Pseudomonas aeruginosa MutL protein functions in *Escherichia coli*

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Escherichia coli MutS, MutL and MutH proteins act sequentially in the MMRS (mismatch repair system). MutH directs the repair system to the newly synthesized strand due to its transient lack of Dam (DNA-adenine methylase) methylation. Although *Pseudomonas aeruginosa* does not have the corresponding *E. coli* MutH and Dam homologues, and consequently the MMRS seems to work differently, we show that the *mutL* gene from *P. aeruginosa* is capable of complementing a MutL-deficient strain of *E. coli*. MutL from *P. aeruginosa* has conserved 21 out of the 22 amino acids known to affect functioning of *E. coli* MutL. We showed, using protein affinity chromatography, that the C-terminal regions of *P. aeruginosa* and *E. coli* MutL are capable of specifically interacting with *E. coli* MutH and retaining the *E. coli* MutH. Although, the amino acid sequences of the C-terminal regions of these two proteins are only 18 % identical, they are 88 % identical in the predicted secondary structure. Finally, by analysing (*E. coli*–*P. aeruginosa*) chimaeric MutL proteins, we show that the N-terminal regions of *E. coli* and *P. aeruginosa* MutL proteins function similarly, *in vivo* and *in vitro*. These new findings support the hypothesis that a large surface, rather than a single amino acid, constitutes the MutL surface for interaction with MutH, and that the N- and C-terminal regions of MutL are involved in such interactions.

Key words: chimaeric MutL, complementation, *Escherichia coli*, mismatch repair system, MutH, *Pseudomonas aeruginosa*.

INTRODUCTION

The MMRS (mismatch repair system) is an important process for the correction of replication errors that escape the polymerase proofreading activity and for preventing homeologous recombination events [1]. In *Escherichia coli*, this repair pathway is initiated by binding of MutS to a mismatch. After the recruitment of MutL, this complex activates the strand discriminating endonuclease MutH, which cleaves the newly synthesized, unmethylated daughter strand at the nearest hemimethylated d(GATC) site, and thereby marks it for a removal and a repair–synthesis process that involves a variety of other proteins. The delay in methylation of the newly replicated d(GATC) sequences by the *E. coli* Dam (DNA-adenine methylase) provides the strand discrimination signal.

Eubacteria and eukaryotes express conserved MutS and MutL homologues [1,2]. However, the *E. coli* MutH/d(GATC) methylation mechanism is found only in a small group of proteobacteria from the γ-subdivision [3] and not in eukaryotes. The mechanism of strand discrimination mentioned above seems to be different in most other prokaryotic and eukaryotic organisms and remains poorly understood.

MutL and its homologues are a family of proteins that have a conserved region of approx. 300 residues at the N-terminus and a divergent C-terminal region of 300–500 residues. A majority of reported mutations in *E. coli* MutL, which affect functionality, are within the conserved N-terminal region [4,5]. In humans, more than 50 % of mutations found in the MutL homologous MLH1, in hereditary non-polyposis colon cancer, are within the equivalently conserved region [6,7].

The physical interaction between *E. coli* MutL and MutH has been demonstrated by two-hybrid assays [8,9], the ability of a MutH affinity column to retain MutL [8], MutL-mediated binding of MutH to a MutS column [10] and chemical and photo cross-linking [11,12]. Two different sites on MutL have been proposed as putative MutH-binding sites for MutL–MutH interaction. The hypothesis that MutL interacts with MutH through the N-terminal (LN40) domain is supported by the fact that LN40 activates endonuclease activity of MutH in the presence of heteroduplex DNA, ATP and MutS, and also by *in vitro* protein–protein chemical and photo cross-linking [11,12]. Based on crystallographic results, a concave surface of LN40 has been proposed to interact with and activate MutH [13]. However, mutations of solvent-exposed residues within this surface resulted in proteins that behaved like wild-type MutL *in vitro* and *in vivo* [5].

The second hypothesis is that the C-terminal region of *E. coli* MutL interacts with MutH. Studies using the yeast two-hybrid system showed that the C-terminal 218 amino acid region of MutL is sufficient for the interaction with MutH and that small deletions of the N- or C-terminal regions of this fragment completely eliminate two-hybrid interaction with MutH [8]. Various studies have indicated that the *E. coli* MutL C-terminal region interacts with the DNA helicase UvrD (DNA helicase II, also involved in the MMRS) [14,15] and mediates MutL dimerization [14,16].

Integrity of the C-terminal region of MutL appears to be crucial for the functioning of *E. coli* MutL because deletion of the last 66, or more, amino acids results in non-functional proteins and all except one of the mutations affecting this region of *E. coli* MutL were nonsense mutations [4].

These results, collectively, suggest that interaction between MutL and MutH involves a large surface of MutL, such that single amino acid substitutions are insufficient to disrupt it [5]. It remains unclear whether this interaction resides on the N- or C-terminal regions of MutL or on both.

In the present study, we used the *E. coli* MutL homologue from *Pseudomonas aeruginosa* to further characterize MutL functioning and MutL–MutH interaction. We analysed *in vitro* and *in vivo* functioning of *E. coli* and *P. aeruginosa* wild-type and chimaeric (*P. aeruginosa*/*E. coli*) MutL, and utilized protein

Abbreviations used: Dam, DNA-adenine methylase; LB, Luria–Bertani; MMRS, mismatch repair system; Pcp, phosphorylcholine phosphatase.

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affinity chromatography to analyse the interactions of the N- or C-terminal regions of *P. aeruginosa* or *E. coli* MutL with *E. coli* MutH.

EXPERIMENTAL

Bacterial strains, plasmids and chemicals

E. coli BL21(DE3) was from Novagen (Madison, WI, U.S.A.); *E. coli* ER 2566 was from New England Biolabs (Beverly, MA, U.S.A.); *P. aeruginosa* wild-type strain (Hex1T) was isolated and characterized in our laboratory [17]; and *E. coli* MutL-deficient strain GM4348 [F-*mutL*459 (KanR)] was generously provided by Dr M. G. Marinus (University of Massachusetts Medical School, Worcester, MA, U.S.A.). Plasmids pTX412, pTX417 and pTX418 (pET-15b derivatives containing the *E. coli mutS*, *mutL* and *mutH* genes respectively) were from Feng and Winkler [18]. Plasmids were purified using the Wizard Plus SV Miniprep DNA purification system (Promega, Madison, WI, U.S.A.). DNA extractions from agarose gels were performed using the QIAEX II Gel Extraction kit (Qiagen, Chatsworth, CA, U.S.A.). Restriction enzymes were from Promega and New England Biolabs. GoTaq DNA polymerase and T4 DNA ligase were from Promega.

Analysis of sequenced bacterial genomes by BLAST

Genomic BLAST (www.ncbi.nlm.nih.gov/sutils/genom_table.cgi) [19] with standard parameters was used to search genomes of bacteria completely sequenced against *E. coli* K12 MutS, MutL, MutH, Dam and UvrD proteins (*E. coli* K12 genome accession no. gi:48994873). In the case of *Buchnera*, *Buchnera aphidicola* str. Bp contains *E. coli* MutS, MutL, MutH and UvrD but not Dam homologues. *B. aphidicola* str. APS and *B. aphidicola* str. Sg have *E. coli* MutS, MutL and UvrD, but neither MutH nor Dam homologues [20].

Amino acid sequence alignment and secondary structure prediction

P. aeruginosa and *E. coli* MutL amino acid sequence alignment was performed using CLUSTAL W (www.ebi.ac.uk/clustalw/). Protein sequences were obtained from NCBI (www.ncbi.nlm.nih.gov/). Secondary structure predictions were obtained using JPRED (www.compbio.dundee.ac.uk/∼www-jpred/). Percentage homology was calculated by dividing the number of amino acids that are identical (amino acid comparison), or which have the same predicted secondary structure (secondary structure comparison), by the total number of amino acids.

Cloning, expression and disruption of the *mutL* gene in *P. aeruginosa*

The *mutL* gene from *P. aeruginosa* was amplified by PCR using primers designed based on the published complete genome sequence of *P. aeruginosa* PAO1 (www.pseudomonas.com/) [21]. The Lpf primer (5′-AGCATATGAGTGAAGCACCGCGTATCC-3′) contains an extra five nucleotides (underlined) to create an NdeI restriction site at the ATG initiation codon of the *mutL* gene. The Lpr primer (5′-CGAGCAACGCCTTGTAATAGAGCA-3′) extends the TGA stop codon 391 bp downstream. The 2298 bp amplified PCR fragment was cloned in p-Geam-T-easy cloning vector (Promega) to generate plasmid pG-Lp.

For expression of *P. aeruginosa* MutL protein in *E. coli*, the NdeI–EcoRI fragment from the pG-Lp plasmid, carrying the coding region of this gene, was cloned in the corresponding restriction sites of the T7 polymerase-driven expression vector pET15b (Novagen) to generate plasmid pET-Lp. This plasmid produced N-terminal His-tagged *P. aeruginosa* MutL protein.

A *P. aeruginosa* MutL-deficient strain (ΔMutL) was generated by replacing the endogenous *mutL* gene (Lp) by homologous recombination, with an interrupted Lp::Km allele. The suicide pKN-Lp::Km plasmid was constructed as follows. The SalI fragment carrying the kanamycin-resistant gene from pUT-miniTn5-Km [22] was cloned in the XhoI site of plasmid pG-Lp, located 673 bp downstream from the transcription start site of *P. aeruginosa mutL* gene, to generate plasmid pG-Lp::Km. The ApaI–SpeI fragment from plasmid pG-Lp::Km, carrying the *Km* gene flanked by *mutL* sequences on both sides, was cloned in the corresponding restriction sites of plasmid pKNG101 [23] to generate plasmid pKN-Lp::Km. This plasmid was inserted into an *E. coli* S17-1 λpir strain, which was used as the donor strain for conjugation in biparental mating with a wild-type strain of *P. aeruginosa* as recipient strain, as described previously [23]. Disruption of the chromosomal copy of the *P. aeruginosa mutL* gene was confirmed by PCR and by phenotypic analyses.

Complementation assay

For *mutL* expression in complementation assays, BglII–EcoRI fragments from plasmid pET-Lp (the present study) and pTX418 (a pET15b derivative plasmid containing the *E. coli mutL* gene) [18] were cloned in the corresponding restriction sites of the broad range replication plasmid PBBRIMCS-5 (p5) [24], to generate plasmids p5-Lp and p5-Lc, containing the *mutL* gene from *P. aeruginosa* and from *E. coli* respectively. We observed previously that fragments BglII–EcoRI or XbaI–EcoRI from plasmid pET-*mutS*, carrying the *P. aeruginosa mutS* gene, cloned in any direction within the plasmid p5, were capable of complementing a *P. aeruginosa* MutS-deficient strain, probably by basal expression of this plasmid [17].

Complementation assays were performed by transforming *E. coli* GM4348 [F-mutL459 (KmR)] and *P. aeruginosa* (present study) MutL-deficient strains with plasmid p5 (no insert), plasmid p5-Lp or plasmid p5-Lc. Overnight cultures of transformed strains were grown from single colonies in LB (Luria–Bertani) media containing 10 or 30 μg/ml gentamicin for p5-, p5-Lp- or p5-Lc-transformed *E. coli* or *P. aeruginosa* strains respectively. Non-transformed controls were grown in LB without antibiotic. To calculate mutation frequencies, appropriate dilutions of overnight cultures were plated on LB (for non-transformed strains) or LB containing 10 or 30 μg/ml gentamicin (for p5-, p5-Lp- or p5-Lc-transformed *E. coli* or *P. aeruginosa* strains respectively) to determine the number of viable cells, or in LB containing 40 μg/ml nalidixic acid (for non-transformed *E. coli* strains), LB containing 100 μg/ml rifampicin (for non-transformed *P. aeruginosa* strains), LB containing 10 μg/ml gentamicin with 40 μg/ml nalidixic acid (for p5-, p5-Lp- or p5-Lc-transformed *E. coli* strains) or LB containing 30 μg/ml gentamicin with 100 μg/ml rifampicin (for p5-, p5-Lp- or p5-Lc-transformed *P. aeruginosa* strains) to score for rifampicin or nalidixic acid resistant cells respectively.

For complementation assays using chimaeric (*E. coli*/*P. aeruginosa*) genes, a similar procedure was followed. Similar results were obtained for three independent complementation assays for all plasmid constructs.

Complementation of the *P. aeruginosa* MutL-deficient strain with the *mutL* gene from *P. aeruginosa* confirmed that the PCR-amplified *P. aeruginosa mutL* gene was functional and that the *P. aeruginosa* MutL-deficient strain had no mutation other than interruption of the *mutL* gene, which could affect the MMRS.

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Expression and purification of *E. coli* MutH, *E. coli* and *P. aeruginosa* MutL, and *E. coli* and *P. aeruginosa* N- and C-terminal regions of MutL

His-tagged *E. coli* MutH and *E. coli* and *P. aeruginosa* MutL proteins were isolated after expression of plasmids pTX417, pTX418 [18] and pET-Lp respectively in a BL21(DE3) strain, as recommended by the pET system manual (Novagen).

For expression of Intein-tagged proteins, the following constructions were performed using the pTYB12 plasmid of the Impact-CN system (New England Biolabs). For an Intein–MutH protein, the NdeI–EcoRI restriction fragment from plasmid pTX417 was cloned in the corresponding restriction sites of plasmid pTYB12, to generate the plasmid pTYB12-H. For Intein–Pcp (phosphorylcholine phosphatase, *P. aeruginosa* PA5292), the NdeI–EcoRI restriction fragment from plasmid pG-Pcp (a pGEM-T Easy vector containing a PCR-amplified fragment of the complete *P. aeruginosa* PA5292 gene, in which an NdeI restriction site was added at the ATG start codon site) was cloned in the corresponding restriction sites of plasmid pTYB12 to generate plasmid pTYB12-Pcp. For Intein–NLc and Intein–NLp fusion proteins, containing the N-terminal region of MutL protein from *E. coli* and *P. aeruginosa* respectively, the NdeI–HincII restriction fragments from plasmids pTX418 (903 bp) and pET-Lp (912 bp) were cloned in the NdeI–SmaI linearized plasmid pTYB12 to generate plasmids pTYB12-NLc and pTYB12-NLp respectively. These constructions produce N-terminal regions with an extra glycine residue at the C-termini of these fragments, corresponding to the GGG codon of the SmaI site that precedes the TGA stop codon. For Intein–CLc and Intein–CLp fusion proteins, containing the C-terminal region of MutL protein from *E. coli* and *P. aeruginosa* respectively, the HincII–EcoRI and XhoI–EcoRV restriction fragments from plasmids pTX418 (1742 bp) and pET-Lp (1422 bp) respectively were cloned in the NruI–EcoRI and XhoI–SmaI linearized pTYB12 plasmid respectively to generate the plasmids pTYB12-CLc and pTYB12-CLp.

Intein-tagged proteins were overexpressed in bacterial strain ER2566 as recommended by the Impact-CN system instruction manual (New England Biolabs). Purified proteins were $> 95\%$ pure as judged by Coomassie Brilliant Blue-stained SDS/PAGE (results not shown). When cited in molar terms, protein concentrations are expressed as monomer equivalents.

Endonuclease activity test

The endonuclease activity of *E. coli* MutH protein was assayed with a supercoiled d(GATC) unmethylated pBKS plasmid. The unmethylated plasmid was purified from an *E. coli* Dam-deficient strain. A supercoiled plasmid was isolated from the nicked species by agarose gel purification using agarACE (Promega; M1741). MutH (15, 30, 73, 145, 363, 725 and 1450 nM) was incubated with 0.3 μg of unmethylated pBKS plasmid/μl in 10 mM Tris/HCl, 10 mM $MgCl_2$, 0.75 mM ATP and 0.05 mg/ml BSA (pH 7.5) for 10 min at 37 °C in the absence or presence of Lc (1, 16, 48, 143 and 430 nM), Lp (2, 21, 52, 129 and 517 nM), Lc–Lp (2, 21, 52, 129 and 550 nM) or Lp–Lc (2, 28, 70, 140 and 560 nM) protein, in a final volume of 35 μl. Cleavage reactions were analysed by 0.6 % agarose gel electrophoresis and DNA was visualized by ethidium bromide staining. Three independent experiments were performed and all gave similar results. One representative experiment is shown in Figure 4.

Cloning, expression and purification of (*E. coli*/*P. aeruginosa*) MutL chimaeric proteins

E. coli/*P. aeruginosa mutL* chimaeric genes were constructed as follows. The ApaLI–EcoRI restriction fragment from plasmid pTX418, containing the C-terminal region of *E. coli* MutL, was replaced by the same restriction fragment from plasmid pET-Lp, containing the C-terminal region of *P. aeruginosa* MutL, and vice versa, to generate plasmids pET-Lc-Lp and pET-Lp-Lc. Expression and purification of the chimaeric proteins were performed as recommended by the pET system manual.

Protein affinity columns

Cultures (500 ml) were used to overexpress the Intein-tagged proteins (I–H, I–Pcp, I–CLc, I–CLp, I–NLc and I–NLp) as recommended by the Impact-CN instruction manual. A clarified cell extract (supernatant), obtained after centrifugation at 12 000 ***g*** for 30 min at 4 °C, of each crude cell extract was used to saturate 800 μl of chitin beads. Unbound material was eliminated by successive washes with 1 M NaCl, 20 mM Tris/HCl (pH 7.5) and 1 mM EDTA with 0.1 % Triton X-100 and then without detergent. Finally, the chitin column containing the adsorbed Intein-tagged protein was equilibrated in buffer A [25 mM Tris/HCl, pH 7.5, 10 % (v/v) glycerol, 2.5 mM 2-mercaptoethanol, 3 mM $MgCl_2$ and 50 mM NaCl]. Similar amounts of different Intein-tagged proteins were overexpressed and then adsorbed on to chitin beads, as confirmed by SDS/PAGE analysis of samples from clarified cell extracts and from saturated columns (results not shown).

His-tagged proteins (300 μg in 2 ml of buffer A) were applied to a chitin/Intein-protein column thrice. Columns were washed with 1.6 ml of buffer A and 0.4 ml fractions were collected. Finally, columns were eluted with 0.8, 1.6 and 0.8 ml of buffer A containing NaCl (final concentration of 500 mM, 1 and 2 M respectively) and then 0.2 ml fractions were collected. Fractions (50 μl for each sample) were analysed by electrophoresis on SDS/PAGE. Stained gels (Coomassie Blue) were scanned and values obtained were plotted using the same arbitrary units for all samples (SigmaPlot program, Smooth 2D option).

Analysis of the LN40(dimer)–MutH docking models

The ten docking models previously selected for the LN40(dimer)–MutH complex, generously provided by Dr P. Friedhoff [12], were analysed using the RasMol program (www.rasmol.org). For this analysis, LN40 molecules of the ten models were analysed to identify those amino acids in which the C-α atom is not more than 4 or 6 Å (1 = 0.1 nm) from any C-α atom of MutH. A similar analysis was performed in MutL to identify those amino acids in which any atom is not more than 3 Å from any atom of MutH. The three analyses gave similar results.

RESULTS

Presence of *E. coli* MutS, MutL, MutH, Dam and UvrD homologues in bacterial sequenced genomes

Completed bacterial genomes widely available were searched by BLAST [19] against the *E. coli* MutS, MutL, MutH, Dam and UvrD proteins. As expected, only a few bacterial species have homologues to the *E. coli* MutH protein (Figure 1). Each of these species also has the corresponding homologues of *E. coli* Dam, MutS, MutL and UvrD proteins, except for *Buchnera*, which does not have the corresponding Dam homologue (Figure 1; see the Experimental section). Within the species lacking the corresponding *E. coli* MutH and Dam homologues, *P. aeruginosa* has the highest sequence homology to *E. coli* MutS, MutL and UvrD proteins (59, 42 and 64 % identical respectively) (Figure 1).

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MutH

Accession	Species	Score
NP_417308.1	Escherichia coli	439
NP_838343.1	Shigella flexneri	433
NP_461922.1	Salmonella typhimurium	397
NP_930886.1	Photorhabdus luminescens	330
NP_404414.1	Yersinia pestis	324
NP_796897.1	Vibrio parahaemolyticus	289
NP_716950.1	Shewanella oneidensis	263
NP_438565.1	Haemophilus influenzae	261
NP_246004.1	Pasteurella multocida	259
NP_777657.1	Buchnera aphidicola	161

Dam

Accession	Species	Score
NP_289926.1	Escherichia coli	572
NP_709160.1	Shigella flexneri	570
NP_458420.1	Salmonella enterica	529
NP_403810.1	Yersinia pestis	390
NP_927457.1	Photorhabdus luminescens	389
NP_232254.1	Vibrio cholerae	353
NP_715929.1	Shewanella oneidensis	333
NP_246159.1	Pasteurella multocida	312
NP_438378.1	Haemophilus influenzae	298

MutS

Accession	Species	Score
NP_417213.1	Escherichia coli	1576
NP_708536.1	Shigella flexneri	1570
NP_457300.1	Salmonella enterica	1500
NP_406817.1	Yersinia pestis	1340
NP_928067.1	Photorhabdus luminescens	1281
NP_438865.1	Haemophilus influenzae	1130
NP_246769.1	Pasteurella multocida	1125
NP_798931.1	Vibrio parahaemolyticus	1122
NP_718984.1	Shewanella oneidensis	1043
NP_252310.1	Pseudomonas aeruginosa	908
NP_820057.1	Coxiella burnetii	885
NP_641638.1	Xanthomonas axonopodis	841
NP_779288.1	Xylella fastidiosa	818
NP_903331.1	Chromobacterium violaceum	815
NP_519272.1	Ralstonia solanacearum	796
NP_880300.1	Bordetella pertussis	759
NP_841737.1	Nitrosomonas europaea	753
NP_240241.1	Buchnera aphidicola	748
ZP_00151389.1	Dechloromonas aromatica	748

MutL

Accession	Species	Score
NP_418591.1	Escherichia coli	1100
NP_710035.1	Shigella flexneri	1090
NP_458795.1	Salmonella enterica	957
NP_404019.1	Yersinia pestis	716
NP_931747.1	Photorhabdus luminescens	686
NP_799198.1	Vibrio parahaemolyticus	516
NP_438240.1	Haemophilus influenzae	477
NP_245841.1	Pasteurella multocida	466
NP_716234.1	Shewanella oneidensis	405
NP_253633.1	Pseudomonas aeruginosa	388
NP_637653.1	Xanthomonas campestris	354
NP_780079.1	Xylella fastidiosa	338
NP_520684.1	Ralstonia solanacearum	316
NP_820082.1	Coxiella burnetii	307
ZP_00149733.1	Dechloromonas aromatica	306
NP_660877.1	Buchnera aphidicola	304
NP_841772.1	Nitrosomonas europaea	304
NP_901012.1	Chromobacterium violaceum	296
NP_885782.1	Bordetella parapertussis	293

UvrD

Accession	Species	Score
NP_418258.1	Escherichia coli	1393
NP_709620.1	Shigella flexneri	1390
NP_462836.1	Salmonella typhimurium	1371
NP_407289.1	Yersinia pestis	1246
NP_931798.1	Photorhabdus luminescens	1227
NP_759917.1	Vibrio vulnificus	1012
NP_716104.1	Shewanella oneidensis	999
NP_245348.1	Pasteurella multocida	954
NP_439344.1	Haemophilus influenzae	941
NP_254130.1	Pseudomonas aeruginosa	892
NP_639365.1	Xanthomonas campestris	811
NP_821025.1	Coxiella burnetii	808
NP_297343.1	Xylella fastidiosa	762
NP_899875.1	Chromobacterium violaceum	652
NP_841514.1	Nitrosomonas europaea	631
NP_884249.1	Bordetella parapertussis	621
NP_273289.1	Neisseria meningitidis	609
NP_871275.1	Wigglesworthia glossinidia	577
NP_520356.1	Ralstonia solanacearum	569

Figure 1 BLAST analysis of bacterial sequenced genomes

BLAST search results using *E. coli* MutH, Dam, MutS, MutL and UvrD proteins as query for search of homologous proteins within completely sequenced bacterial genomes. Only the highest homologues to *E. coli* proteins for each bacterial species are indicated. Black vertical lines indicate bacterial species that do not have the MutH/Dam system and the first species within this group is underlined. For each protein the third column indicates the score (bits).

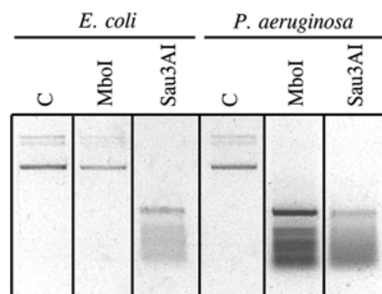

Figure 2 Analysis of adenine methylation within d(GATC) sequences

Plasmids purified from *E. coli* or *P. aeruginosa* wild-type strains were digested with MboI or Sau3AI restriction enzymes, which are sensitive and insensitive to adenine methylation respectively, and then separated on 0.8 % agarose gel. DNA was visualized by ethidium bromide staining. The negative of the photograph is shown. C, control plasmid from *E. coli* or *P. aeruginosa* not digested.

Absence of d(GATC) adenine methylation in *P. aeruginosa*

Homologues to the *E. coli* Dam methylation protein have not been found in the genome of *P. aeruginosa* [21]. We confirmed the absence of Dam methylation by analysing the methylation state of d(GATC) sequences in plasmids purified from *P. aeruginosa*, using restriction enzymes sensitive and non-sensitive to adenine methylation. MboI and Sau3AI restriction enzymes recognize the same sequence, d(GATC). MboI will not cut the sequence if the adenine residue is methylated, whereas Sau3AI is insensitive to adenine methylation. Plasmids purified from *P. aeruginosa* and from Dam+ *E. coli* were digested with Sau3AI and MboI restriction enzymes. While the plasmid purified from Dam+ *E. coli* DNA was digested only with Sau3AI, the plasmid purified from *P. aeruginosa* was digested equally with MboI and Sau3AI (Figure 2), confirming lack of Dam methylation in *P. aeruginosa*. Similar results were obtained when genomic DNA from wild-type *P. aeruginosa* and Dam+ *E. coli* were analysed with the same restriction enzymes (results not shown).

Comparative analysis of MutL proteins from *E. coli* and *P. aeruginosa*

Analysis of sequence homology between *E. coli* and *P. aeruginosa* MutL proteins revealed, as expected, that the N-terminal regions (amino acids 1–335) are highly homologous (61 % identical and 80 % considering similar amino acids), whereas the C-terminal regions are only 18 % identical (35 % considering similar amino acids; Figure 3). However, the predicted secondary structure (see the Experimental section) gave very similar homology for the N- and C-terminal regions (91 and 88 % identical respectively; Figure 3).

Amino acids important for the functioning of *E. coli* MutL protein have been identified after isolation of hydroxylamine-induced mutations, which impart a dominant-negative phenotype to the wild-type strain for increased spontaneous mutagenesis [4], or by amino acid substitution, guided by *E. coli* N-terminal MutL three-dimensional structure [5]. Among these mutations, 21 change amino acids that are located in the N-terminal region and one in the C-terminal region. The amino acids located in the N-terminal region are all conserved in the *P. aeruginosa* MutL protein (19 are identical and two have conservative changes), whereas the amino acid located in the C-terminal region is only weakly similar in *P. aeruginosa* (Figure 3).

Alanine scanning mutagenesis of Mlh1p revealed a region insensitive to mutagenesis (approx. 100 residues) [25]. It was postulated that this region, predicted to form a random coil or unstructured domain, could serve as a flexible linker between the C- and N-terminal domains. Analysis of *E. coli* MutL mutants in which different portions of this unstructured region are deleted revealed that part of this linker region can be eliminated without affecting the *in vivo* functioning of MutL [14]. The predicted secondary structure of *P. aeruginosa* MutL protein revealed the presence of a similar unstructured linker region (Figure 3).

In vivo complementation of MutL-deficient strains

Studies on complementation of MMRS-deficient *E. coli* strains with corresponding genes from other bacteria showed

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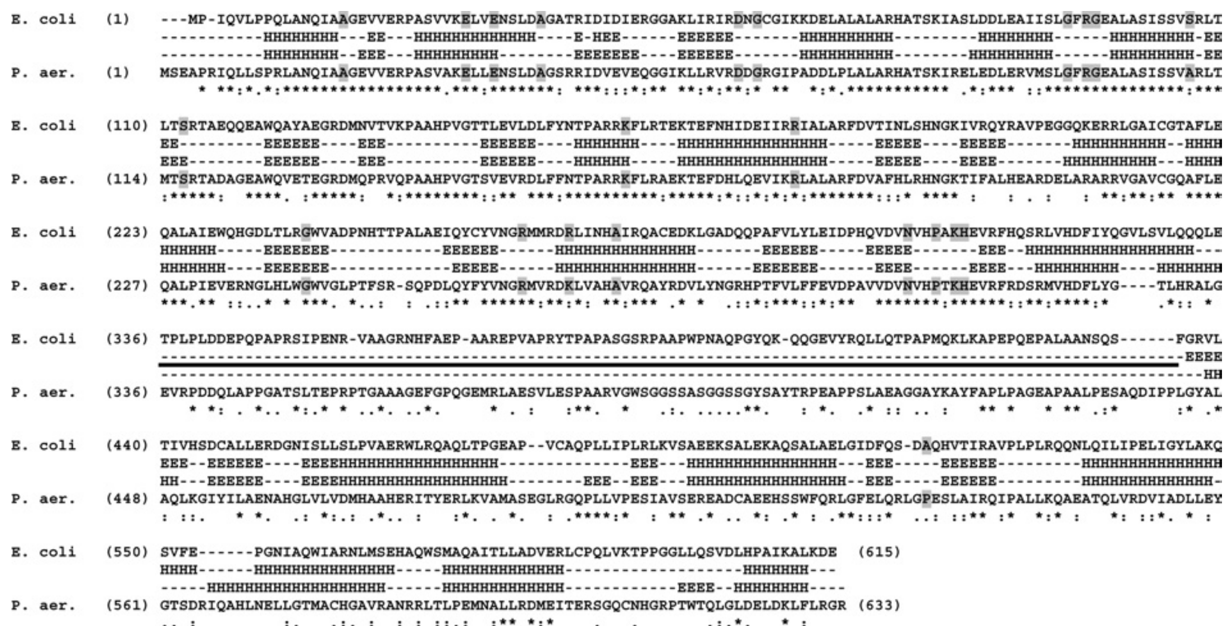

Figure 3 ***E. coli* and *P. aeruginosa* MutL amino acid sequence comparison**

Amino acid sequences were aligned using CLUSTAL W. Amino acids for which mutation in *E. coli* MutL results in non-functional protein are indicated with grey boxes. Amino acids that are identical (*), strongly similar (:), weakly similar (.) or different ( ) are indicated. Predicted secondary structures are shown between the amino acid sequences. H, helix; E, β-sheet. Black line indicates an unstructured linker region between the N- and C-terminal regions.

successful complementation in the case of *mutS* gene from *Salmonella typhimurium* [26] and *mutH* genes from *Haemophilus influenzae* and *Vibrio cholerae* [27], and partial complementation with an incomplete *mutS* gene from *Pseudomonas putida* [28] and *mutL* and *mutS* genes from *H. influenzae* [29]. No complementation was observed with the *mutS* or *uvrD* genes from *P. aeruginosa* [30], *mutS* or *mutL* genes from *Streptococcus pneumoniae* [31], or *mutS* gene from *Thermus aquaticus* [32].

It is not clear *a priori* if the *mutL* gene from *P. aeruginosa* will function in the context of the *E. coli* MMRS, since *P. aeruginosa* does not have the corresponding *E. coli* MutH and Dam homologues.

Functioning of MutL protein from *P. aeruginosa* was assessed by an *in vivo* complementation assay using a MutL-deficient *E. coli* strain. The complementation assay is based on the mutator phenotype that occurs when the bacterium lacks the MutL protein. As shown in Table 1, the *mutL* gene from *P. aeruginosa* complemented the *E. coli* MutL-deficient strain to a similar extent as the *mutL* gene from *E. coli* (92 and 99 % respectively). The 10-fold increase of antibiotic-resistant cells in *P. aeruginosa* ΔMutL compared with *E. coli* ΔMutL (Table 1) is not due to the use of different antibiotics, since *E. coli* strains analysed with rifampicin gave results similar to those obtained with nalidixic acid (results not shown).

The *mutL* gene from *E. coli* was likewise capable of complementing the *P. aeruginosa* MutL-deficient strain. In this case, complemented strains repaired more than 99 % of the mutations. As negative and positive controls, *E. coli* and *P. aeruginosa* MutL-deficient strains were transformed with empty vector or complemented with *mutL* gene from *E. coli* and *P. aeruginosa* respectively (Table 1). These results show that MutL from *P. aeruginosa* restores functioning of the MMRS of an *E. coli* MutL-deficient strain.

Table 1 ***In vivo* complementation of *E. coli* and *P. aeruginosa* MutL-deficient strains with wild-type and chimaeric *mutL* genes**

Strain	Plasmid*	Complemented strains (%)	No. of antibiotic-resistant cells/ 10^8 cells†	Complementation (%)‡
E. coli WT	–	–	1 ± 0.8	99
E. coli Δ MutL	p5	0	234 ± 72	0
E. coli Δ MutL	p5-Lc	100	1 ± 0.9	99
E. coli Δ MutL	p5-Lp	100	19 ± 8	92
E. coli Δ MutL	p5-Lc-Lp	100	8 ± 6	96
E. coli Δ MutL	p5-Lp-Lc	100	10 ± 8	93
P. aeruginosa WT	–	–	5 ± 3	99
P. aeruginosa Δ MutL	p5	0	3.000 ± 200	0
P. aeruginosa Δ MutL	p5-Lp	90	5 ± 2	99§
P. aeruginosa Δ MutL	p5-Lc	74	25 ± 6	99§

*p5, empty plasmid; p5-Lc, p5 plasmid with *E. coli mutL* gene; p5-Lp, p5 plasmid with *P. aeruginosa mutL* gene; p5-Lc-Lp, p5 plasmid with chimaeric (*E. coli*/*P. aeruginosa*) *mutL* gene; p5-Lp-Lc, p5 plasmid with chimaeric (*P. aeruginosa*/*E. coli*) *mutL* gene.

†Results are means $\pm$ S.D. for three independent cultures. Antibiotic-resistant cells were scored using nalidixic acid for *E. coli* and rifampicin for *P. aeruginosa*.

‡The percentage of complementation was calculated considering the number of antibiotic-resistant cells obtained in the absence of MutL as 0 % of complementation, for each bacterial species.

§The percentage of complementation was calculated considering only complemented strains.

P. aeruginosa MutL protein activates *E. coli* MutH endonuclease activity *in vitro*

In vivo complementation of the *E. coli* MutL-deficient strain indicates that the MutL protein from *P. aeruginosa* interacts with and activates the *E. coli* mutH protein. We analysed the *in vitro* capacity of *P. aeruginosa* MutL protein to stimulate *E. coli* MutH endonuclease activity. MutH endonuclease activity was

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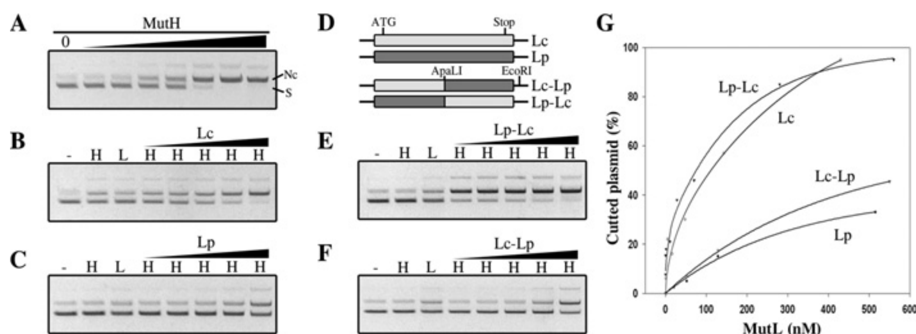

Figure 4 ***In vitro*** **activation of** ***E. coli*** **MutH endonuclease activity**

(**A**) Unmethylated d(GATC) plasmid was digested with increasing amounts of *E. coli* MutH protein (see the Experimental section), separated on 0.6 % agarose gel and visualized with ethidium bromide. S, supercoiled plasmid; Nc, nicked circular plasmid. (**B**, **C**, **E** and **F**) Unmethylated d(GATC) plasmid was incubated with buffer (–), with 70 nM *E. coli* MutH (H), with 500 nM of the indicated MutL protein (L) or with 70 nM *E. coli* MutH and increasing amounts of the indicated MutL protein (H/MutL) (see the Experimental section), before separation on 0.6 % agarose gel. (**D**) Schematic representation of *E. coli* (Lc), *P. aeruginosa* (Lp) and chimaeric MutL proteins containing the *E. coli* N-terminal region and *P. aeruginosa* C-terminal region (Lc–Lp) or vice versa (Lp–Lc). ApaLI and EcoRI indicate positions of the corresponding restriction sites used for the construction of chimaeric *mutL* genes. (**G**) Ethidium bromide-stained gels (**B**, **C**, **E** and **F**) were scanned and the amount of Nc plasmid was plotted as a percentage. The amount of Nc plasmid obtained with MutH alone (70 nM) was defined as 0 %. The difference between the total amount of plasmid used and the amount of Nc plasmid obtained with MutH alone is taken as 100 %. Negatives of ethidium bromide photographs are shown.

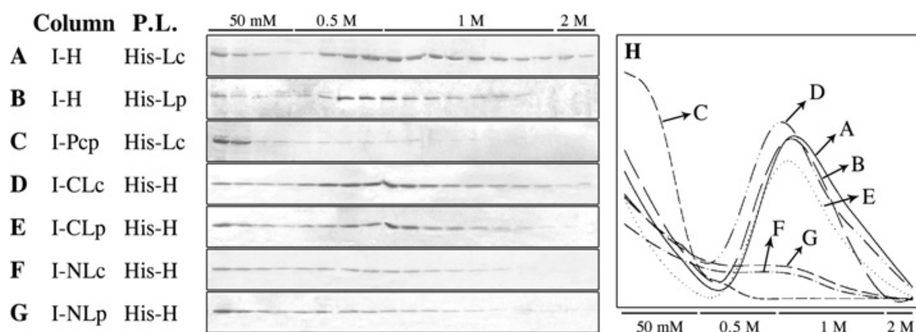

Figure 5 Analysis of MutL–MutH interaction on protein affinity columns

(**A**–**G**) Intein-tagged proteins were adsorbed on to chitin beads and used as affinity ligands. Different His-tagged proteins were loaded on to each column and then eluted with increasing amounts of NaCl, as indicated (see the Experimental section). Gels shown in (**A**–**G**) were scanned and the values plotted in (**H**) (see the Experimental section). Column, protein adsorbed on to chitin beads. P.L., protein loaded on to the column. I, Intein-tagged proteins; His, His-tagged proteins; H, *E. coli* MutH protein; CLc and CLp, MutL C-terminal regions of *E. coli* and *P. aeruginosa*; NLc and NLp, N-terminal regions of *E. coli* and *P. aeruginosa*.

monitored by studying the conversion of the supercoiled into the nicked circular form of d(GATC) unmethylated plasmid. At high enzyme concentrations, the *E. coli* MutH protein was able to cleave unmethylated plasmid DNA, whereas at low protein concentration, MutH alone showed almost no DNA cleavage activity (Figure 4A). As described previously [8,11], addition of *E. coli* MutL protein and ATP to the incubation system stimulates *E. coli* MutH endonuclease activity (Figures 4B and 4G). Addition of *P. aeruginosa* MutL protein and ATP also stimulated *E. coli* MutH endonuclease activity, although to a lesser extent (Figures 4C and 4G). These results show that MutL from *P. aeruginosa* activates *E. coli* MutH endonuclease activity in the *in vitro* mismatch-MutS independent system, although less efficiently compared with the *E. coli* MutL protein.

MutH is specifically retained by the C-terminal regions of *E. coli* and *P. aeruginosa* MutL proteins on affinity chromatography

As described above, both the N- and C-terminal regions of *E. coli* MutL have been proposed to interact with MutH. We analysed *in vitro* interaction between *E. coli* MutH protein and the N- and C-terminal regions of *P. aeruginosa* and *E. coli* MutL proteins using protein affinity columns. MutH and the N- and C-terminal regions of *P. aeruginosa* and *E. coli* MutL proteins were expressed as fusion proteins. Intein-tagged proteins were overexpressed, adsorbed on to chitin beads through the Intein-tag and used as an affinity ligand. Purified His-tagged proteins (*E. coli* His–MutH, and *P. aeruginosa* and *E. coli* His–MutL) were loaded on to the affinity columns, then eluted with a series of salt washes as described previously [8] (see the Experimental section).

A fraction of the loaded *E. coli* MutL protein was retained on the MutH affinity column after the 50 mM NaCl wash step and was then eluted with 0.5–1 M NaCl (Figures 5A and 5H). A similar result was obtained when *P. aeruginosa* MutL protein was loaded on to a similar MutH affinity column (Figures 5B and 5H). As control, a similar experiment was performed but using the Intein–Pcp (Pcp from *P. aeruginosa*, PA5292) fusion protein adsorbed on to chitin beads as an affinity column. In this case, the loaded *E. coli* MutL protein was found principally in the flowthrough and 50 mM NaCl wash (Figures 5C and 5H).

To identify the MutL region responsible for the *in vitro* affinity interaction with MutH, the N- and C-terminal regions of MutL from *P. aeruginosa* (I–NLp and I–CLp) and *E. coli* (I–NLc and I–CLc) were overexpressed, fused to the Intein protein, adsorbed on to chitin beads through the Intein-tag and used as affinity ligands. A fraction of the loaded *E. coli* MutH protein was retained on affinity columns containing the C-terminal region of MutL from *E. coli* (I–CLc) or *P. aeruginosa* (I–CLp) after the 50 mM NaCl wash step and was then eluted with 0.5–1 M NaCl (Figures 5D, 5E

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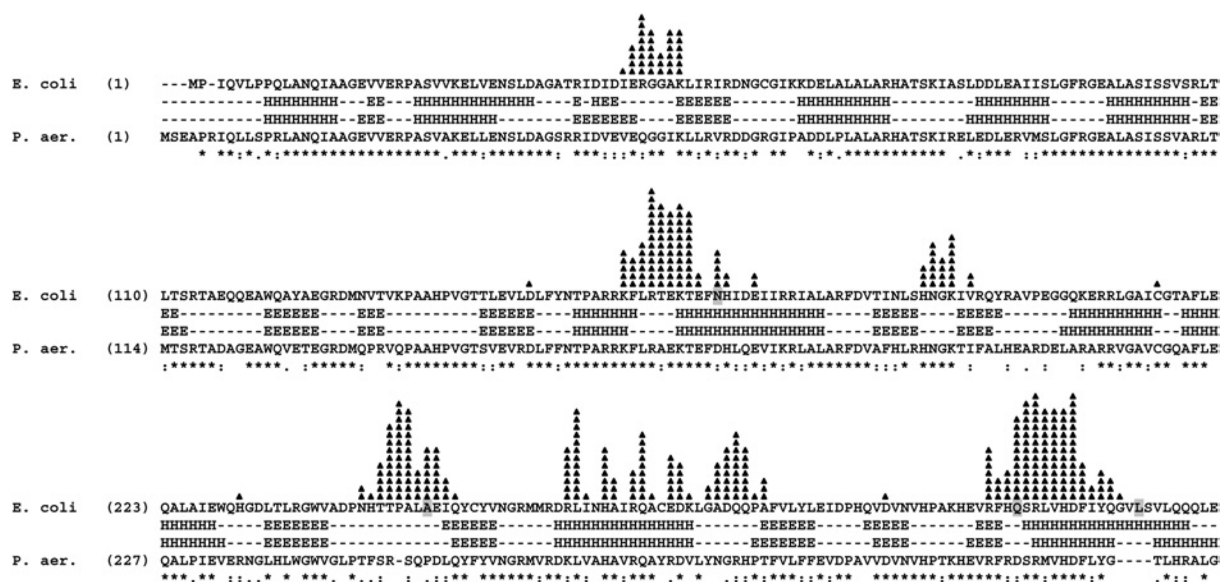

Figure 6 Identification of putative amino acids involved in MutL–MutH interaction

Selected docking models for interaction of *E. coli* LN40(dimer)–MutH [12] were analysed using the RasMol program (see the Experimental section). MutL amino acids in which the C-α is not more than 6 Å from any C-α of MutH are indicated. The number of black triangles indicates how many times each amino acid was identified by this analysis. The four amino acids identified on *E. coli* MutL protein by cross-linking (Asn[169], Ala[251], Gln[314] and Leu[327]) [12] are indicated with grey boxes. Amino acids that are identical (*), strongly similar (:), wealhy similar (.) or different () are indicated. Predicted secondary structures are shown between the amino acid sequences. H, helix; E, β-sheet.

and 5H). The amount of MutH retained by the C-terminal regions was similar to that retained by the full-length proteins. In contrast, when the *E. coli* MutH protein was loaded on to affinity columns containing the N-terminal region of MutL from *E. coli* (I–NLc) or *P. aeruginosa* (I–NLp), only a small fraction of the applied *E. coli* MutH protein was retained and subsequently eluted with 0.5–1 M NaCl (Figures 5F, 5G and 5H). These results show that the C-terminal regions of *P. aeruginosa* and *E. coli* MutL proteins specifically interact with and retain MutH in these protein affinity columns.

Analysis of *E. coli–P. aeruginosa* MutL chimaeric proteins

Based on the above results, it is not clear why, *in vitro*, MutL from *P. aeruginosa* stimulates *E. coli* MutH endonuclease activity less efficiently compared with *E. coli* MutL protein (see Figures 4B, 4C and 4G). One possibility is that although the C-terminal region of *P. aeruginosa* interacts with *E. coli* MutH protein, the highly divergent amino acid sequence of *P. aeruginosa* MutL protein affects stimulation of MutH endonuclease activity, performed by the MutL N-terminal region. However, the *P. aeruginosa* N-terminal region could also be involved. It was proposed recently that the interaction site between the N-terminal region of *E. coli* MutL and MutH could be mapped on to a region comprising Asn[169], Ala[251], Gln[314] and Leu[327] residues of MutL [12]; however, these amino acids are only weakly conserved in *P. aeruginosa* MutL protein (Figure 6).

To analyse whether the N- or C-terminal region of *P. aeruginosa* MutL protein was responsible for the decreased stimulatory effect of *E. coli* MutH endonuclease activity *in vitro*, we generated chimaeric proteins containing the *E. coli* N-terminal region and the *P. aeruginosa* C-terminal region (Lc–Lp), or vice versa (Lp–Lc) (Figure 4D). *In vivo* analysis showed that both these chimaeric proteins are capable of complementing the MMRS of an *E. coli* MutL-deficient strain. Transformed *E. coli* MutL-deficient strains expressing Lc–Lp or Lp–Lc were capable of repairing 96 and 93 % of the mutations respectively (Table 1).

In vitro, the Lp–Lc chimaeric protein stimulated *E. coli* MutH endonuclease activity similar to *E. coli* MutL protein (Figures 4E and 4G). The Lc–Lp chimaeric protein also stimulated *E. coli* MutH endonuclease activity, but to a lesser extent, similar to *P. aeruginosa* MutL protein (Figures 4F and 4G). These results indicate that (i) the C-terminal region of *P. aeruginosa* MutL protein is responsible for the decreased *in vitro* stimulatory effect on *E. coli* MutH endonuclease activity, and (ii) the N-terminal region of *P. aeruginosa* MutL protein functions, *in vivo* and *in vitro*, similarly to the N-terminal region of *E. coli* MutL protein.

Analysis of the MutH interface of the N-terminal region of MutL

It was recently proposed, based on a combination of site-directed mutagenesis and site-specific cross-linking, that the MutH interaction site of LN40 maps on to a region comprising Asn[169], Ala[251], Gln[314] and Leu[327] residues [12]. From 10 000 docking models generated for the complex LN40(dimer)–MutH, ten were selected on the basis of cross-linking results [12].

The chimaeric Lp–Lc protein behaved similar to wild-type *E. coli* MutL protein *in vivo* and *in vitro*, showing that the N-terminal regions of *P. aeruginosa* and *E. coli* MutL proteins function in the same manner. The N-terminal region of *P. aeruginosa* MutL protein has conserved the 21 amino acids essential for the functioning of *E. coli* MutL protein (see Figure 3). However, the four amino acids identified on the surface of the *E. coli* LN40 dimer by cross-linking experiments with MutH (positions 169, 251, 314 and 327) are only weakly conserved in *P. aeruginosa* MutL protein (Figure 6). Our analysis of the ten models previously selected for the LN40(dimer)–MutH complex [12] revealed interesting features. LN40 molecules of the ten docking models were analysed to identify amino acids in which the C-α atom is not more than 4 or 6 Å from any C-α atom of MutH, or amino acids in which any atom is no more than 3 Å from any atom of MutH. Considering these distances (3–6 Å), the amino acids identified could also be involved in the interaction between LN40 and MutH. The three analyses gave similar results. In Figure 6 (showing

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only amino acids in which the C-α atom is not more than 6 Å from any C-α atom of MutH), the identified amino acids are clustered in several groups. Some of these clusters are in regions that are highly conserved between *E. coli* and *P. aeruginosa* (Figure 6), suggesting that selected models involving these regions could also model the interaction between the N-terminal region of *P. aeruginosa* MutL protein and *E. coli* MutH protein. Comparative analysis between *E. coli* and *P. aeruginosa* MutL proteins will better identify regions and/or amino acids important for interaction between MutL and MutH proteins.

DISCUSSION

We have shown that MutL from *P. aeruginosa* is capable of complementing an *E. coli* MutL-deficient strain. This result was surprising considering that (i) *P. aeruginosa* does not have *E. coli* MutH and Dam homologues and therefore the mechanism of DNA strand discrimination for mismatch repair in *P. aeruginosa* is presumably different from that of *E. coli*; (ii) MutS and UvrD proteins from *P. aeruginosa* do not complement the corresponding *E. coli* mutant strains [30]. In fact, MutS produced a dominant-negative effect when introduced into a wild-type *E. coli* strain, increasing the mutation frequency 50 times [30]. Oliver et al. [30] reported that *P. aeruginosa mutL* gene failed to complement an *E. coli* MutL-deficient strain. However, in that study, the *P. aeruginosa mutL* gene was introduced with its own promoter region into the *E. coli* MutL-deficient strain, and it is possible that the *P. aeruginosa mutL* promoter was non-functional in *E. coli* or that the expression level obtained was insufficient to complement the *E. coli* MutL-deficient strain.

It has been proposed that the *E. coli mutH* methylation-based system evolved from a restriction modification system [3]. If this evolution was recent, perhaps the *E. coli* and *P. aeruginosa* MutL proteins did not have time to diverge and so they are both capable of complementing *E. coli* and *P. aeruginosa* MutL-deficient strains. Another possibility is that the regions that allow these proteins to work in both species are structurally important for MutL, or for interaction with other proteins (MutS, UvrD, Vsr endonuclease, etc.), and are therefore stable during evolution.

The *E. coli* MutL protein and the chimaeric construct having *P. aeruginosa* N-terminal region were both functional *in vivo* and also capable of stimulating *in vitro* the *E. coli* MutH endonuclease activity, showing that the N-terminal regions of *P. aeruginosa* and *E. coli* MutL proteins function similarly. Recently, a model for LN40–MutH complex was proposed [12]. Although the four amino acids of *E. coli* LN40 proposed for MutL–MutH interaction are only weakly conserved in *P. aeruginosa* MutL, our analysis revealed that some of the proposed LN40(dimer)–MutH models could involve amino acid regions that are highly conserved in *P. aeruginosa* MutL.

E. coli MutL mutants with deletion of the C-terminal region (amino acids 67–285) have a dominant-negative effect when introduced into a wild-type strain [4]. MutH and UvrD interact with the last 218 amino acid segment of *E. coli* MutL [8,14,15], and removal of a small number of residues from either the N- or C-terminus of this segment terminates this interaction [8]. Our results are consistent with these and support the hypothesis that the C-terminal region of MutL is involved in the interaction with MutH. We showed, using protein affinity chromatography, that the C-terminal regions of *E. coli* and *P. aeruginosa* MutL are capable of specifically interacting with and retaining *E. coli* MutH. Although the amino acid sequences of the C-terminal regions of *E. coli* and *P. aeruginosa* MutL have low homology (18 %), the predicted secondary structure showed high homology (88 %). These results, and the fact that no MutL point mutation capable of preventing MutL–MutH interaction has been isolated so far, support the hypothesis that the MutH interface on MutL involves a large surface or a particular structure.

MutH is capable of interacting with the C-terminal region of MutL without any particular requirement (ATP, MutS, DNA, etc.) (the present study and [8]), suggesting that a part of cellular MutL forms a complex with MutH. Such a MutL–MutH cellular soluble complex would have functional implications.

According to the sliding clamp model [33], several sliding clamps are generated at a mismatch site after the binding of MutS and exchange of ADP with ATP. MutL, with no binding to ATP, then interacts with MutS(ATP) sliding clamps. The function of MutL is to physically connect the MutS sliding clamps with MutH endonuclease and UvrD helicase. MutS(ATP) and MutS-(ATP)–MutL sliding clamps are both capable of ATP hydrolysis-independent diffusion along duplex DNA. However, MutL binding enhances unloading of MutS sliding clamps 10-fold [33]. MutH does not affect MutS mispair binding activity, MutS or MutL ATPase activity, or formation/release of MutS–MutL complex [33]. Therefore interaction of MutS with preformed MutL–MutH complex, rather than two-stage interaction (first with MutL, then with MutH), will make MutH more likely to exert its enzymatic activity before dissociation of the clamp.

This hypothesis can be extended to UvrD, which, similar to MutH, interacts with the last 218 amino acid segment of *E. coli* MutL without any particular requirement [14,15]. The use of *uvrD* mutant strains to obtain purified MutL, not contaminated with UvrD further supports the putative MutL–UvrD soluble complexes *in vivo* [10].

The *E. coli* MMRS proceeds through recognition of a hemimethylated d(GATC) site by MutH or through the presence of a nick within the double-stranded DNA molecule [34,35]. Therefore UvrD function is independent of the formation and/or activation of a MutS–MutL–MutH complex. The proposed model is capable of explaining both cases. MutS–MutL–MutH sliding clamps will slide through the DNA until a hemimethylated d(GATC) site is found, generating a nick in the unmethylated DNA strand, or until it dissociates. If a MutS–MutL–UvrD sliding clamp is formed, this complex will slide through the DNA until a nick is found or until it dissociates. Both kinds of complex can be generated continuously and can operate independently or consecutively.

Results of the present and previous studies suggest that both the N- and C-terminal regions of MutL are involved in the interaction with MutH. However, further genetic and biochemical experiments are necessary to understand how MutL interacts with and activates MutH at the molecular level within the *E. coli* MMRS.

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