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Identification of a Novel Inhibition Site in Translocase MraY Based upon the Site of Interaction with Lysis Protein E from Bacteriophage ϕX174

Maria T. Rodolis,[a] Agnes Mihalyi,[a] Amy O'Reilly,[b] Justinas Slikas,[a] David I. Roper,[b] Robert E. W. Hancock,[c] and Timothy D. H. Bugg*[a]

Translocase MraY is the site of action of lysis protein E from bacteriophage ϕX174. Previous genetic studies have shown that mutation F288L in transmembrane helix 9 of *E. coli* MraY confers resistance to protein E. Construction of a helical wheel model for transmembrane helix 9 of MraY and the transmembrane domain of protein E enabled the identification of an Arg-Trp-x-x-Trp (RWxxW) motif in protein E that might interact with Phe288 of MraY and the neighbouring Glu287. This motif is also found in a number of cationic antimicrobial peptide sequences. Synthetic dipeptides and pentapeptides based on the RWxxW consensus sequence showed inhibition of particulate *E. coli* MraY activity (IC_{50} 200–600 μM), and demonstrated antimicrobial activity against *E. coli* (MIC 31–125 μg mL^{-1}). Cationic antimicrobial peptides at a concentration of 100 μg mL^{-1} containing Arg-Trp sequences also showed 30–60% inhibition of *E. coli* MraY activity. Assay of the synthetic peptide inhibitors against recombinant MraY enzymes from *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Micrococcus flavus* (all of which lack Phe288) showed reduced levels of enzyme inhibition, and assay against recombinant *E. coli* MraY F288L and an E287A mutant demonstrated either reduced or no detectable enzyme inhibition, thus indicating that these peptides interact at this site. The MIC of Arg-Trp-octyl ester against *E. coli* was increased eightfold by overexpression of *mraY*, and was further increased by overexpression of the *mraY* mutant F288L, also consistent with inhibition at the RWxxW site. As this site is on the exterior face of the cytoplasmic membrane, it constitutes a potential new site for antimicrobial action, and provides a new cellular target for cationic antimicrobial peptides.

Introduction

Translocase MraY catalyses the first lipid-linked step of bacterial peptidoglycan biosynthesis, the reaction of UDPMurNAc-pentapeptide with lipid carrier undecaprenyl phosphate to yield undecaprenyl-diphospho-MurNAc-pentapeptide (lipid intermediate I; Scheme 1).[1] MraY is a low-abundance membrane protein containing ten transmembrane helices. The *Escherichia coli* enzyme has been overexpressed and solubilised in active form,[2] and the *Bacillus subtilis* enzyme has been purified to homogeneity.[3] The crystal structure of MraY from *Aquifex aeolicus* ($MraY_{AA}$) was recently solved at 3.3 Å by Chung and colleagues.[4] The active site contains three conserved aspartic acid residues, each of which has been shown to be essential for activity in the *E. coli* enzyme (Asp115, Asp116, and Asp267).[5]

MraY is the site of action for several groups of uridine-containing natural product antibiotics: mureidomycin/pacidamycin uridine-peptides, liposidomycin/caprazamycin liponucleosides and muraymycin uridine-peptides.[6] These agents are thought to bind in place of the uridine diphospho-MurNAc-pentapeptide at the active site of MraY,[2,7] on the cytoplasmic face of the cytoplasmic membrane.

Genetic studies by Bernhardt et al. have shown that MraY is also the site of action of protein E (lysis protein) from bacteriophage ϕX174, a 91-residue protein that causes lysis of the *E. coli* host during the lifecycle of this bacteriophage.[8,9] Mutation F288L in transmembrane helix 9 of MraY causes resistance to protein E, both in the wild-type host,[8] and when overexpressed on a pBAD30 vector, with the protein E gene on a λ prophage.[10] Although other mutations (P170L, ΔL172, G186S and V291M) also conferred resistance in the latter experimental model, resistance was only observed at high levels of expression of these mutants.[10] Mendel et al. have found that a synthetic peptide, E_{pep}, containing the 37-residue transmembrane domain of E inhibits particulate *E. coli* MraY enzyme activity (IC_{50} 0.8 μM), but not detergent-solubilised MraY, and concluded that protein E inhibits MraY through a protein–protein interaction distant from the active site.[11] In contrast, Zheng et al. found that full-length recombinant His_6-tagged E inhibits both solubilised and particulate *E. coli* MraY, and non-competitive inhibition ($K_i = 0.5$ μM) was observed with both

[a] Dr. M. T. Rodolis, A. Mihalyi, J. Slikas, Prof. T. D. H. Bugg
Department of Chemistry, University of Warwick
Gibbet Hill Road, Coventry CV4 7AL (UK)
E-mail: T.D.Bugg@warwick.ac.uk

[b] A. O'Reilly, Dr. D. I. Roper
School of Life Sciences, University of Warwick
Gibbet Hill Road, Coventry CV4 7AL (UK)

[c] Prof. R. E. W. Hancock
Centre for Microbial Diseases and Immunity Research
University of British Columbia
2259 Lower Mall Research Station, Vancouver BC V6T 1Z3 (Canada)

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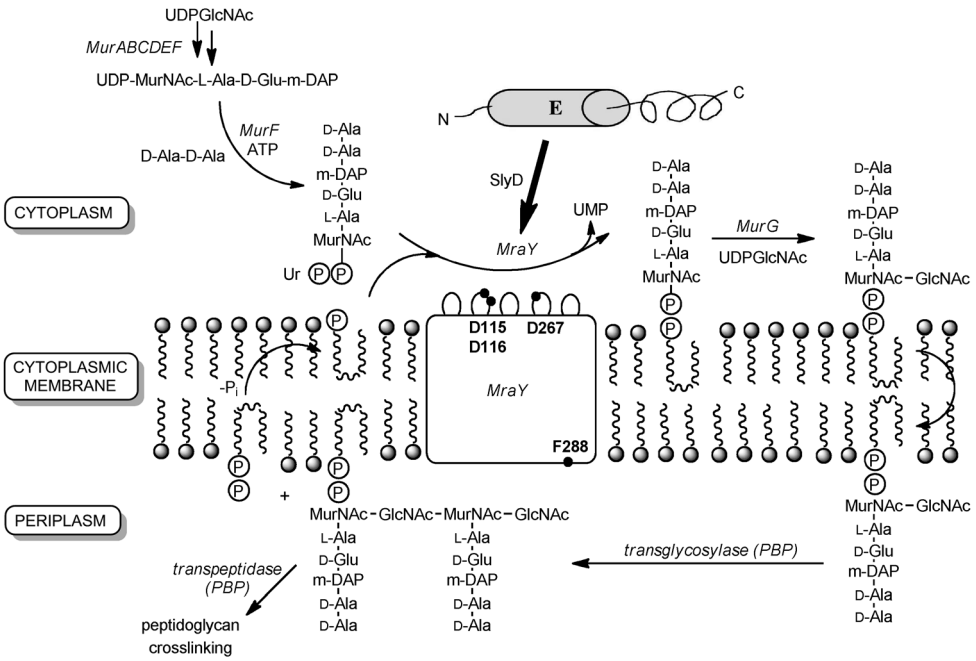


Scheme 1. Lipid-linked cycle of peptidoglycan biosynthesis in *E. coli*. MraY catalyses the reaction of UDPMurNAc-pentapeptide with undecaprenyl phosphate to generate lipid intermediate I. Also shown are the locations of active site residues D115, D116 and D267 on cytoplasmic loops, and F288 whose mutation to Leu causes resistance to E. Lysis by protein E also requires peptidyl-prolyl isomerase SlyD.[8]

lipid and sugar-nucleotide substrates.[12] A recent alanine-scanning study of protein E by Tanaka et al. found that only mutation at Pro19 completely prevented E-mediated lysis, although minor effects on the timing of cell lysis were found with mutations at several other positions.[13] Hence, there are conflicting experimental data regarding the precise interaction between protein E and MraY, though it is clear from genetic analysis that Phe288 of MraY is essential for this interaction.

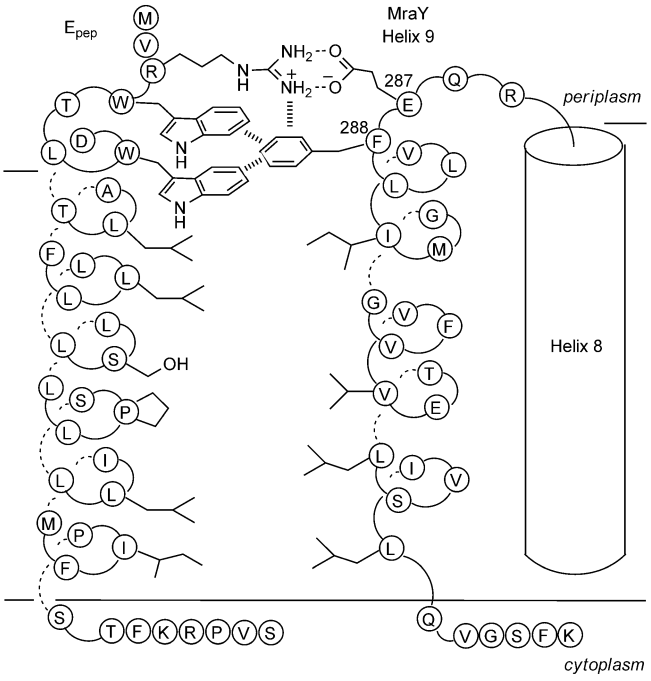
Here we propose a hypothesis for an interaction site based upon an Arg-Trp-x-x-Trp motif (where x is any amino acid) found in protein E and in several cationic antimicrobial peptides. We demonstrate that synthetic peptides based on this motif show inhibition of *E. coli* MraY activity in vitro, and have antimicrobial activity against *E. coli*, and that MraY enzyme inhibition by these peptides depends on the presence of Phe288 and Glu287 in MraY.

Results and Discussion

Hypothesis for an interaction site between protein E and MraY

Phe288 of *E. coli* MraY, mutation of which to Leu is known to cause resistance to protein E,[8] is located in transmembrane helix 9 (TM9), near the extracellular face of the membrane. We modelled the prospective interaction between these two proteins, by aligning an α-helical-wheel-based model of TM9 next to the known α-helix formed by the transmembrane domain of protein E (Scheme 2).[11] The α-helix of TM9 was proposed to start at Phe288, and could terminate at either Val299 (before Glu300) or Leu306 (before Gln307). Although inclusion of Glu300 within a transmembrane domain would be unusual, there are known precedents in multihelix proteins;[14,15] we extended TM9 to Leu306 to include hydrophobic residues 302 and 304–306 within the transmembrane region, and to provide a similar length to that of the TM domain of protein E. The crystal structure of TM9 in *A. aeolicus* MraY ($MraY_{AA}$) reveals a substantial kink at Ala292; this allows favourable interactions between Glu298 (equivalent to Glu300 of *E. coli* MraY) and the active site.[4] The location of this kink results in isolation of Phe286 (*E. coli* Phe288) in a smaller helix, TM9a, close to the exterior face of the membrane.[4]

This model revealed several interesting features. Situated opposite Phe288 of MraY at the extracellular face of the membrane are the two indole side chains of protein E: Trp7 (in the membrane) and Trp4 (probably at the interface), which might form favourable π-stacking interactions with the aromatic side chain of Phe288. Further-



Scheme 2. Model for interaction site between RWxxW motif of E_{pep} and Phe288 and Glu287 of *E. coli* MraY, showing possible noncovalent interactions.

more, Arg3 of protein E could form a favourable electrostatic interaction with Glu287 in the TM8–TM9 turn of MraY, and a possible π–cation interaction with Phe288. Glu287 is highly conserved in MraY (see Figure S4 in the Supporting Information), whereas Phe288 is found only in MraY sequences from Enterobacteriaceae and a few other Gram-negative bacteria (e.g., *Haemophilus influenza*). Pro21 of protein E (mutation of which leads to loss of lytic activity)[13] would strongly impede helicity of this region, and is predicted to lie on the same face of the TM α-helix, facing MraY, close to Leu302 of MraY.

According to our model, the protein E triad Arg3, Trp4 and Trp7 would form a motif that could provide an interaction site for Glu287 and Phe288 of MraY. Analysis of the amino acid sequences of protein E in 48 microviridae bacteriophages related to ϕX174 (Figure S5) revealed three groups of sequences, corresponding to three groups of phages reported by Rokyta et al.[16] In 16 out of 21 sequences of group 1 (ϕX-174-like) this RWxxW motif was conserved, with the remainder showing replacements of Arg3 and/or Trp7. In group 2 (α3-like) phages, Trp4 was conserved, Arg3 was replaced by His in two sequences, and Trp7 was found in six of 12 sequences. In group 3 (G4-like) phages, Trp4 was conserved, but Trp7 was replaced by Ser, and His was found at position 3 in 13 of 15 sequences, with an additional conserved Glu at position 2.

Remarkably, the Arg-Trp dipeptide motif is also found close to the N or C terminus of several cationic antimicrobial peptides (Table 1). The sequence of indolicidin, a well-studied antimicrobial peptide isolated from bovine neutrophils, contains RWPWW starting at position 2 of the C-terminal peptide sequence.[17] Derivatives of indolicidin containing optimised sequences for antimicrobial activity such as the clinical candidate peptide MX226 (Omiganan) contain the same RWxxW motif,[18] and it has been observed that Arg and Trp predominate in the sequences of high-activity compounds obtained from peptide libraries of this kind.[18–20] The lactoferricin B fragment contains RWQW starting at position 5, and it has been demonstrated that both Trp6 and Trp8 of this peptide are essential for antimicrobial activity.[21] The N-terminal sequence of cecropin A contains the sequence KWKSF, and it was reported that the Trp residue in this sequence is essential for activity.[22] The N-terminal sequence of tritrpticin contains the related sequence RFPWW.[23] The physical properties of Arg and Trp side-chains are thought to contribute to their relatively high occurrence in antimicrobial peptides.[24] Although some cationic antimicrobial peptides are known to insert into and form pores in bacterial membranes, there is considerable evidence to suggest that there are alternative mechanisms of action for these antimicrobial agents.[25] The appearance of the same motif in cationic antimicrobial peptides therefore suggests a possible link with the bacteriophage E protein mechanism of bacterial cell lysis.

Table 1. Occurrence of RWxxW or similar motifs close to the N or C termini of cationic antimicrobial peptides.

Name	Sequence	*E. coli* MIC [μg mL^{-1}]	Ref.
protein E	MV**RW**TL**W**DTLAFLLL	lytic	
indolicidin	C-R**RW**PW**W**PWKWPLI	12.5–25	[17]
MX226	C-KR**RW**PW**W**PWRLI	38	[18]
HHC8	C-RK**RW**WW**W**IK	6.0–12	[18]
HHC10	K**RW**WK**W**IRW	1.5	[18]
HHC36	K**RW**WK**W**WR	2.7–5.4	[18]
HHC45	C-**RW**KK**W**RKW	3.0–23	[18]
lactoferricin B	EKCR**RW**QWRMKKLG	24	[21]
cecropin	**KW**KL**F**KKIEK	1.0–2.0	[22]
tritrpticin	VR**RF**PW**W**WPFLRR	20	[23]

Synthesis of Arg-Trp containing peptides

To investigate this hypothesis experimentally, a series of dipeptides and pentapeptides were synthesised, containing elements of the Arg-Trp and RWxxW consensus motifs. Dipeptides Arg-Trp-OMe, Arg-Gly and Gly-Trp-OMe were synthesised by solution-phase synthesis with Boc protecting groups, in 20–50% yields. The corresponding octyl carboxylic esters H_2N-Arg-Trp-oct, H_2N-Arg-Gly-oct and H_2N-Gly-Trp-oct and the N-octyl amides octyl-Arg-Trp-OMe, octyl-Arg-Gly-OH and octyl-Gly-Trp-OMe were also synthesised (it was hypothesised that the octyl group would help to localise the dipeptide in the cytoplasmic membrane).

The set of pentapeptides RWGLW, GWGLW, RGGLW, RWGGW and RWGLG (each side chain of interest replaced with Gly) was synthesised by solid-phase chemical synthesis, in 50–87% yield. 2-Chlorotrityl solid-phase resin[26] was found to be superior to Wang resin for solid-phase synthesis of peptides containing the Arg-Trp sequence, presumably because of the bulky side chains on both these amino acids. Two hexapeptides, ERWGGW and EHWGGG, were also synthesised (69 and 75% yields, respectively) to mimic the related Glu-His-Trp sequence found near the N terminus of the group 3 (G4-like) microviridae bacteriophages.

Inhibition of translocase MraY activity by Arg-Trp-containing peptides

The set of synthetic Arg-Trp containing peptides was assayed as inhibitors of overexpressed particulate *E. coli* MraY, by using a continuous fluorescence assay that we reported previously,[2] with the modified substrate UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys(N^{ε}-dansyl)-D-Ala-D-Ala.

Inhibition of *E. coli* MraY was observed with pentapeptides RGGLW (IC_{50} 210 μM) and RWGLW (IC_{50} 590 μM; Table 2). Pentapeptides RWGLG, RWGGW and GWGLW showed background fluorescence, which interfered with the fluorescence assay; hence, these peptides were assayed against *E. coli* MraY by using a radiochemical assay.[2,5] Inhibition of MraY was observed in each case (IC_{50} 209–274 μM). These data showed that MraY was inhibited by synthetic peptides based on the RWxxW motif, but the structure–activity data suggested that no single amino acid residue in the motif was critical for MraY enzyme inhibition.

Of the synthetic dipeptides, inhibition was observed with only H_2N-GW-octyl ester (IC_{50} 790 μM), thus indicating some se-

Table 2. Activity of synthetic peptides based on RWxxW consensus motif as inhibitors of *E. coli* MraY (IC_{50}) determined by continuous fluorescence assay, and antimicrobial activity against *E. coli* K12.

Peptide sequence	*E. coli* MraY IC_{50} [μM]	Antimicrobial MIC against *E. coli* K12 [μg mL^{-1}]
RWGLW	590 ± 100	–
RGGLW	210 ± 40	–
RWGLG	274 ± 30[a]	–
RWGGW	233 ± 25[a]	–
GWGLW	209 ± 20[a]	–
EHWGGG	460 ± 30	–
ERWGGW	n.i.	–
H_2N-RW-oct	> 1000	31
H_2N-GW-Oct	790 ± 160	–
H_2N-RW-OMe	n.i.	–
H_2N-GW-OMe	n.i.	–

[a] Radiochemical assay, peptides showing background fluorescence. n.i., no inhibition at 1 mg mL^{-1}. No enzyme inhibition observed for *N*-octyl-RW-OMe, *N*-octyl-GW-OMe, Arg-Gly, RG-oct, or *N*-octyl-RG.

lectivity. No inhibition of MraY was observed with Gly-Trp-OMe or *N*-octyl-Gly-Trp. Inhibition of *E. coli* MraY was also observed for hexapeptide EHWGGG (IC_{50} 460 μM).

To investigate the specificity of MraY inhibition, the *mraY* genes from *B. subtilis*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus* were also overexpressed, as described in the Experimental Section, and the corresponding recombinant MraY enzymes were expressed in *E. coli*. In addition, membranes from *Micrococcus flavus*, which contain naturally enhanced levels of MraY,[27] were used to assay the *M. flavus* MraY activity. In each of these enzymes Phe288 is replaced by Leu or Ile (Table 3). In the *P. aeruginosa* and *S. aureus* MraY enzymes, a Phe residue is found either three or four residues further in the sequence, corresponding to approximately one turn of the MraY TM9 α-helix; *B. subtilis* and *M. flavus* sequences contain no aromatic residues in this region.

No inhibition of *P. aeruginosa* or *M. flavus* MraY, which lack Phe288, was observed with RGGLW or RWGLW; however, inhibition of *S. aureus* MraY was observed with RWGLW (IC_{50} = 320 μM), and inhibition of the *B. subtilis* enzyme was observed with RGGLW (IC_{50} = 310 μM) and RWGLW (IC_{50} = 950 μM). The synthetic dipeptide H_2N-GW-oct inhibited all the MraY enzymes tested (IC_{50} = 0.15–1.8 mM). Little or no inhibition was observed with other dipeptides. Hexapeptide EHWGGG inhibited *M. flavus* MraY (IC_{50} = 175 μM), *S. aureus* MraY (IC_{50} = 440 μM) and *P. aeruginosa* MraY (IC_{50} = 340 μM), but not *B. subtilis* MraY.

To explore structure–activity relationships in more detail, F288L and E287A mutants of *E. coli* MraY were constructed and expressed in *E. coli*. Both recombinant enzymes showed catalytic activity comparable to that of wild-type MraY, and membranes containing overexpressed recombinant MraY were assayed with the synthetic peptides and the 37-amino acid protein E-derived peptide E_{pep}. E_{pep} showed no inhibition of the F288L mutant, consistent with this mutation providing resistance against ϕX174 protein E.[9] No inhibition of the F288L mutant enzyme was observed with RWGLW, RGGLW, EHWGGG or H_2N-GW-oct, consistent with interaction of these peptides with Phe288. E_{pep} showed inhibition of the E287A mutant, but with sevenfold reduced potency (IC_{50} 48 μM; wild-type 6.9 μM). No inhibition of the E287A mutant was observed by RWGLW, RGGLW or EHWGGG, consistent with interaction with Glu287. Weak inhibition of E287A MraY was observed with H_2N-GW-oct (IC_{50} 2.1 mM), 2.5-fold reduced potency compared with wild-type MraY (IC_{50} 790 μM).

Several cationic antimicrobial peptides containing sequences similar to the RWxxW consensus sequence were assayed as inhibitors of *E. coli* MraY. Because of the background fluorescence of these peptides, they were assayed with 100 μg mL^{-1} radiolabelled MraY. The cationic peptide indolicidin[17] showed 30% inhibition, whereas indolicidin derivatives (MX226, Kai47, Kai50) showed 52–62% inhibition (Table 4). Two further Arg-Trp-containing peptides (Sub6 and 1002) showed 58 and 41% inhibition, respectively. These data support the hypothesis that Arg-Trp-containing antimicrobial peptides inhibit MraY. In this series of peptides, the highest inhibitory activity was observed for peptides containing the tripeptide sequence Arg-Trp-Trp.

Table 4. Inhibition of *E. coli* MraY by cationic antimicrobial peptides at 100 μg mL^{-1}, by radiochemical assay.

Peptide name	Peptide sequence	Inhibition [%]
indolicidin	C-R**RW**PW**W**PWKWPLI	30
Kai47	C-K**RW**KW**W**RFKWKIF	52
Kai50	C-R**RW**WR**W**WRWKWRLI	61
MX226	C-KR**RW**PWPWRLI	55
Sub6	C-**RW**WKIWVIRWWR	58
1002	N-VQ**RW**LIVWRIRK	41

Table 3. Inhibition (IC_{50}) of overexpressed MraY from *E. coli*, *P. aeruginosa*, *B. subtilis*, *S. aureus* and *M. flavus*, and F288L and E287A site-directed mutants of *E. coli* MraY by selected synthetic peptides, assayed by continuous fluorescence.

Organism	MraY sequence near Phe288	IC_{50} [μM] RWGLW	RGGLW	GWOct	EHWGGG	E_{pep}
E. coli	RQ**EF**LLVIM	590	210	790	460	6.9
P. aeruginosa	RQ**E**IVL**F**IM	n.i.	n.i.	360	340	n.i.
B. subtilis	KL**E**ILLVII	950	310	155	n.i.	n.i.
S. aureus	NQ**E**LSLI**F**I	320	n.i.	1800	440	n.i.
M. flavus	RT**E**ILVAVL	n.i.	n.i.	490	175	n.i.
E. coli mutant F288L	RQ**EL**LLVIM	n.i.	n.i.	n.i.	n.i.	n.i.
E. coli mutant E287A	RQ**AF**LLVIM	n.i.	n.i.	2100	n.i.	48

n.i.: no inhibition at 1 mg mL^{-1}.

Antimicrobial activity of Arg-Trp containing peptides

The synthetic peptides were tested for antimicrobial activity in a microtitre plate growth assay. With *E. coli* K12, growth inhibition was observed only with

dipeptide H_2N-RW-oct (MIC 31 μg mL^{-1}; Table 2). This dipeptide derivative also showed antimicrobial activity against *Pseudomonas putida* and *P. aeruginosa*, and against Gram-positive *S. aureus* and *B. subtilis* (Table 5). Antimicrobial activity against *B. subtilis* was also observed for dipeptide derivative H_2N-GW-oct (MIC 16 μg mL^{-1}), and weak antimicrobial activity against *B. subtilis* was shown by pentapeptide RWGGW (MIC 125 μg mL^{-1}).

Table 5. Antimicrobial MICs for synthetic dipeptides and pentapeptides.

Peptide	MIC [μg mL^{-1}]				
	E. coli	*P. putida* strain mt-2	*B. subtilis*	*P. aeruginosa* strain PA0001	*S. aureus* MRSA strain JE2
H_2N-GW-Oct	–	–	16	n.t.	n.t.
H_2N-RW-oct	31	31	8	40	30
H_2N-RW-OMe	–	–	125	n.t.	n.t.
RWGGW	–	–	125	n.t.	n.t.

n.t.: not tested.

Overexpression of *mraY* protects against RW-oct antibacterial action

As the Arg-Trp-octyl ester and Arg-Trp-containing antimicrobial peptides showed antimicrobial activity against *E. coli* K12, we investigated whether overexpression of *mraY* or *mraY* mutants would confer antimicrobial resistance. It has been reported that overexpression of *mraY* confers resistance to lysis by ϕX174 protein E,[10] consistent with the formation of a 1:1 protein complex between MraY and E.

Overexpression of recombinant *mraY* from a pET52b vector in *E. coli* C43 by induction with 0.5 mM IPTG was found not to cause any significant effect on growth over 8 h. Therefore, the MIC value for each peptide was measured in the presence of 0.5 mM IPTG. For the Arg-Trp-octyl ester, the MIC increased from 31 to 250 μg mL^{-1} for *E. coli* C43 overexpressing *mraY*. (Addition of 0.5 mM IPTG to *E. coli* C43 containing empty vector pET52b showed unchanged MIC.) Overexpression of the F288L *mraY* mutant in *E. coli* C43 resulted in a higher MIC (500 μg mL^{-1}); overexpression of the E287A mutant gave a MIC of 300 μg mL^{-1} (the same as that observed for overexpression of wild-type *mraY*). Hence, overexpression of *mraY* protects against the antibacterial effects of H_2N-RW-oct, consistent with inhibition of MraY in vivo, even though no in vitro inhibition of MraY was observed at 1 mM Arg-Trp-octyl. The higher MIC value for the overexpressed F288L mutant is consistent with binding to Phe288, as the mutant enzyme would bind H_2N-RW-oct poorly so could effectively complement native *mraY*. The possibility that H_2N-RW-oct might cause membrane permeabilisation was also tested by examining uptake of the nonpolar fluorescent probe 1-*N*-phenylnaphthylamine[28] into growing *P. putida* cells. Whereas cells treated with 125 μg mL^{-1} EDTA showed incorporation of fluorescent dye over 20 min, treatment with 125 μg mL^{-1} H_2N-RWOct gave no significant increase in fluorescence (Figure S6), thus indicating that H_2N-RWOct does not cause membrane disruption.

The effect of *mraY* overexpression was also tested with Arg-Trp-containing antimicrobial peptides. As shown in Table 6, MIC values were in fact reduced two- to fourfold for seven cationic antimicrobial peptides with overexpressed *mraY*, the opposite effect to that for H_2N-RW-oct. Overexpression of F288L *mraY* also gave an increased MIC (relative to overexpressed wild-type *mraY*) for indolicidin (twofold) and for peptides 1020 (fourfold) and 1002 (twofold); MIC values for MX226, Kai47, Kai50 and Sub6 were unchanged. Overexpression of E287A *mraY* gave an increased MIC value (relative to overexpressed wild-type *mraY*) for indolicidin (fourfold) and peptide Sub6 (twofold), whereas the MIC values for MX226, Kai47, Kai50, 1002 and 1020 were unchanged. Reduction of MIC in the presence of overexpressed *mraY* implies that, in these cases, MraY assists the antimicrobial action of the antimicrobial peptides. As antimicrobial peptides tend to demonstrate a multi-modal mechanism of action,[25,29] it is possible that binding to the protein E site of MraY provides a mechanism for membrane insertion for these peptides.

Table 6. Effect of overexpression of *mraY*, and F288L and E287A mutant *mraY* genes, on *E. coli* MIC for selected peptides [μg mL^{-1}].

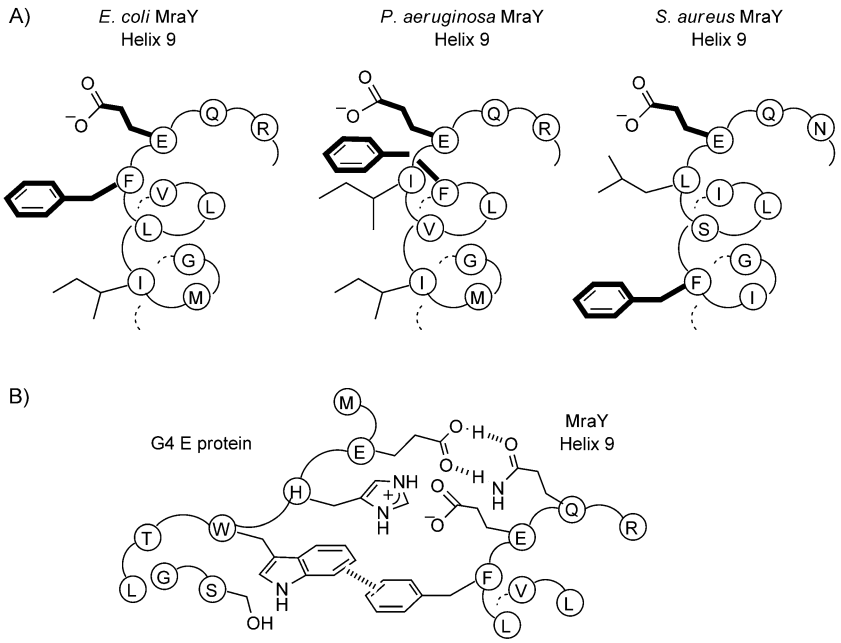
Peptide sequence	Name	*E. coli* empty pET52b	+wild-type *mraY*	+*mraY* F288L mutant	+*mraY* E287A mutant
N-RW-oct		31	250	500	300
C-R**RW**PW**W**PWKWPLI	indolicidin	62	16	31	62
C-K**RW**KW**W**RFKWKIF	Kai47	31	16	16	16
C-R**RW**WR**W**WRWKWRLI	Kai50	31	16	16	16
C-KR**RW**PWPWRLI	MX226	150	62	62	62
C-**RW**WKIWVIRWWR	Sub6	16	8	8	16
N-VQ**RW**LIVWRIRK	1002	8	2	4	2
N-VRLRI**RW**WVL	1020	16	4	16	4

Conclusions

From the likely structures of TM9 of *E. coli* MraY and the transmembrane region of protein E, we propose a model for the interaction of the RWxxW motif with Phe288 of MraY (mutation of which causes resistance to protein E)[8] and to neighbouring Glu287. The inhibition of particulate *E. coli* MraY in vitro by synthetic dipeptides and pentapeptides containing this consensus sequence, as well as the lack of inhibition of the F288L and E287A MraY mutants, is fully consistent with this model. This motif provides an effective binding site for Phe288, as, in addition to potential π–π interactions between the two Trp residues and Phe288, the guanidinium side chain of Arg3 could form a π–cation interaction with Phe288, an interaction that has been proposed to stabilise helix–helix interactions in membranes.[30] There are likely to be other interaction sites between protein E and MraY within the membrane: Tanaka and Clemons replaced each amino acid in protein E with Ala, and showed that Pro21 and Pro29 are essential for cell lysis.[13] They found that replacement of either Arg3 or Trp4 with Ala gave no functional phenotype, but replacement of Trp7 by Ala led

to delay in the onset of cell lysis.[13] Our MraY enzyme inhibition data with synthetic pentapeptides (Table 2) suggest that no single amino acid in the RWxxW motif is essential for interaction with MraY; this might explain why no change in phenotype was observed upon replacement of Arg3 or Trp4 of protein E with Ala. Rather it appears to be the combined effect of all three amino acids that is responsible for the interaction.

As Phe288 and Glu287 are predicted to lie on the exterior face of the cytoplasmic membrane, this site should be relatively accessible from the cell surface, and could therefore represent a novel site for antimicrobial action. It is therefore interesting that one of the dipeptide derivatives, H_2N-RW-oct, showed antibacterial activity against *E. coli* (MIC 31 μg mL^{-1}). Overexpression of *mraY* protected *E. coli* eightfold against H_2N-RW-oct, and overexpression of the F288L and E287A *mraY* mutants protected to yet higher degrees, again consistent with interaction at this site in vivo. It is of interest that H_2N-RW-oct demonstrated much higher antibacterial activity than did H_2N-GW-oct, which showed higher MraY inhibitory activity in vitro. We rationalise this as showing the importance of Arg or Lys residues in cationic antibacterial peptides for binding to the lipid headgroups in the bacterial cytoplasmic membrane.[25]



Scheme 3. Rationalisation of structure–activity data through α-helical wheel models.

As Phe288 is conserved in only the Enterobacteriaceae (and a few closely related Gram-negative bacteria), one might expect that Arg-Trp-containing peptides would selectively inhibit only *E. coli* MraY; however, some inhibition of MraY enzymes from other bacteria was observed (Table 3). In particular, H_2N-GW-oct inhibited every assayed recombinant MraY, and H_2N-RW-oct had antimicrobial activity against *P. aeruginosa* and *S. aureus*. This might be attributable to Glu287, which is conserved in all MraY sequences, and in some cases to a Phe residue on the next turn of TM9 (Scheme 3A), perhaps close enough to interact productively. This suggests that agents that target this site might show broader antimicrobial spectra. Strøm et al. reported that Arg-Trp-benzyl ester showed antimicrobial activity against *S. aureus*;[31] this might be through the same mechanism of action as the Arg-Trp-octyl ester in our study.

The synthetic hexapeptide EHWGGG, based on the protein E sequence of G4-like microviridae bacteriophages, also inhibited all recombinant MraY enzymes except that of *B. subtilis*. We propose that the additional Glu residue in this peptide can form a favourable hydrogen bond with Gln286 in the TM8–TM9 loop (Scheme 3B), which is conserved in most MraY sequences except that of *B. subtilis* (Thr in *M. flavus* MraY, which could also form a hydrogen bond). A Gln–Glu interaction was reported in helix–helix interactions in membrane proteins.[32] The imidazolium side chain of His could also form a hydrogen bond with Glu287 and a π–cation interaction with Phe288 (Scheme 3B). There is also a report of synthetic dipeptide derivatives based on His–Trp that exhibited antibacterial activity;[33] this might be related to our observation.

The initial observation that the RWxxW motif is found in a number of cationic antimicrobial peptides[17–24] suggested that this site might also be accessed by these antibacterial peptides. We found that a number of cationic antimicrobial peptides containing Arg-Trp were able to inhibit MraY in vitro, but that overexpression of *mraY* increased the potency of antimicrobial activity against *E. coli* (rather than decreasing it as we observed for RW-oct). Cationic antimicrobial peptides are known to have multiple sites of action, both within the cytoplasmic membrane and towards intracellular targets;[25] therefore, insertion into the cytoplasmic membrane is a rate-limiting step in their mechanism of action.[25,34] Our rationalisation is that interaction of cationic antimicrobial peptides with the integral membrane protein MraY assists membrane insertion of these peptides, and hence overexpression of *mraY* renders *E. coli* more susceptible to these agents. A similar effect was demonstrated for the cationic lantibiotic peptide Nisin, which targets lipid II in cell wall biosynthesis as well as mediating lipid-II-mediated pore formation.[35] Alternatively, overexpression of *mraY* might increase susceptibility to one of the other known peptide targets, either membrane-associated or cytoplasmic (and thus requiring traversal of the membrane).[36] Interaction with MraY is therefore another mode of action for cationic antimicrobial peptides containing Arg-Trp, and might help to explain the relatively high occurrence (and preferred use) of Arg-Trp in short antimicrobial peptides.[18,24]

One unsolved question is why the binding of agents at Phe288/Glu287 on the extracellular face of the cytoplasmic membrane leads to MraY inhibition, given that the MraY active site is on the inner face of the cytoplasmic membrane. Mendel et al. proposed in 2006 that binding of protein E to MraY by a protein–protein interaction might block an essential protein–protein interaction between MraY and another peptidoglycan biosynthetic enzyme (or a cell-division protein) in the cytoplasmic membrane.[11] In the crystal structure of *A. aeolicus* MraY,[4] the corresponding residues (Phe286 and Glu285) are on an exposed face of MraY, at the membrane interface (Figure 1).

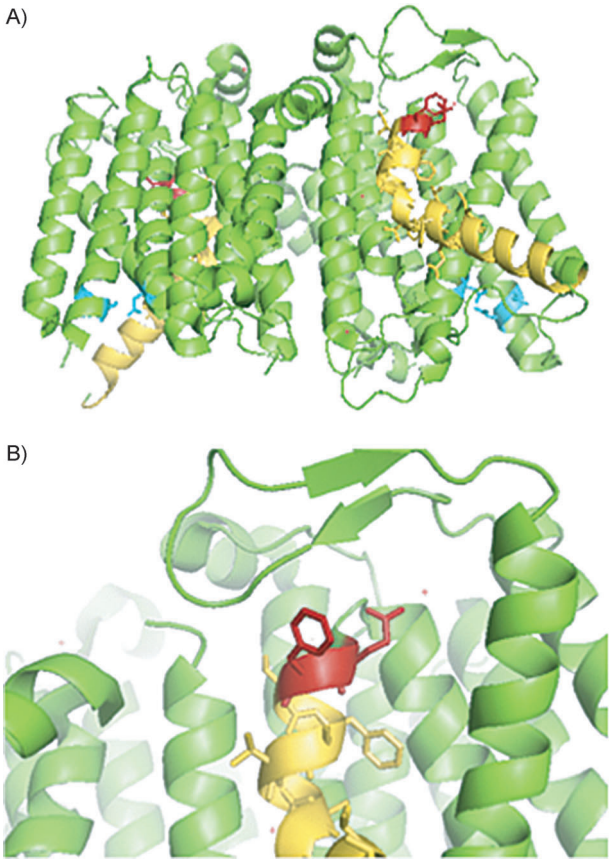


Figure 1. Phe286 and Glu285 (red) and transmembrane helix 9 (yellow) in *A. aeolicus* MraY. A) MraY dimer; B) detail. Views prepared by using PyMOL molecular graphic software. PDB ID: 4J72.

This site could therefore be a site of interaction with another extracellular peptidoglycan biosynthesis protein or a cell-division protein. It is interesting to note that helix 9 of MraY, which we hypothesise forms contacts with protein E, contains an unusual sharp kink, and points out into the cytoplasmic membrane. This sharp kink might explain the requirement for Pro21 and Pro29 in protein E; these would create a bent α-helical conformation.

Interestingly, this site can be accessed in two different ways in nature. Protein E targets this site from the inside of the cell (Scheme 4) by using peptidyl-prolyl isomerase SlyD as an accessory protein,[8] whereas cationic antimicrobial peptides target this site from the exterior of the cell. Hence this site appears to be a "weak spot" in cell wall assembly; nature has targeted this more than once during evolution.

Experimental Section

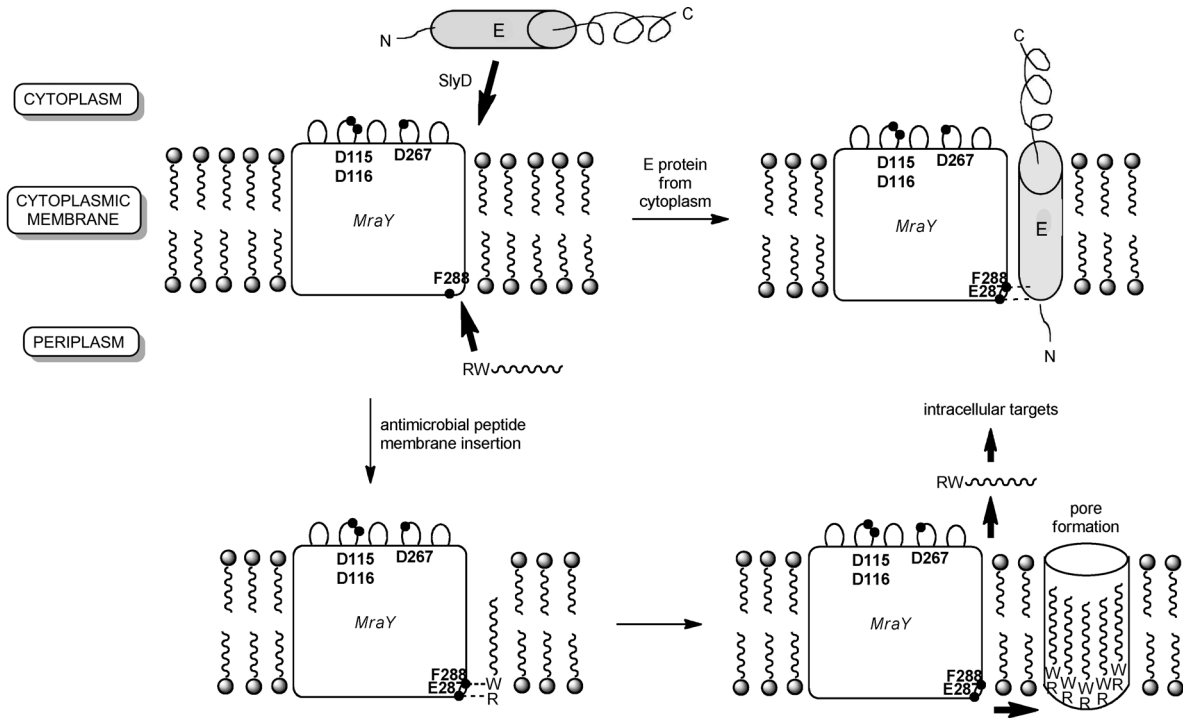
Synthetic dipeptides H_2N-RW-oct and H_2N-GW-Oct were synthesised by solution-phase coupling L-Trp octyl ester to Boc-L-Arg or Boc-Gly by using HATU or EDC, respectively, followed by Boc deprotection with trifluoroacetic acid (1%). Synthetic peptides RWGLW, RGGLW, GWGLW, RWGGW, RWGLG, EHGGG and ERWGGW were synthesised by solid-phase peptide coupling on 2-chlorotrityl resin.[26] Antimicrobial peptides were synthesised by Fmoc solid-phase chemistry at the Centre for Brain Health, University of British Columbia, Canada and were 95% pure. Synthetic procedures and spectroscopic characterisation data are in the Supporting Information. Reagents and biochemical were purchased from Sigma-Aldrich, except where noted otherwise.

MraY inhibition assays

Overexpression of MraY enzymes: E. coli MraY was overexpressed as previously described[5] from plasmid pJFY3c, transformed into *E. coli* C43(DE3).[37] Assays were carried out with inner-membrane preparations (described below), as inhibition of *E. coli* MraY by E_{pep} was previously observed only with particulate MraY (not detergent-solubilised enzyme).[11] Membranes from this construct showed specific activity of 2.27 fluorescence absorbance units (FAU) per min per mg protein, six times higher than for membranes from wild-type *E. coli* C43; we previously reported 28-fold overexpression of MraY activity in *E. coli* JM109.[2] A construct containing *P. aeruginosa* MraY overexpressed as a C-His_6 fusion protein (a gift from Prof. Roger Levesque, Université Laval, Québec, Canada); membranes containing overexpressed *P. aeruginosa* MraY showed specific activity of 4.91 FAU min^{-1} mg^{-1} (protein). *B. subtilis mraY* and *S. aureus mraY* genes were cloned into pET52b (Novagen) and expressed as N-terminal StrepTag fusion proteins in *E. coli* C43(DE3).[37] Expression of *B. subtilis* and *S. aureus* MraY was verified by Western blotting of the overexpressed membranes, by using α-StrepTactin HRP (BioRad, bands at 32–33 kDa; see the Supporting Information); membranes containing overexpressed *B. subtilis* or *S. aureus* MraY showed specific activities of 2.71 and 2.98 FAU min^{-1} mg^{-1} (protein) respectively. Inner membranes from *M. flavus*, containing naturally enhanced levels of MraY,[27] were also prepared and showed specific activity of 0.71 FAU min^{-1} mg^{-1} (protein).

E. coli mraY mutants F288L and E287A were generated by using a Quikchange II Site-Directed Mutagenesis Kit (Stratagene; primers in the Supporting Information). DNA sequences of mutant clones were confirmed by DNA sequencing. The mutant *mraY* genes were cloned into pET52b (Novagen) and expressed in *E. coli* C43(DE3). F288L and E287A MraY mutants were compared with wild-type *E. coli* mraY expressed from the same vector and in the same host.

Overexpression and isolation of membrane-bound MraY: A culture (500 mL) of each overexpression strain (containing *mraY* or *mraY* constructs) was grown in lysogeny broth (LB) containing ampicillin (100 μg mL^{-1}) at 37 °C to OD_{600} = 0.6, then induced with IPTG (1 mM) and allowed to grow for 4 h at 37 °C with shaking. The cells were centrifuged (4400 *g*, 15 min, 4 °C), and the pellet was transferred to a preweighed Falcon tube and resuspended in buffer (3 mL g^{-1} pellet; Tris (50 mM pH 7.5), β-mercaptoethanol (2 mM) and $MgCl_2$ (1 mM)). Egg white lysozyme (2.5 mg), and bovine pancreas DNAse I (25 μg) were added to each cell suspension (1 mL). The cells were then lysed by using a cell disruptor (TS Series Cabi-



Scheme 4. Two routes for access to Phe288 in MraY, for protein E through membrane insertion from the cytoplasm, or for cationic antimicrobial peptides through membrane insertion from the periplasm. Membrane insertion assisted by MraY thereby leads to intramembrane or intracellular effects. The locations of active-site residues D115, D116, D267 are shown. E287 and F288 are shown here to interact with the RWxxW motif.

net; Constant Systems Ltd, Daventry, UK), then centrifuged (24000 *g*, 20 min, 4 °C). The supernatant was then isolated and centrifuged (60000 *g*, 1 h, 4 °C) in an ultracentrifuge. The membrane pellet was homogenised in the above buffer (< 2 mL) and flash frozen in liquid N_2 (300 μL aliquots).

MraY assays: UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys(N^{ε}-dansyl)-D-Ala-D-Ala was prepared by following the procedure of Brandish et al.[2] The MraY-catalysed reaction was monitored on an LS55 fluorimeter (λ_{ex} 340 nm, λ_{ex} 530 nm; PerkinElmer). To monitor the formation of dansyl-lipid I, membrane-bound *E. coli* MraY (15 μL of 0.6 mg mL^{-1} stock) was incubated with UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys(N^{ε}-dansyl)-D-Ala-D-Ala (17.5 μM), lipid carrier undecaprenyl phosphate (39 μM) or heptaprenyl phosphate (59 μM), with $MgCl_2$ (20 mM) in Tris buffer (83 mM, pH 7.5), in a total volume of 0.5 mL.

The final protein concentrations in membranes containing overexpressed *E. coli*, *P. aeruginosa*, *S. aureus*, *M. flavus* or *B. subtilis* MraY in this continuous assay were 0.6, 0.1, 0.28, 0.3, 0.3 and 0.9 mg mL^{-1}, respectively, determined by using a Bio-Rad Protein Assay kit. Specific activities for the overexpressed *E. coli*, *P. aeruginosa*, *S. aureus*, *M. flavus* and *B. subtilis* MraY enzymes were 2.3, 4.9, 3.0, 0.7, and 2.7 FAU min^{-1} mg^{-1} (protein), respectively. MraY inhibitors (40–800 μg mL^{-1}) were added, in duplicate assays; IC_{50} values were determined from plots of activity against inhibitor concentration. Radiochemical assays were carried out by following the method of Brandish et al.,[2] with UDP-MurNAc-L-Ala-γ-D-Glu-L-Lys(N^{ε}-dansyl)-^{14}C-D-Ala-^{14}C-D-Ala (3.4 nCi), heptaprenyl phosphate (27 μg mL^{-1}) and *E. coli* (C43) membranes containing overexpressed protein (40 μg protein) in Tris (100 μL; 90 mM, pH 7.5) containing $MgCl_2$ (23 mM), glycerol (4.0%, *v/v*), DMSO (2.3%, *v/v*) and Triton X-100 (0.1%). Reactions were stopped by addition of pyridinium acetate (50 μL; 6 M, pH 4.2), and lipid-linked products were extracted into 1-butanol (100 μL) and quantitated by scintillation counting.

MIC determination by the microtitre broth dilution technique: Inhibitors were tested for growth inhibition of *P. putida*, *E. coli*, *B. subtilis*, *P. aeruginosa and S. aureus* in lysogeny broth by using the NCCLS protocol (http://isoforlab.com/phocadownload/csli/M7-A7.pdf) in a 96-well microtitre plate. Optical density measurements (OD_{595}) were made with a GENios plate reader (Tecan, Männedorf, Switzerland). The inhibitor concentration that reduced the growth by 50% was recorded as the MIC of the compound.

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Keywords: antibiotics · bacteriophage · biosynthesis · lysis protein E · MraY · peptidoglycans

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*M. T. Rodolis, A. Mihalyi, A. O'Reilly, J. Slikas, D. I. Roper, R. E. W. Hancock, T. D. H. Bugg**

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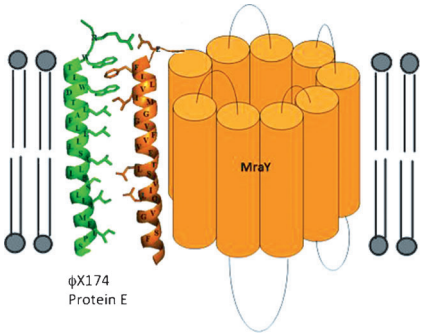
Identification of a Novel Inhibition Site in Translocase MraY Based upon the Site of Interaction with Lysis Protein E from Bacteriophage ϕX174



Site of natural interest: An interaction site is identified between the Arg-Trp-x-x-Trp motif in bacteriophage ϕX174 protein E and Phe288 and Glu287 of translocase MraY, based on synthetic peptide structure–activity data and mutant MraY enzymes.