile. Aliquots (0.5 μ l) were mixed on a steel target with 1 μ l of 2,5-dihydroxybenzoic acid (Aldrich) solution (20 mg/ml in 20% acetonitrile, 0.5% trifluoroacetic acid), and the droplet was left to dry at room temperature. The mass spectra were recorded on an ABI-MDS SCIEX 4800 matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer. The $[MH]^+$ molecular ions were measured in reflector and tandem (Lift) mode; the accuracy of mass peak measurement was 0.1 Da for parent ions and 0.5 Da for daughter ions.

RESULTS

Chromosomally encoded RimL contributes to the basal level of resistance to McC, its analogs, and nonhydrolyzable aminoacyl adenylates. To determine the role of Rim NATs in McC resistance, *E. coli* K-12-based strains lacking individual *rim* genes were constructed. Three strains carrying double *rim* deletions ($\Delta rimI \Delta rimJ$, $\Delta rimI \Delta rimL$, and $\Delta rimJ \Delta rimL$) and a triple $\Delta rimI \Delta rimJ \Delta rimL$ mutant were also constructed. All strains were viable and grew equally well in rich medium at 37°C (data not shown). Susceptibilities of mutant strains to (i) McC, (ii) McC(1120), an 1,120-Da McC maturation intermediate lacking the propylamine modification (11), (iii) two aminoacyl sulfamoyl adenylates (aaSAs) active against *E. coli* (aspartyl sulfamoyl adenylate DSA and leucyl sulfamoyl adenylate (LSA) (12), (iv) two synthetic McC(1120) analogs with an MRTGNA peptide ("X") corresponding to the first six amino acids of McC heptapeptide coupled to DSA and LSA (12), and (v) albomycin were next determined. Drops of solutions containing various compounds were deposited on lawns of wild-type or mutant *E. coli*, and the sizes of growth inhibition zones were recorded after overnight incubation at 37°C. McC and its analogs were used at 100 and 350 μ M concentrations. AaSAs, which lack the transport peptide, were tested at the much higher concentration of 10 mM in order to obtain observable growth inhibition zones. Albomycin was used at a 100 μ M concentration. Results obtained with the triple mutant strain are shown in Fig. 2. As can be seen, the mutant cells were more sensitive to McC, its chemical analogs, or aaSAs than, but were as sensitive to albomycin as, the wild-type cells. The extent of protection afforded by chromosomal *rim* genes varied for different

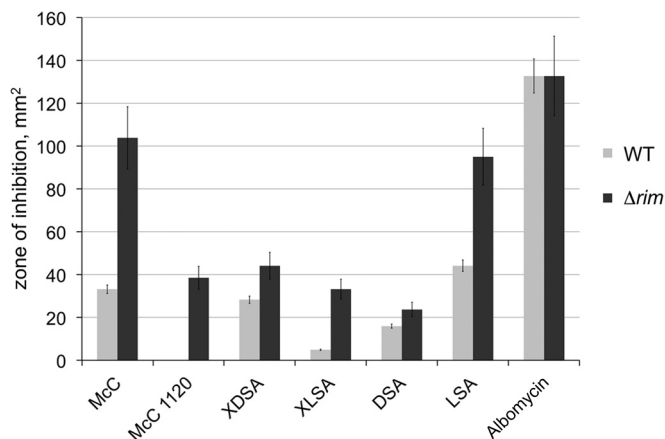


FIG 2 Cells lacking *rim* genes are hypersensitive to McC, its maturation intermediates, chemical analogs, and nonhydrolyzable aminoacyl adenylates but not to albomycin. Five-microliter drops of solutions containing the indicated compounds were deposited on lawns of wild-type or isogenic mutant $\Delta rimI \Delta rimJ \Delta rimL$ *E. coli* cells. After overnight growth at 37°C, the areas of growth inhibition zones were determined. The bars show mean values obtained in three independent experiments; the error bars show standard deviations. The compounds tested included the following: McC (a mature form with amino-propyl modification [Fig. 1]); McC(1120), an 1,120-Da McC maturation intermediate lacking the propylamine modification; XDSA and XLSA, which are chemically synthesized McC(1120) analogs containing an MRTGNA hexapeptide C-terminally fused to aspartyl or leucyl sulfamoyl adenylates, respectively; aminoacyl sulfamoyl adenylates DSA and LSA carrying aspartyl (Fig. 1) and leucyl aminoacyl moieties, respectively; and, finally, albomycin.

compounds. Protection was marginal for DSA but very obvious for McC and McC(1120).

Deletions of *rimI* and/or *rimJ* had no effect on sensitivity to any of the compounds tested (data not shown). Further, the $\Delta rimI \Delta rimL$ and $\Delta rimJ \Delta rimL$ double mutants or the $\Delta rimI \Delta rimJ \Delta rimL$ triple mutant were no more sensitive than cells lacking *rimL* only (data not shown). The result thus suggests that RimL contributes to the basal level of *E. coli* resistance to McC, its analogues, and nonhydrolyzable aminoacyl-adenylates but not to albomycin. In contrast, RimI and RimJ do not contribute to cellular resistance to any of the compounds tested.

Cell extracts from wild-type and various *rim* mutant cells were prepared, supplemented with McC, and incubated for 15 min. Elsewhere, we show that a 15-min incubation is sufficient for complete processing of McC by cellular peptidases at the conditions of the assay (4). Indeed, the tRNA^{ASP} aminoacylation reaction was strongly inhibited when measured after the processing was complete in all extracts (Fig. 3A). However, the tRNA^{ASP} aminoacylation activity was partially recovered in extracts of wild-type cells that were incubated with McC for longer periods of time (Fig. 3A). The recovery of tRNA^{ASP} aminoacylation activity in extracts prepared from $\Delta rimI$, $\Delta rimJ$, or $\Delta rimI \Delta rimJ$ cells closely followed that observed in the wild-type cell extract (Fig. 3A). In contrast, no such recovery was observed in extracts prepared from $\Delta rimL$, $\Delta rimI \Delta rimL$ or $\Delta rimJ \Delta rimL$ double mutants or from $\Delta rimI \Delta rimJ \Delta rimL$ triple mutant cells: the tRNA^{ASP} aminoacylation activity in these extracts appeared to be inhibited irreversibly (Fig. 3A). The results thus indicate that cellular RimL contributes to the basal level of *E. coli* resistance to McC by inactivating processed McC.

The addition of McC(1120) to wild-type cell extract led to par-

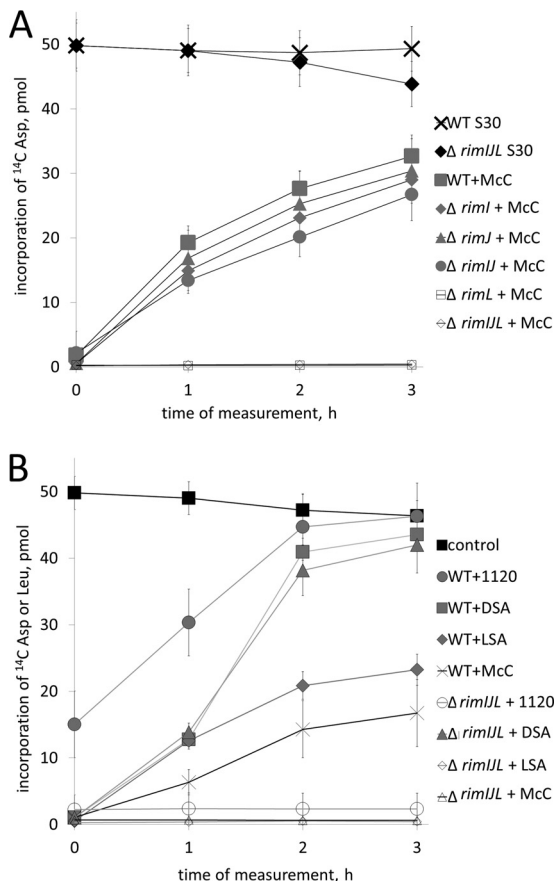


FIG 3 RimL detoxifies processed McC and LSA *in vitro*. (A) AspRS-catalyzed aminoacylation of tRNA^{Asp} in S30 extracts prepared from wild-type *E. coli* and the indicated *rim* mutants. Extracts were supplied with intact McC and incubated for 15 min to allow processing. Aliquots of extracts were removed, and tRNA^{Asp} aminoacylation reactions were carried out. The first aliquots were removed right after the initial 15-min incubation needed for McC processing. This time point is labeled “0.” The amounts of aminoacylated tRNA^{Asp} (measured as incorporation of [¹⁴C] Asp in TCA-precipitable material) are shown. Control shows the time course of aminoacylation levels in wild-type S30 cell extracts without any additions. Data from three independently performed experiments (mean values and standard deviations) are shown. (B) *E. coli* S30 extracts prepared from wild-type or a triple *rim* deletion *E. coli* strain were combined with intact McC, McC(1120), DSA, or LSA. Extracts containing McC or McC(1120) were incubated for 15 min to allow processing before initiating tests for tRNA^{Asp} aminoacylation (times are indicated as indicated for panel A); in extracts containing DSA or LSA, measurements of tRNA^{Asp} or tRNA^{Leu} aminoacylation, respectively, were initiated immediately after the addition of inhibitors. Data from three independently performed experiments (mean values and standard deviations) are shown.

tial inhibition of tRNA^{Asp} aminoacylation at the time of the first measurement after a 15-min preincubation to allow processing (Fig. 3B). Upon further incubation, complete recovery from inhibition by McC(1120) was observed. In extracts of triple mutant cells, McC(1120) fully inhibited tRNA^{Asp} aminoacylation without subsequent recovery of AspRS activity (Fig. 3B). We conclude that RimL inactivates processed McC(1120) more efficiently than processed McC. The incomplete initial inhibition by McC(1120) in wild-type cell extract is therefore likely due to a partial detoxification of processed McC(1120) that must be occurring simultaneously with the processing of intact McC(1120) during the initial 15-min incubation.

The effect of the DSA addition on aminoacylation of tRNA^{Asp} in extracts from wild-type or triple mutant cells was also tested. Since DSA does not require processing, the first measurement of AspRS activity was performed immediately after the addition of DSA. As can be seen (Fig. 3B), the AspRS activity in both extracts was initially inhibited by the addition of DSA but eventually completely recovered from the inhibition. The absence of requirement for Rim proteins for recovery from DSA inhibition agrees with *in vivo* data showing that the contribution of intracellular Rim proteins to DSA (or XDSA) resistance is only slight (Fig. 2). LSA, a sulfamoyl adenosine inhibitor of LeuRS, irreversibly inhibited aminoacylation of tRNA^{Leu} in triple mutant cell extracts, but partial recovery from inhibition was observed in wild-type cell extracts (Fig. 3B), again in agreement with *in vivo* data, which showed that cells lacking *rimL* were significantly more sensitive to LSA than wild-type cells (Fig. 2).

Effects of Rim proteins on resistance to McC and related compounds. Our results suggest either that RimI and RimJ are not necessary for detoxification of McC and toxic aaSAs or that intracellular amounts of these proteins are too low to affect McC and aaSA resistance. PET-based expression plasmids overproducing *E. coli* RimI, RimJ, or RimL were constructed. SDS-PAGE analysis indicated that all three proteins were overproduced at high (and similar) levels (data not shown). Sensitivity of *E. coli* BL21(DE3) cells harboring Rim-overproducing plasmids to McC, a collection of bioactive aaSAs (DSA, LSA, KSA, ASA, GSA, FSA, and PSA), and albomycin was tested. We used all bioactive aaSAs available to us since we considered that RimI and/or RimJ may be specific for certain aminoacyl adenylates. Previously, we showed that cells overproducing MccE^{CTD} are resistant to McC, DSA, LSA, KSA, ASA, GSA, FSA, and albomycin but are sensitive to PSA (6). This result was reproduced (Table 1). Cells overproducing RimL were indistinguishable from MccE^{CTD}-overproducing cells (Table 1). In contrast, cells overproducing RimI or RimJ were as sensitive as cells harboring the PET vector plasmid to every compound tested (Table 1).

The inability of overproduced RimI or RimJ to provide resistance to McC could have been due to poor solubility and/or folding of recombinant proteins. To rule out this possibility, recombinant Rim proteins were purified from cytoplasmic extracts of appropriate overproducing cells to apparent homogeneity. Control experiments established that each protein was acetylating its cognate substrates and was therefore properly folded and active (see Materials and Methods). The addition of recombinant MccE^{CTD} or RimL—but not RimI or RimJ—to extracts of $\Delta rimI \Delta rimJ \Delta rimL$ cells inhibited with McC led to almost complete

TABLE 1 Sensitivity of cells overproducing Rim proteins and YhhY to McC, albomycin, and aaSAs^a

Protein	McC	Albomycin	DSA	LSA	KSA	GSA	ASA	FSA	PSA
RimL	R	R	R	R	R	R	R	R	S
RimI	S	S	S	S	S	S	S	S	S
RimJ	S	S	S	S	S	S	S	S	S
YhhY	S	S	S	R	S	S	PR	PR	S

^a R, absence of visible growth inhibition zones at the concentration of inhibitor used; S, clear growth inhibition zones of the same size as observed on control cell lawn formed by cells carrying a pET28a plasmid vector; PR (partial resistance), presence of turbid growth inhibition zones whose diameter was at least twice less than that on control lawn.

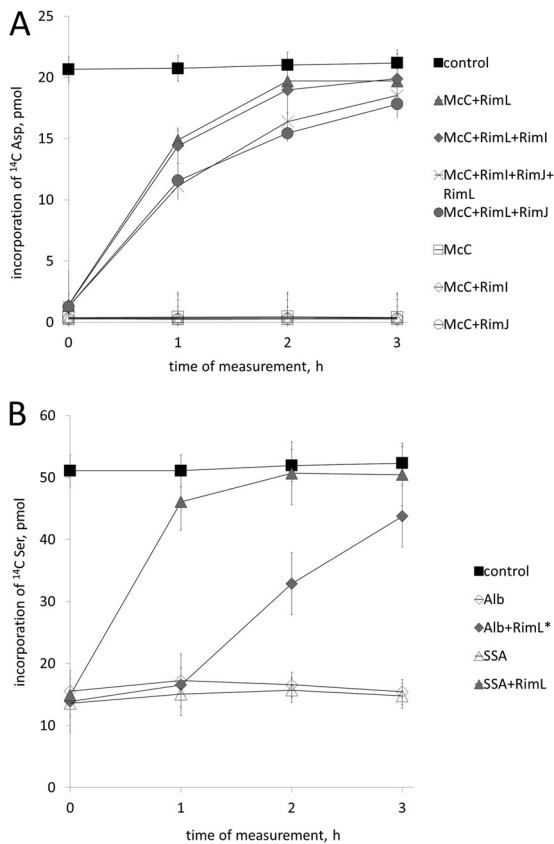


FIG 4 RimL protein is sufficient for McC, McC(1120), DSA, XDSA, LSA, and albomycin resistance *in vitro*. Wild-type *E. coli* S30 extracts were supplemented with McC (A) or albomycin (B) and incubated to allow processing. Next, the indicated purified Rim proteins were added to reaction mixtures (time “0”), and incubation was continued. At various time points after addition of Rim proteins, reaction aliquots were withdrawn and tRNA^{Asp} (A) or tRNA^{Ser} (B) aminoacylation reactions were carried out. In panel B, a 20-fold-higher concentration of RimL (labeled with an asterisk) was used in reaction mixtures containing albomycin than in those containing SSA, which was used as a control. Data from three independently performed experiments with each inhibitor are shown (with means and standard deviations).

recovery of tRNA^{Asp} aminoacylation after a 3-hour incubation (Fig. 4A). Simultaneous addition of RimI and RimJ had no effect, and neither RimI nor RimJ stimulated RimL-mediated recovery of tRNA^{Asp} aminoacylation (Fig. 4A).

The addition of RimL to albomycin-inhibited extracts led to recovery of tRNA^{Ser} aminoacylation (Fig. 4B). No such effect was observed with RimI or RimJ (data not shown). Since ~20-fold-larger amounts of RimL were required to recover tRNA^{Ser} aminoacylation in albomycin-inhibited extracts than to achieve full recovery of tRNA^{Asp} aminoacylation in McC-inhibited extracts, processed albomycin is a much poorer RimL substrate than processed McC, in agreement with *in vivo* data (Fig. 2) and earlier findings with MccE^{CTD} (6).

Purified Rim proteins were combined with acetyl-CoA, a donor of acetyl groups, and the rate of production of free CoA generated during the acetylation reaction was determined in the presence of processed McC, DSA, ESA, and LSA as described in Materials and Methods. The results, presented in Table 2, demonstrate that production of CoA from acetyl-CoA was observed with every aaSA tested in the presence of RimL. A similar result was obtained in the presence of

MccE^{CTD}, as expected (6). In contrast, no CoA production was detected in reaction mixtures that contained RimI or RimJ.

RimL-catalyzed acetylation of aaSAs was confirmed by MALDI-TOF MS analysis of reaction products (Fig. 5 and data not shown). In all cases, a mass peak corresponding to the reaction substrate disappeared upon incubation with pure RimL and acetyl-CoA. Instead, a new peak whose *m/z* value was increased by 42 atomic units, corresponding to a transfer of an acetyl group, was observed. Thus, just like MccE^{CTD}, RimL can utilize aaSAs as the substrates for acetylation with little specificity with respect to the amino acid attached to the nucleotide moiety. RimI and RimJ are inactive in this reaction.

Identification of an additional *E. coli* NAT capable of acetylating aminoacyl adenylates. MALDI-TOF MS analysis of wild-type cells extract that recovered from LSA inhibition revealed that a mass peak corresponding to LSA (*m/z* = 460) disappeared. Instead, a peak corresponding to acetylated LSA (*m/z* = 502) was observed (Fig. 5). Interestingly, incubation in triple *rim* mutant cell extracts also led to the accumulation of detectable amounts of acetylated form of LSA, though the peak corresponding to unmodified LSA was also present (Fig. 5). Incubation of wild-type or *rim* mutant cell extracts with DSA led to the disappearance of the mass peak with an *m/z* of 462, corresponding to DSA (Fig. 5). No peak corresponding to acetylated or any other modified form of DSA was observed. The lack of accumulation of acetylated DSA was not caused by the inability of RimL to acetylate DSA, since acetylation products were readily detected upon incubation of either DSA or LSA with extract prepared from RimL-overproducing cells (Fig. 5). The results thus suggest that in wild-type cells DSA is efficiently degraded in a RimL-independent way, in agreement with results of *in vivo* sensitivity tests (Fig. 2) and *in vitro* results (Fig. 3B).

Accumulation of a +42 peak in LSA-inhibited extracts lacking Rim proteins suggested that there exists yet another, non-Rim, NAT that can acetylate LSA. Bioinformatics analysis of the *E. coli* genome reveals several genes coding for putative or experimentally proven acetyltransferases different from the Rim proteins. The products of two such genes, *yhhY* and *speG*, are most closely related to Rim proteins (Fig. 6A) and were investigated further. A *yhhY* deletion strain, a quadruple mutant strain lacking the three *rim* genes and the *yhhY* gene, and a *yhhY* expression plasmid were generated. The corresponding *speG* deletion strains and an expression plasmid were also made. The sensitivity of *yhhY* or *speG* deletion strains to various inhibitors used in the experiments described above was not different from those of control (wild-type or triple *rim* mutant) strains (data not shown). *SpeG* overproduction had no effect on the sensitivity of cells to McC and aaSAs (data not shown). In contrast, *YhhY*-overproducing cells became com-

TABLE 2 *In vitro* substrate specificity of *E. coli* Rim and YhhY acetyltransferases

Substrate	Sp act ^a (μmol/mg/min)			
	RimI	RimJ	RimL	YhhY
DSA	0	0	2.39	0
ESA	0	0	1.54	0
LSA	0	0	2.11	28
ISA	ND	ND	2.5	17
Albomycin	0	0	0	ND

^a Amount of product in the presence of 1 mM acetyl-CoA and 0.5 to 10 mM substrates (see Materials and Methods); ND, not determined.

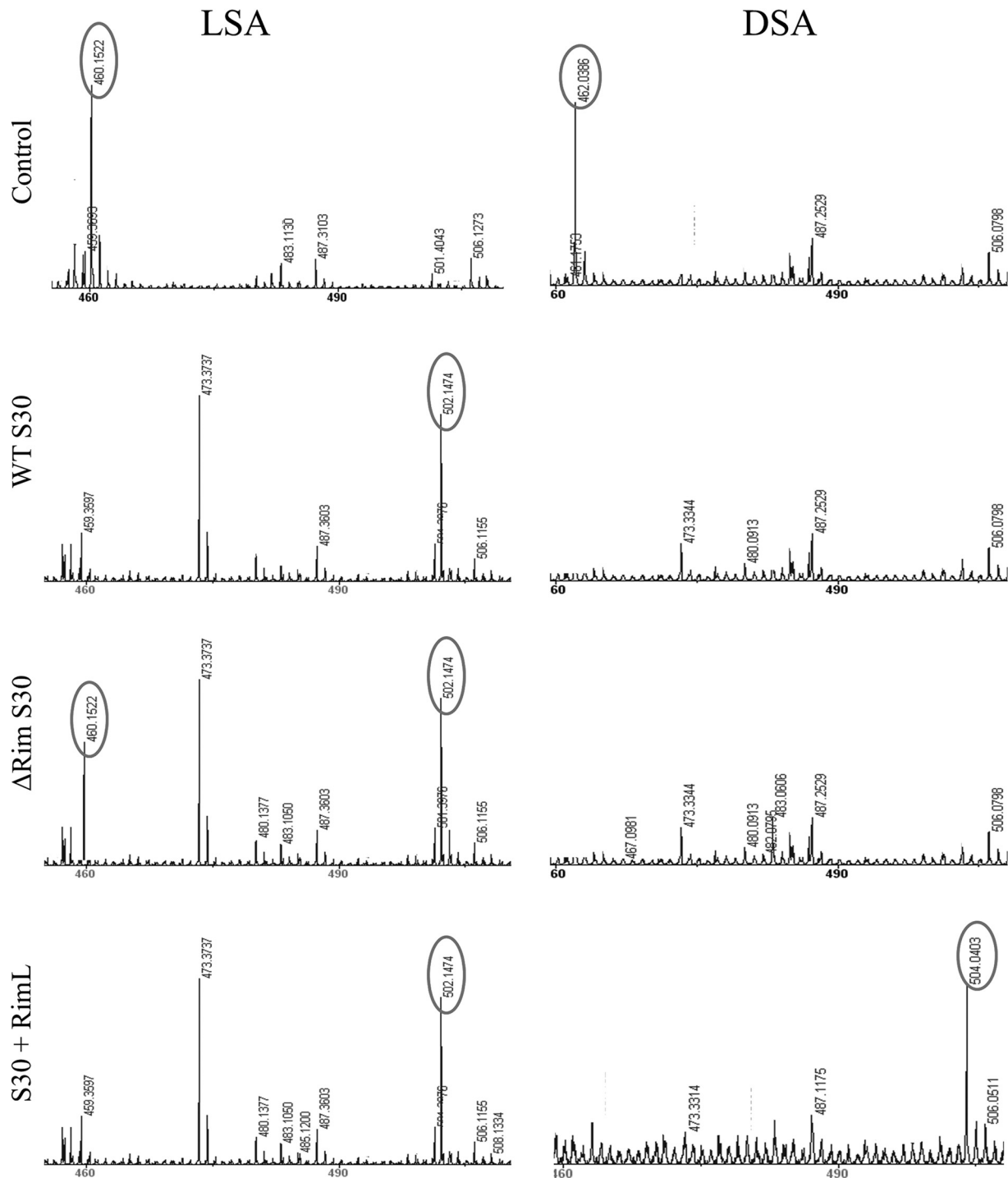


FIG 5 Mass spectrometric analysis of LSA and DSA incubated in various cell extracts. LSA and DSA were incubated with a buffer (control) or with S30 extracts prepared from wild-type, Δrim , or RimL-overproducing cells, and reactions were subjected to MALDI-TOF MS analysis. Only relevant portions of mass spectra are shown. The peaks corresponding to mass ions described in the text are in ovals.

pletely resistant to LSA and were partially resistant to ASA and FSA but remained as sensitive to McC, DSA, KSA, GSA, and PSA as control cells harboring an empty vector plasmid (Table 1). The results thus indicate that YhhY, when overproduced, can indeed provide resistance to some nonhydrolyzable aminoacyl adenylates.

S30 extracts from triple *rim* and quadruple deletion (triple *rim* and *yhhY*) were incubated with LSA, followed by MALDI-TOF MS analysis. While partial conversion of LSA to a +42 mass ion with an *m/z* of 502 was detected in triple mutant cells, the LSA peak remained intact in the quadruple mutant cell extract and no peak with *m/z* of 502 was detected (Fig. 6B). We therefore con-

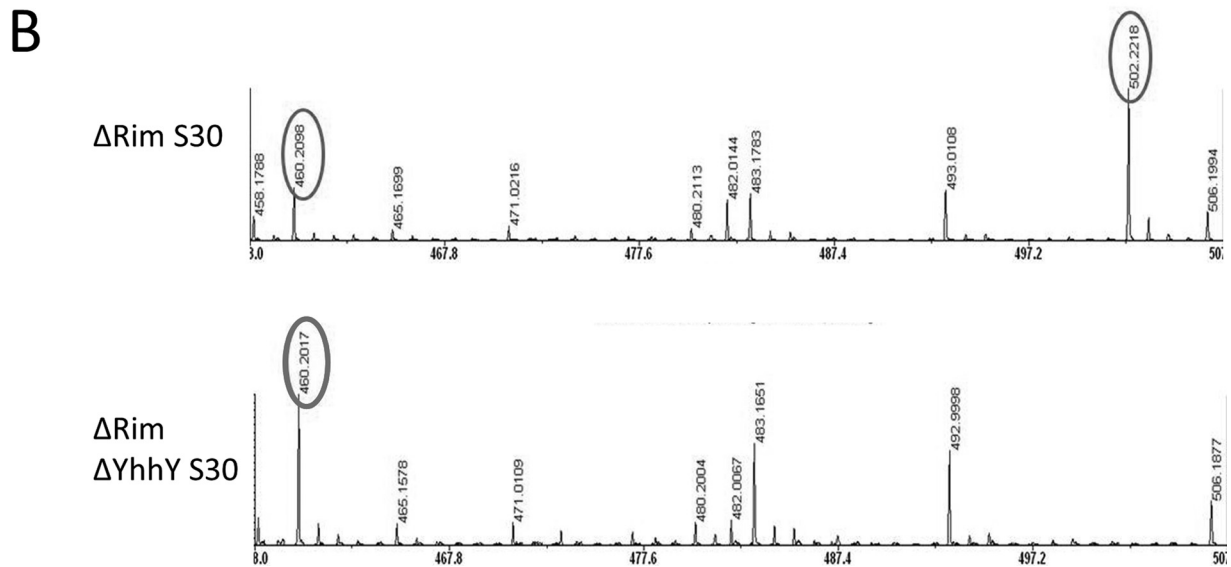
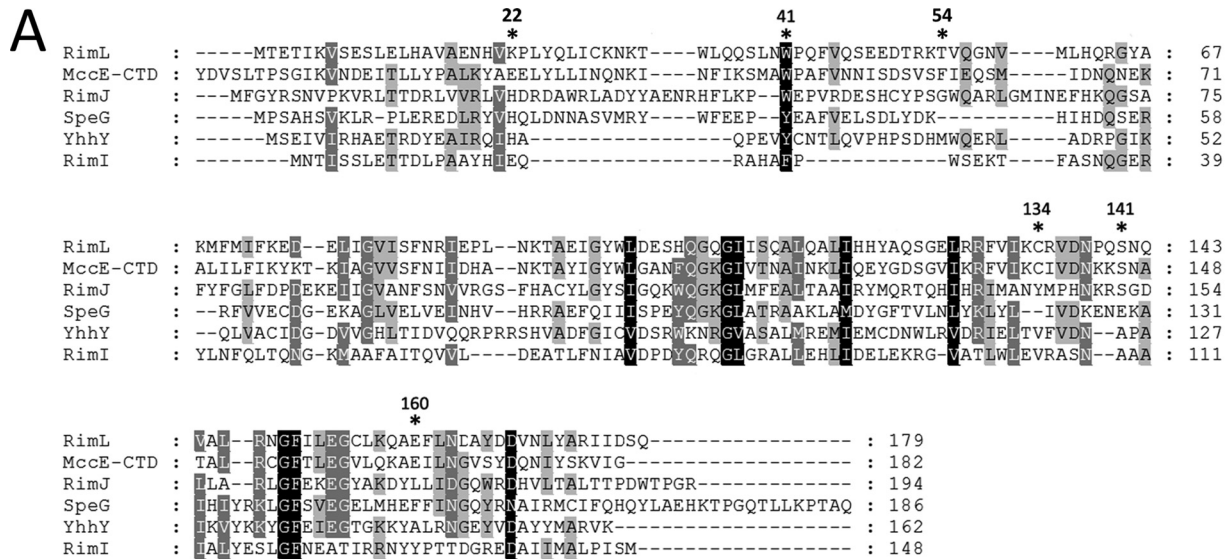


FIG 6 YhhY is an acetyltransferase that detoxifies nonhydrolyzable aminoacyl adenylates *in vivo* and *in vitro*. (A) A multiple sequence alignment of *E. coli* Rim proteins, MccE^{CTD}, SpeG, and the YhhY protein. The extent of sequence conservation is indicated by background shading. Catalytic and substrate binding residues are marked with asterisks. Residues 22, 41, and 54 of RimL correspond to known adenine-binding residues of MccE^{CTD} and likely adenine-binding residues of YhhY: RimL Lys²² corresponds to His²¹ of YhhY; Trp⁴¹ corresponds to Trp⁴⁵³ of MccE (Trp⁴⁶ of MccE^{CTD}) and Trp⁴¹ of YhhY, and Thr⁵⁴ corresponds to Phe⁴⁶⁶ of MccE (Phe⁵⁹ of MccE^{CTD}) (17). Residue Cys¹³⁴ of RimL forms a disulfide bond with acetyl-CoA; Ser¹⁴¹ and Glu¹⁶⁰ act as general acid/base catalysts of the acetyltransferase reaction. Alignment was built using MUSCLE software, and the figure was prepared using GENEDEC. (B) LSA was incubated with extracts prepared from indicated cells and subjected to MALDI-MS analysis. Only relevant portions of mass spectra are shown.

clude that endogenous YhhY is responsible for acetylation of LSA in the absence of RimL.

The YhhY protein was purified, and its ability to acetylate several substrates was determined. The results, presented in Table 2, indicate that YhhY was approximately 10 times more active than RimL in acetylation of LSA and ISA but was unable to utilize DSA and ESA as acetylation substrates, in agreement with *in vivo* results.

DISCUSSION

The principal result of this work is the demonstration that the *Escherichia coli* RimL protein contributes to microcin C resistance by acetylating the amino group of the processed Mcc aspar-

tate moiety. In this respect, RimL appears to be very similar to the C-terminal domain of MccE acetyltransferase encoded by the plasmid-borne Mcc biosynthesis/immunity gene cluster *mcc* (6). As is the case with MccE^{CTD}, the nature of the amino acid appears to be unimportant for RimL activity, since all aminoacyl sulfamoyl adenylates containing the primary amine group tested in this work (i.e., Ala, Asp, Gly, Phe, Lys, Glu, Leu, and Ile sulfamoyl adenylates) are efficiently acetylated. The nature of the bond between the aminoacyl and nucleotide moiety is also unimportant, since compounds containing either phosphoamide or sulfamoyl are recognized well. Processed albomycin, which contains a pyrimidine nucleotide instead of adenosine, is also acetylated by RimL, albeit

much less efficiently. Like the MccE acetyltransferase, RimL does not use free amino acids or intact McC as the substrates.

Structural analysis indicates that the promiscuity of MccE^{CTD} with respect to aminoacyl moieties of its substrates is due to tight binding to substrate nucleotidyl moiety through stacking interactions with two aromatic amino acids of the protein, Trp⁴⁵³ and Phe⁴⁶⁶, and the absence of contacts with the aminoacyl moiety of the substrate (17). The available crystal structure of RimL from *Salmonella* (16) reveals a conservation of only one of the π -stacking residues, Trp⁴¹, which corresponds to MccE Trp⁴⁵³ (MccE^{CTD} Trp⁴⁶) (Fig. 6A). Though a second aromatic residue corresponding to MccE Phe⁴⁶⁶ (MccE^{CTD} Phe⁵⁹) is missing, the other face of the active site of RimL is lined with hydrophobic amino acids such as Leu²⁴, Met⁶⁹, and Val¹⁸¹; a histidine side chain (His⁵⁴) is also placed in close proximity. In the *Salmonella* RimI crystal structure (18), a duet of similar stacking residues—Trp²⁷ and His³²—can be identified. However, the adenine-binding site in RimI is occluded by the carboxylate of Glu¹⁹ and the amide side chain of Asn⁶⁸. Steric occlusion by these residues could contribute to the observed inability of RimI to acetylate aminoacyl adenylates by preventing their binding.

In the active site of YhhY predicted by alignment with the MccE^{CTD} structure, His²¹ and the indole ring of Trp⁴¹ are predicted to stack on either side of the substrate adenine ring analogous to Trp⁴⁵³ and Phe⁴⁶⁶ of MccE^{CTD}. Thus, a purine nucleobase binding motif created by two parallel planar aromatic amino acid side chains appears to be a common mechanism for engagement of aminoacyl adenylates by NATs. The narrower specificity of YhhY toward its substrates (the obvious preference for compounds with amino acids containing nonpolar side chains) suggests that this protein contains an additional hydrophobic pocket that accepts this part of the substrate.

Interestingly, our data reveal that though purified RimL can effectively acetylate and therefore detoxify DSA, another system efficiently removes DSA from *E. coli* cell extracts whether RimL is present or not. This yet-to-be identified system appears to degrade DSA, which completely disappears from the extracts, and mass spectrometric analysis fails to reveal peaks that could correspond to modified DSA. This additional system is not functional against McC(1120), whose processing product is identical for DSA except for a phosphoamide bond instead of sulfamoyl.

There are three Rim proteins encoded by the *E. coli* genome. RimI was discovered as an enzyme responsible for acetylation of ribosomal protein S18 (19), and RimJ acetylates ribosomal protein S5 (20), while RimL acetylates the L12 protein, converting it to L7 (21). The importance of the Rim proteins and the acetyl transfer reactions that they catalyze for cell physiology is not clear at present. *In vitro*, L7 forms a more stable complex with the L10 protein than does L12 (22). The L7/L12 ratio is higher in cells grown in poor medium (23), suggesting that RimL activity is activated by nutrient deprivation. These conditions also stimulate McC production by cells harboring the *mcc* operon (24). Be that as it may, none of the Rim proteins or combinations thereof is essential for viability at 37°C (though temperature sensitivity was noted for a *rimL rimJ* deletion mutant [18–20]). In fact, Rim proteins may have functions that are unrelated to *N*-acetylase activity. For example, RimJ plays an important role in the process of assembly of the 30S ribosomal subunits (25), and this involvement does not depend on the function of acetyltransferase. The data presented here indicate that some Rim proteins and other *E. coli* NATs of

unassigned functions may acetylate aminoacyl nucleotides that accumulate either during cell metabolism or as a result of antibiotic treatment and thus contribute to bacterial cell fitness. Moreover, these proteins can also serve as an important source of antibiotic resistance.

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